Progress in Biological Control

P. Narayanasamy

Biological Management of Diseases of Crops

Volume 1: Characteristics of Biological Control Agents

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Volume 1: Characteristics of Biological Control Agents

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Progress in Biological Control

Series Preface

Biological control of pests, weeds, and plant and animal diseases utilising their natural antagonists is a well-established and rapidly evolving field of science. Despite its stunning successes world-wide and a steadily growing number of applications, biological control has remained grossly underexploited. Its untapped potential, however, represents the best hope to providing lasting, environmentally sound, and socially acceptable pest management. Such techniques are urgently needed for the control of an increasing number of problem pests affecting agriculture and forestry, and to suppress invasive organisms which threaten natural habitats and global biodiversity.

Based on the positive features of biological control, such as its target specificity and the lack of negative impacts on humans, it is the prime candidate in the search for reducing dependency on chemical pesticides. Replacement of chemical control by biological control – even partially as in many IPM programs – has important positive but so far neglected socio-economic, humanitarian, environmental and ethical implications. Change from chemical to biological control substantially contributes to the conservation of natural resources, and results in a considerable reduction of environmental pollution. It eliminates human exposure to toxic pesticides, improves sustainability of production systems, and enhances biodiversity. Public demand for finding solutions based on biological control is the main driving force in the increasing utilisation of natural enemies for controlling noxious organisms.

This book series is intended to accelerate these developments through exploring the progress made within the various aspects of biological control, and via documenting these advances to the benefit of fellow scientists, students, public officials, policy-makers, and the public at large. Each of the books in this series is expected to provide a comprehensive, authoritative synthesis of the topic, likely to stand the test of time.

Heikki M.T. Hokkanen, Series Editor

Preface

Various crop plants have been domesticated, after careful selection from innumerable wild plant species over several millennia, because of their potential for higher yield and better quality of the produce. Crop production systems have been developed primarily to fulfill philosophic and economic objectives of feeding humans and animals and providing better livelihood for the growers. Microbial plant pathogens continue to be a scourge of mankind from the prehistoric period, as the causative agents of numerous devastating diseases of plants that provide food, feed, fiber and all other materials essentially required for man and animals. Continuous and sustained efforts have been made to minimize the quantitative and qualitative losses of crops due to diseases incited by the microbial plant pathogens – oomycetes, fungi, bacteria, phytoplasmas, viruses and viroids – in different ecosystems. Managing crop diseases through development of cultivars resistant to diseases has been successful only to a limited extent, because of the unavailability of dependable sources of resistance genes for incorporation into susceptible cultivars. Application of chemicals is being practiced for several centuries and selective chemicals with systemic action could provide protection against microbial pathogens for short periods only. Development of resistance in plant pathogens to chemicals, accumulation of chemical residues in grains and food materials and environmental pollution due to indiscriminate use of chemicals gave negative signals for their continued use for crop protection. Biological management of crop diseases has emerged as an attractive, alternative approach for minimizing the incidence and severity of diseases of crops caused by microbial pathogens.

Biological management of crop diseases involves the utilization of biotic and/or abiotic agents that act through one or more mechanisms to reduce the infection potential of microbial pathogens directly and/or indirectly by activating the host defense systems to reduce the disease incidence and/intensity. The biotic agents include oomycetes, fungi, bacteria and viruses that suppress the development of crop diseases caused by microbial pathogens in various crops. The abiotic agents such as solar energy, heat, ultraviolet light, organic amendments, organic and inorganic compounds and naturally-derived substances of plant and animal origin also possess

the ability to restrict the development of crop pathogens through direct and/indirect effects, as the biotic agents. Although innumerable biotic and abiotic agents have been demonstrated to have high level of biocontrol activity in in vitro assays, very few have been found to have the expected level of biosuppressive activity under field conditions where they have to compete with the pathogens for the available nutrients and niche for survival. In the recent years, due to growing concern and awareness for protecting the environment and the need for providing chemical-free food to the consumers, several formulations based on microbial antagonists and resistance inducers (plant activators) have been commercialized. Consistency in their performance under various agroclimatic zones is one of the major requirements to make the production of biocontrol products as a viable economic industrial venture. The advantages of biocontrol strategies and their limitations are discussed in detail in two volumes of this treatise. The investigations to study the nature and characteristics of the biological control agents are presented in the first volume. The possibilities of integrating different biological strategies with crop disease management systems are highlighted in the second volume.

The information presented in this book represents extensive literature search (over 2,500 citations) and it is aimed to provide a comprehensive knowledge to the upper level undergraduate and graduate students, researchers and teachers associated with teaching courses as a component of biological management of diseases of crops in the Departments of Plant Pathology, Plant Protection, Microbiology, Molecular Biology, Botany, Ecology, Agriculture and Horticulture. Provision of several protocols appended at the end of relevant chapters to assist the researchers in planning their experiments is a unique feature of this book.

Coimbatore, India P. Narayanasamy

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Introduction

Biological management of diseases of crops has the foundation on the research efforts to identify the various ecofriendly approaches (i) to mitigate the ill effects of infection by microbial plant pathogens – oomycetes, fungi, bacteria, phytoplasmas, viruses and viroids, by using antagonistic microorganisms and naturally-derived materials of plant and animal origin, (ii) to reduce or replace the use of synthetic chemicals and (iii) to integrate the compatible and synergistic strategies for enhancing the effectiveness of disease suppression. These approaches are expected to protect the environment and to create the possibilities of increasing the yields of crops and supplying chemical residue-free farm produce to the consumer.

1.1 Concepts and Aims of Biological Management of Crop Diseases

Crops in different ecosystems have been under biotic and abiotic stresses that constitute a major constraint in realizing the full yield potential of cultivars of various crops. The extent of loss induced, may vary depending on the nature of the cause and availability of environments favorable for the development and persistence of disease-inducing agent(s). Among the biotic causes, microbial plant pathogens play a predominant role in adversely affecting the crop production systems leading to huge quantitative and qualitative losses in attainable yield levels. In order to minimize the incidence and severity of diseases of crops, the effectiveness of various short- and long-term strategies has been assessed in different geographical locations. No single disease management strategy or combination of strategies could be advocated for successful cultivation of crops with desirable profit margin to the growers. However, with growing concern for environmental pollution and presence of chemical residues in grains, vegetables, fruits and other food materials, intensive efforts have been made to restrict the use of plant protection chemicals which were earlier considered as indispensable requirement for containing the plant diseases.

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Crop management methods have been developed primarily to maintain soil fertility at high levels and to enhance the crop yields by manipulating application of fertilizers, tillage and irrigation practices and maintaining appreciable levels of organic matter in the soil. Crop management practices have significant influence on the incidence and severity of diseases caused by microbial pathogens. It is essential that disease management strategies are compatible with the current cultural practices adopted in a given geographical location to have the acceptance of the farmers. Biological management tactics applicable for crop disease management seek to be a component of the synergistic orchestrated symphony, the benefit of which will reach both the producer and consumer. Concepts of biological management of diseases have evolved from time to time, based on the information and techniques to assess the nature of interactions between the pathogens and other organisms and the plants. The term 'biological control' or 'biocontrol' is applied in a narrow sense to indicate the control of "one organism by another organism" (Beirner 1967). This term has been used also in a wider sense to indicate the "use of natural or modified organisms, genes or gene product to reduce the effects of undesirable organisms (pathogens) and to favor desirable organisms such as crops, trees, animals, beneficial insects and microorganisms". This broad definition was proposed in the Report of the Research Briefing Panel on Biocontrol in Managed Ecosystems (Cook 1987). It is well known that no organism can exist independently and interdependence is the basic factor that decides the survival and persistence of life forms through subsequent generations. Restrictive application of the term 'biological control' to one organism controlling another organism appears to be unrealistic. The development of a microorganism controlling another depends on several factors that favor its antagonistic activity. Further, some microorganisms and naturally-derived organic compounds act on the host plants in the same manner by inhibiting pathogen development directly or enhancing the level of resistance in host plants against the target pathogens indirectly. Both biotic and abiotic agents that have been commercialized are applied to crops against several diseases and both types have direct and indirect effects on the pathogen and host plants. Hence, biological management is defined as the utilization of biotic and abiotic agents that act through one or more mechanisms to reduce the potential of the pathogen directly or indirectly by activating host defense systems to reduce the disease incidence and/ intensity. Several investigations have shown that combination of biotic and abiotic agents results in synergism, improving the effectiveness of disease control.

Green revolution leading to higher crop yields achieved in several countries including developing countries was possible by applying different crop management strategies such as use of high yielding varieties, high doses of fertilizers and frequent and indiscriminate use of pesticides. Adoption of these strategies was also responsible for emergence of new problems that reduced yields in due course. As regards crop disease management, development of resistance in pathogens to fungicides and bactericides became a serious constraint for reaching the intended targets of high yield. The need for finding out effective alternative strategies for minimizing the incidence and intensity of crop diseases was fully realized. In addition, the possibility of restricting the use or replacing the synthetic chemicals that were apparently

found to be highly effective against some pathogens had to be explored. The studies on the compatibility of biotic agents with fungicides or bactericides revealed that it would be possible to select strains of biocontrol agents (BCAs) showing tolerance to synthetic chemicals and they could be applied in combination with the chemicals resulting in restricted use of the chemicals. Thus the aims of the biological management of crop diseases are to (i) select the most effective biotic agents; (ii) identify the abiotic agents that can act individually or in combination with biotic agents; (iii) assess their effects on growth promotion in treated plants; and (iv) examine the possibility of reducing the use or replacing the chemicals without compromising the effectiveness of disease control.

1.2 Landmarks in the Development of Biological Disease Management Systems

Occurrence of diseases of crops of economic importance is known even in prehistoric times as indicated by examination of fossils about 25,000 years old (Klausner 1987; Chu et al. 1989). Although concepts about the nature of the causes of plant diseases underwent changes due to the availability of information, efforts to contain the diseases were made constantly to reduce the losses. Several ancient conventional practices were adopted to preserve soil fertility. Crop rotation, application of green manures, tillage and irrigation methods had been followed with a view to reducing disease incidence by increasing the period of absence of susceptible crops, encouraging the microbial activities and altering soil moisture conditions to make them unfavorable for pathogen development (Grigg 1974; Katan 2010). Injuries to branches and stems of plants due to pruning were protected with a mixture of cow dung and urine to prevent infection of apple canker (Austen 1657) indicating that the points of pathogen entry had to be sealed with protectants.

Antagonism of one microorganism by another microorganism was observed by many earlier researchers. But the practical utility of this phenomenon became a reality only after the discovery of Alexander Fleming (1929). Production of penicillin and its application in medicine provided great impetus to initiation of numerous studies on antagonists of plant pathogens. Investigations to determine the usefulness of application of antagonistic fungi for the suppression of damping-off of pine seedlings (Hartley 1921) and potato scab disease (Millard and Taylor 1927) appear to be the pioneering attempts that laid the foundation for the development of biological management as a feasible strategy. Mycoparasitism of the pathogen *Rhizoctonia solani* by *Trichoderma (Gliocladium) virens* was first reported by Weindling and Fawcett (1936). The evidence for the existence of soil suppressive to soilborne pathogens was obtained by Henry (1931) and the transferability of microbiota responsible for soil suppressiveness to conducive soil was demonstrated by Shipton et al. (1973). The role of ectomycorrhizal fungi in increasing the resistance of pine plants against *Phytophthora cinnamomi* was investigated by Marx (1969). The possibility of commercial exploitation of cross-protection phenomenon for

reducing the incidence of *Citrus tristeza virus* (CTV) was demonstrated in Brazil by pre-immunization of susceptible plants with attenuated strain of CTV (Costa and Miller 1980).

Antagonistic microorganisms could compete with and inhibit the development of the microbial plant pathogens present in the soil by producing antibiotics. The plant growth-promoting rhizobacteria (PGPRs), *Pseudomonas* spp. and *Bacillus* spp. are excellent antibiotics producers. Hence, they may have the potential for suppressing the development of crop diseases caused by microbial pathogens (Cook and Rovira 1976). Fluorescent *Pseudomonas* spp. are present abundantly in the rhizosphere and their ability to protect plants against microbial plant pathogens and to promote growth of plants has been demonstrated. *Bacillus* spp. produce endospores that are resistant to adverse environmental conditions, facilitating their survival for long periods. These bacteria produce different kinds of antibiotics, siderophores and growth-promoting compounds that could have a major role in disease-suppressive and growth-promotion effects on plants treated with them. Many PGPRs are able to transcend the endodermis barrier, cross the root cortex to the vascular system and subsequently thrive as endophytes in stem, leaves, tubers and other organs (Bell et al. 1995; Hallman et al. 1997).

1.3 Nature and Characteristics of Biological Control Agents

Biological control or biocontrol agents (BCAs) effective against diseases caused by microbial plant pathogens – oomycetes, fungi, bacteria, phytoplasma, viruses and viroids – may be divided into two groups: (i) biotic agents and (ii) abiotic agents. They exhibit varying degrees of biocontrol potential depending on the host-pathogen combination and the environmental conditions in the geographical location concerned.

1.3.1 Biotic Biological Control Agents

Biotic biological control agents include living fungi, bacteria and viruses that have inhibitory effects on the microbial pathogens through various mechanisms of action such as antagonism, competition for nutrients and niches, prevention of colonization of host tissues by the pathogen and induction of resistance in plants against the diseases to be controlled. The fungal biocontrol agents belong to different taxonomic groups and they exist in the form of several strains, varieties or races which differ in their biocontrol potential. Various methods based on the biological, biochemical, immunological and genetic characteristics of the biocontrol agents are employed to identify and differentiate the strains of fungal BCAs with high biocontrol activity. The assessment of biocontrol activity is performed in the laboratory, greenhouse and under field conditions. Similar assessments are carried out in the case of

mycorrhizal fungi also (Chap. 2). The fungal BCAs possess one or more mechanisms of biocontrol activity against target pathogens. They may produce chitinolytic enzymes and antibiotics that effectively inhibit the pathogen development. They may also compete with the pathogens for available nutrients and also prevent the host tissue colonization by the pathogen. In addition, some of them indirectly act on the pathogen by inducing resistance in host plants and increase the plant growth as well (Chap. 3).

The bacterial biocontrol agents are less variable in their morphological characteristics compared with the fungal biocontrol agents. Hence, several biochemical, physiological, immunological and nucleic acid-based techniques are required for their characterization and identification of the strains or isolates that are the most efficient in suppressing the development of the microbial plant pathogens and diseases symptoms induced by them. Metabolic fingerprinting and polymerase chain reaction-based techniques have been found to be more useful for the identification and monitoring their population after application in the greenhouse and field. The genes governing biosynthesis and their expression during the interaction between the biocontrol agents and pathogens have been studied by employing nucleic acid-based techniques. The assessment of biocontrol activities of bacterial BCAs have been performed in in vitro, greenhouse and field conditions by employing a wide range of methods (Chap. 4). The antagonistic activities of the bacterial biocontrol agents depends on their ability to produce various kinds of antibiotics, biosurfactants, volatile organic compounds, cell wall-degrading enzymes (CWDEs) and other toxic compounds that suppress the development of microbial pathogens. Production of siderophores to interfere with iron utilization by the pathogens and ability to colonize the plant tissues and niches aggressively denying access to the pathogens have been shown to be other mechanisms of biocontrol activity of some bacterial BCAs to suppress pathogen development. Systemic acquired resistance (SAR) and induced systemic resistance (ISR) are routes of enhancing host plant resistance by the bacterial BCAs especially, plant growth-promoting rhizobacteria (PGPRs). The PGPRs have the ability to enhance the plant growth, in addition to protection to the plants against diseases caused by microbial pathogens in various crops (Chap. 5).

As the viruses can infect all organisms including humans, the possibility of employing viruses as biological control agents against fungal and bacterial plant pathogens has been explored. The phenomenon of hypovirulence involves infection of fungal pathogens whose virulence is progressively reduced and consequent reduction in severity of disease induced by the fungal pathogens. Several mycoviruses have been characterized and classified into three families. Bacteriophages, the viral pathogens of bacteria have been shown to have specificity of infection of specific bacterial plant pathogens. Bacteriophages specific to *Xanthomonas* spp., *Erwinia* spp. and *Ralstonia solanacearum* have been isolated and their efficacy has been well demonstrated. Mild strains of a virus is able to cross-protect susceptible plants against severe strains of the same or related viruses. Naturally occurring mild strains and attenuated strains have been successfully employed to reduce the incidence and/severity of diseases like *Citrus tristeza virus* (CTV) and *Papaya ringspot virus*

(PRSV). Engineered mild strains of PRSV have been employed to provide high level of protection to papaya cultivars against severe strains of PRSV (Chap. 6). In order to improve the performance of selected species or strains of the fungal, bacterial and viral biocontrol agents, different approaches such as selection of efficient strains from naturally occurring variants, induction of mutation in specific genes of the BCA, transformation, protoplast fusion, antibody-mediated protection, production of nonpathogenic mutants and engineering mild strains of viruses for cross-protection and utilization of pathogen-derived resistance (PDR) have been adopted with varying degrees of success (Chap. 7).

1.3.2 Abiotic Biological Control Agents

Abiotic biological control agents are derived from diverse sources of organic and inorganic origin. They may be applied to the soil, seeds, plants and harvested produce to reduce the incidence and severity of diseases caused by microbial pathogens. Organic amendments such as composts, green manures, vegetable wastes etc., may contain antagonistic organisms or they may differentially favor the development of antagonistic microorganisms, in addition to improving the soil fertility. Plant extracts and secondary metabolites like essential oils have been shown to have high level of disease-suppressive activity. A few of them are available as commercial products. The glucosinolates from the members of the family Cruciferaceae have been demonstrated to suppress the development of soilborne pathogens. Chitosan, derived from the crab shell, stimulates the proliferation of the antagonists of soilborne pathogens and it could induce resistance in plants against several pathogens including those causing postharvest diseases. Synthetic organic compounds such as salicylic acid, acibenzolar-*S*-methyl (ASM), benzothiadiazole (BTH) and ß-aminobutyric acid (BABA) have been used for treating seeds and plants. The extent of protection provided by them depends on the concentration, frequency of application and the host-pathogen combinations. They appear to induce resistance in plants against various diseases affecting plants and harvested produce. Among the inorganic compound, phosphate salts and silicon have given promising results and the consistency of their performance has to be ascertained under field conditions (Chap. 8).

The information presented in this book in an easily understandable style has been distilled from an extensive literature search to include latest findings and concepts on biological management of crop diseases. Various aspects of biotic and abiotic biological control agents and the crop production strategies that have significant impact on the success of crop disease control are discussed describing some case studies in detail. The information presented in this book is expected to provide a comprehensive knowledge to the upper level undergraduate and graduate students. Researchers and teachers in the Departments of Plant Pathology, Plant Protection, Microbiology, Molecular Biology, Botany, Ecology, Agriculture and Horticulture will find the information presented in the book to be useful for furthering their

research investigations. Incorporation of several protocols appended at the end of relevant chapters to assist the researchers in planning their experiments is a unique feature of this book.

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Chapter 2
Detection and Identification of Fungal Biological Control Agents

Agricultural and horticultural crops and silvicultural plantations are affected in various ecosystems by different abiotic and biotic stresses. The extent of loss suffered, depends on the nature of the causes, and existence of favorable environments and disease-inducing potential of the cause(s) in a particular geographical location. In the case of biotic causes, microbial pathogens have a predominant role in adversely affecting crop production systems, leading to huge losses in quantity and quality of the harvested produce. Numerous studies have been carried out to assess the pathogenic potential of microbial plant pathogens, to determine extent of variability of microbial plant pathogens and to effectively contain the spread of the diseases caused by them, in order to minimize the losses and to sustain the production systems. Among the various short- and long- term strategies for the development of effective disease management systems, the usefulness of biocontrol agents (BCAs) in reducing the incidence and intensity of diseases caused by microbial plant pathogens has been demonstrated by several studies (Narayanasamy 2002, 2011).

Biocontrol agents effective against diseases caused by microbial plant pathogens may be divided into two groups: (i) biotic agents and (ii) abiotic agents. Biotic biocontrol agents are the nonpathogenic living organisms that exhibit antagonistic potential against microbial pathogens – oomycetes, fungi, bacteria, phytoplasmas, viruses and viroids – that cause numerous economically important diseases in various agroecosystems. They are able to suppress the pathogen development through one or more mechanisms. Abiotic agents are derived from inorganic or organic sources. The abiotic agents may also act directly on the microbial pathogens by inhibiting their growth and/reproduction or indirectly act on the host plants by activating their defense systems. They may be classified based on their chemical constitution or on the mode of action on the test microorganisms. Some of the abiotic agents may exhibit direct and indirect mechanisms of action on the microbial pathogens. A few of the abiotic agents have been synthesized for application against the microbial pathogens and are available as commercial products.

Microbial plant pathogens are known to exist in the form of several races, biotypes and strains that differ in their pathogenic potential, sensitivity to chemicals

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and environmental conditions. Likewise, the biocontrol agents that can be employed against microbial pathogens with the aim of reducing the incidence and severity of diseases caused by them, do exhibit variations in their biocontrol efficacy. In the case of biotic agents, strains or subspecies of the same morphologic species have been found to vary in their biocontrol efficacy, due to their differing sensitivity to environmental conditions, adaptations to new locations, rhizosphere competence and nature and quantity of antibiotics, enzymes or other toxic metabolites produced by them. It is, therefore, essential to establish the precise identity of the biotic biocontrol agents to compare the results obtained at different locations and periods after application of the BCA under testing/observations. Various biological, biochemical and genomic characteristics are determined to detect, identify and differentiate the biotic BCAs and their strains or isolates. Following identification of the fungi isolated, their biocontrol potential has to be assessed simultaneously by applying different screening methods performed in in vitro, greenhouses and fields to select the most efficient species/strains. The relative usefulness of different characteristics of BCAs in establishing their identity is discussed in this chapter.

2.1 Methods of Detection and Identification

Fungal biocontrol agents (BCAs) are present in various habitats like soil, air and different organs/tissues of plants. The BCAs have to be isolated and maintained in suitable culture media prior to their identification. Unculturable BCAs are maintained on natural live hosts/plants.

2.1.1 Cultural Methods

Fungal biocontrol agents are isolated using appropriate medium that favors its development and providing required environmental conditions. Many BCAs may develop well in media used for isolation of fungal plant pathogens. But some may need selective medium that favors the BCA differentially. *Trichoderma* spp., although considered as soil fungi, could be isolated from the above ground plant tissues of *Theobroma cacao* (cocoa) as endophytic organism. They are reisolated from surface-sterilized cocoa stem tissues, including the bark and xylem, apical meristem and to a lesser degree from leaves. Scanning electron microscopic observations showed the colonization of glandular tips on the surface of sterilized stems of cocoa seedlings inoculated with *T. harzianum* (DIS 219f), *T. hamatum* (DIS219b), *T. ovalisporum* (Dis 70a) and *T. koningiopsis* (Dis 172ai). These fungi could enter glandular trichomes during colonization of cocoa stems, allowing systemic colonization of cocoa tissues (Bailey et al. 2009).

Some of the general and selective media used for isolating different fungal BCAs are presented in Appendix 2.1. Fungal BCAs have to be provided optimum environmental conditions for their mycelial growth and sporulation. Temperature and pH are the important factors necessary for their development and production

of enzymes, antibiotics and toxins that may be involved in the biocontrol activity against the target phytopathogens. After allowing sufficient period, the fungi developing in the media are examined under light microscope to record their morphological characteristics of asexual reproductive structures like sporangia, conidia and spore-bearing structures like acervuli and pycnidia. The presence of sexual spores, if any, is also recorded. Identification based on morphologic characteristics requires considerable experience in and knowledge of classical taxonomy. The fungal BCAs are distributed in different classes, families and genera of fungi. Some of them are placed in the same genus or families that include phytopathogenic fungi also. Hence, more precise techniques depending on characteristics other than morphologic characters are also required for precise identification of fungal BCAs. A semi-selective medium for the isolation of the fungal BCA *Penicillium oxalicum* was developed. The antibiotic nystatin (0.0.06 g/l) included in the medium inhibited the growth of all fungi except *P. oxalicum*. The selectivity of the medium PoIM was evaluated by identifying the types of naturally occurring microorganisms in natural soils. Potato dextrose agar (PDA) recovered yeasts, mycelial fungi and bacteria, while PoIM supported only the growth of a few yeasts and bacteria and some fungi that could be identified by colony characters. Use of the PoIM semiselective medium in conjunction with the polymerase chain reaction (PCR) assay resulted in accurate and reliable detection and identification of *P. oxalicum* (Larena and Melgarejo 2009a, b).

Isolation and quantification of microorganisms by plate count may be useful to measure soil biodiversity. The plate count technique has certain advantages over cultivation-independent methods like facilitation for taxonomic, genetic and functional studies on isolated microorganisms. *Trichoderma* spp. and *Gliocladium* spp. are not only important as biocontrol agents but also as inducers of greater plant growth. The efficiency of different culture media for the isolation of *Trichoderma* spp. and *Gliocladium* spp. was assessed. Potato dextrose agar medium amended with Rose Bengal, chloramphenicol and streptomycin sulfate was found to be the most effective for the isolation of these fungal BCAs in soil samples (Gil et al. 2009). Following isolation of potential biocontrol agents to be applied for the control of phytopathogens, screening of the fungal isolates is carried out to assess their biocontrol activity in vitro generally, along with tests to establish their identity and relatedness to strains already available. Morphologic characteristics of some of the more intensively studied fungus-like and fungal BCAs, that may be useful for the identification (Cook and Baker 1983) are briefly described.

1. ***Pythium oligandrum***
   Class : Oomycetes; Genus : *Pythium*; Order : Peronosporales; Family : Pythiaceae.
   Sporangia are either terminal or intercalary; subspherical, 25–45 μ in diameter. Zoospores (20–50) are formed in thin-walled vesicles that release them on maturity. Zoospores are longitudinally grooved, reniform and biflagellate. The sexual spores, oospores are hyaline or yellow, subspherical with thick wall that bear spiny pointed protuberances. The oospores are long-lived and highly resistant to adverse environmental conditions. The BCA overwinters as oospores which germinate when favorable conditions are available.

2. ***Ampelomyces quisqualis***
   Class: Deuteromycetes; Family: Dematiaceae; Genus: *Ampelomyces* (earlier known as Cicinnobolus).

   Asexual spores are formed in superficial pycnidia with thin walls. Conidia are non-septate, pale brown, thin-walled and smooth. They may be straight or curved or cylindrical or fusiform in shape. No sexual spore is formed in general.

3. ***Cladosporium cladosporioides***
   Class: Deuteromycetes; Family: Dematiaceae; Genus: *Cladosporium* (sexual stage: Mycosphaerella).

   Asexual spore-bearing structures, conidiophores are erect and pigmented producing irregular branches at the apical ends. Conidia are hyaline (colorless) or pigmented, smooth or rough ellipsoidal and non-septate and they are formed in chains.

4. ***Coniothyrium minitans***
   Class: Deuteromycetes; Family: Sphaeropsidaceae; Genus: *Coniothyrium*.

   Globose pycnidia are superficial and smooth and they are covered by a black carbonaceous envelope. Pycnidiospores are released through the ostiole (opening) as black slimy mass. Individual spores are brown, elliptical and smooth. Pycnidia are formed inside the sclerotia of the fungal pathogens like Sclerotinia sclerotiorum and *Botytis cinera.*

5. ***Fusarium oxysporum***
   Class: Deuteromycetes; Family: Tuberculariaceae; Genus: *Fusarium*.

   The fungi in this genus produce three kinds of spores in the asexual phase of their life cycle. Microconidia are single-celled, hyaline, smaller in size, oval-ellipsoidal, cylindrical or straight and produced abundantly. Macroconidia are larger in size, thin-walled, sickle-shaped with 3–6 cells, pointed at both ends. They are produced on conidiophores formed in specialized structures known as sporodochia. Chlamydospores are formed due to thickening of certain cells of the vegetative hyphae. Thick-walled chlamydospores are either terminal or intercalary and are resistant to adverse environmental conditions. Non-pathogenic strains are present along with the pathogenic strains in the same niche in roots, stems and other plant tissues.

6. ***Gliocladium virens***
   Class: Deuteromycetes; Family: Moniliaceae; Genus: *Gliocladium* (perfect stage: Hypocrea gelatinosa).

   Frequently the genus *Gliocladium* has been misidentified as *Trichoderma* spp. The conidiphores form side branches at the apical ends and they produce flask-shaped phialides on which conidia formed. The conidia are smooth and elliptical in shape. Thin-walled, globose chlamydospores are also formed facilitating overwintering of the BCA.

7. ***Myrothecium verrucaria***
   Class: Deuteromycetes; Family: Tuberculariaceae; Genus: *Myrothecium.*

   Branched conidiophores are present in large numbers in the sporodochium. Black mass of conidia surrounded by white floccose margin is seen in the

sporodochium. Individually conidia are fusiform with one pointed end and another end protruding, fan-tailed and truncate.

8. ***Penicillium oxalicum***
   Class: Deuteromycetes; Family: Moniliaceae; Genus: *Penicillium*.
   Long brush-like conidia bearing conidiophores have branches that end in sterigmata. Conidia are borne on the sterigmata in chains. They are smooth and elliptical in shape.

9. ***Phialophora graminicola***
   Class: Deuteromycetes; Family: Dematiaceae; Genus: *Phialophora* (perfect stage: Gaeumannomyces).
   Conidiophores are hyaline to brown and have branches which end in flask-shaped phialides. Hyaline or slightly yellowish single-celled conidia are produced from the phialides.

10. ***Sphaerellopsis filum (syn: Darluca filum)***
    Class: Deuteromycetes (perfect stage: Eudarluca caricis); Family: Dematiaceae; Genus: *Sphaerellopsis*.
    Specialized spore-bearing structures, pycnidia have ostiole (opening) through which conidia are released. Septate hyaline to pale brown conidiophores with phialides at the terminal end are formed from the inner walls of pycnidia. Hyaline to pale brown conidia with a septum and elliptical in shape are borne on phialides.

11. ***Trichoderma viride (perfect stage: Hypocrea rufa)***
    Class: Deuteromycetes; Family: Moniliaceae; Genus: *Trichoderma*.
    Many species of *Trichoderma* have been reported to be effective against several microbial plant pathogens. Some isolates of *T. viride* were earlier identified as *Gliocladium virens* and they produce gliotoxin and viridin. *Trichoderma* spp. are effective mycoparasites of fungal pathogens. Conidiophores are erect, septate and branched, producing phialides at the terminal ends. Conidia formed from phialides are single-celled, green, globose or ovoid in large numbers often gathering to form ball-like groups. Chlamydospores may be intercalary or rarely terminal and help in the perpetuation of the fungus during adverse conditions.

12. ***Trichothecium roseum***
    Class: Deuteromycetes; Family: Moniliaceae; Genus: *Trichothecium*.
    Hyaline and septate conidiophores formed singly or in groups are erect, straight or flexuous, simple or branched. Hyaline, smooth, oblong two-celled conidia are produced on the conidiophores in fragile chain-like cluster.

13. ***Verticillium lecanii***
    Class: Deuteromycetes; Family: Dematiaceae; Genus: *Verticillium*.
    Erect, septate conidiophores bear solitary or whorls of 3–4 phialides. Conidia are cylindrical or ellipsoidal are grouped as parallel bundles or heads.

Biological or conventional methods of identification of BCAs in plants or soil require isolation of organisms in pure cultures followed by microscopic examination

to determine morphological characteristics of reproductive structures which may be produced after several days or even weeks. Thus, results may be available after lapse of considerable interval after receiving the samples. Further, identification of fungal BCAs based on morphological characteristics may lead to erroneous conclusions, because of the similarity in morphological characteristics of isolates that differ in the biocontrol efficacy. The imperative need for additional tests can be clearly understood by the results of several studies. *Pythium contiguanum* (= *P. drechsleri*) isolated from salt-marsh in Algeria was characterized by its contiguous inflated type of contiguous sporangia, smooth- walled oogonia and mostly monoclinous antheridia. It produced both spherical and sickle-shaped appressoria. Since the fungus with its earlier name *P. drechsleri* is known to be a phytopathogen, it was necessary determine the molecular characteristics, in addition to morphologic characteristics recorded. *P. contiguanum* was morphologically very similar to *P. periilium*. The ITS1 sequences of the nuclear ribosomal DNA of *P. contiguanum* showed homogeneity of 92 % to *P. periilium*, 86 % to *P. torulosum* and 78.5 % to *P. vanterpoolii*. Based on the differences in the nucleotide sequences, *P. contiguanum* was described as a new taxon. This BCA was found to be mycoparasite on *Botrytis cinerea* causing gray mold disease of grapevine. Further, *P. contiguanum* was not pathogenic to grapevine (Paul 2000). Another species *P. bifurcatum* isolated from wheat field was also found to be a mycoparasite of *B. cinerea*. The antheridial cells of *P. bifurcatum* are sometimes bifurcated into two cells earning the name for this fungus. Based on the differences in the sequences of ITS region of nuclear ribosomal DNA of this fungus, it was considered to be a new species of *Pythium* (Paul 2003).

Pseudozyma flocculosa, an effective antagonist of powdery mildew pathogens infecting greenhouse crops, was initially classified under the genus *Sporothrix* (Traquair et al. 1988). Some species of *Sporothrix* like *S. schenkii* are known to be human pathogens (Bennett 1990). Hence, the registration of *P. flocculosa* had to overcome the serious concern expressed by governing authorities. The objections and doubts raised could be resolved by the physiological, biochemical and molecular studies. The information provided by these studies particularly based on the sequence analysis of the larger nuclear subunit of rDNA, conclusively established that this fungal BCA species should be reclassified under the genus *Pseudozyma* (Boekhout 1995). Further studies using molecular markers rDNA for identification of *P. flocculosa* confirmed that *P. flocculosa* was genetically distinct from *Sporothrix*. By employing the molecular markers, specific isolates could be selected for efficiency as biocontrol agents (Avis et al. 2001). Hence, the conventional methods are now frequently complemented by biochemical and/molecular methods to allow classification of the new BCA into existing or new taxa.

2.1.2 Biochemical Methods

In order to enhance the dependability and level of accuracy of identification of fungal BCAs, biochemical methods have been successfully applied. These methods may be useful to differentiate the isolates or strains of the same morphologic species

or closely related species based on the variations in the biochemical constituents or nature of enzymes or metabolites produced by them.

Pythium oligandrum, a biocontrol agent, possesses multiple modes of action through direct and indirect effects (stimulation of defense reaction and growth promotion in host plants). The strains of this BCA produce oospores to allow root colonization favoring persistence and synthesize tryptamine (plant growth enhancer) and oligandrin (plant protective elicitor). Production of tryptamine was determined by capillary electrophoresis method. Formation of oligandrin was detected by Western blotting technique. The strains of *P. oligandrum* produced tryptamine in the presence of tryptophan, a precursor of indole-acetic acid (IAA) and metabolism. The strains LMS 1.01.631 and CBS 530.74 produced high amounts of tryptamine. Rabbit anti-oligandrin serum recognized the protein (oligandrin) in the cultures of *P. oligandrum* strains, but not in the cultures of other *Pythium* spp. tested (Vallance et al. 2009). The non-target effects of *Clonostachys rosea* (IK726) and *Pseudomonas fluorescens* (DR54) were assessed after introducing them by seed and soil inoculation. The soils were assayed for the BCAs and different features of the indigenous soil microbiota. *C. rosea* and *P. fluorescens* declined by a factor 20 and 10^6 respectively after a period of over 190 days. In general, the non-target effects were small and transient. Phospholipid fatty acid (PFA) analysis indicated the perturbations of the soil microbial community structure following the treatments with the BCAs and also reversal of altered conditions to status quo ante reflecting the decline of introduced BCA populations. The PFA seems to be useful for in situ monitoring of BCA non-target effects on the soilborne microorganisms (Johansen et al. 2005).

Trichoderma spp. are well distributed globally in soils and organic matter. Many species are used as biocontrol agents and are also used for the production of cellulases and other enzymes. They produce many secondary metabolites including mycotoxins (Sivasithamparam and Ghisalberti 1998). Fungal strains have been differentiated by applying chemical image analysis techniques to chromatographic matrices of crude fungal culture extracts separated by high performance liquid chromatography (HPLC) with diode array detection (DAD). *Trichoderma* strains (44) were characterized by secondary metabolite profile using HPLC-DAD. The strains except one could be correctly assigned to *T. atroviride* (9 strains), *T. viride* (3 strains), *T. harzianum* (10 strains), *T. citrinoviride* (12 strains) and *T. longibrachiatum* (9 strains). One strain of *T. hamatum* was identified by rDNA sequencing technique which confirmed the identity of 43 strains identified by HPLC-DAD technique (Thrane et al. 2001). Antagonistic activity of the fungal BCAs may be associated with the abilities to produce cell-wall-degrading enzymes (CWDEs). β-1,3-Glucanase is an important CWDE involved in mycoparasitism of *Trichoderma* spp. A simple microplate-based method was used to assay the activity of β-1,3-glucanase from different isolates. This microassay required only 130 µl as against 1,150 µl of the enzyme needed for the standard β-1,3-glucanase macroassay in 20 isolates of *Trichoderma* spp. The results indicated that the optimized microassay developed in this study could be used to screen the isolates of *Trichoderma* spp. and select the most efficient producers of β-1,3-glucanase that may be effective in controlling the target pathogens (Ramada et al. 2010). Identification of *Trichoderma*

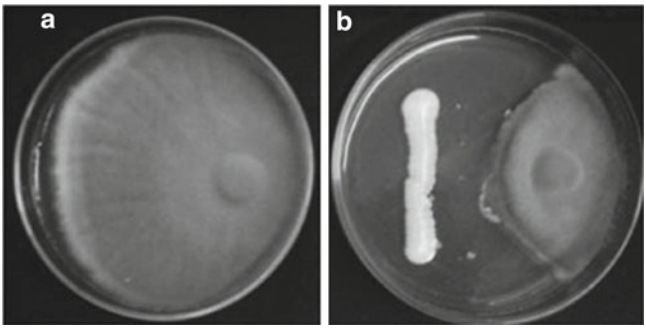

Fig. 2.1 Inhibition of mycelial growth of *Colletotrichum sublineolum* on potato dextrose agar (PDA) by the yeast *Torulapspora globosa* (Courtesy of Rosa et al. 2010 and with kind permission of Springer Science + Business B.V., Heidelberg, Germany)

atroviride based on the profiles of volatile metabolites was attempted. A solid phase microextraction (SPME) coupled to gas chromatography-mass spectrometry (GC-MS) was employed to profile microbial volatile organic compounds (MVOCs) in the head space of cultures of *T. atroviride.* The MVOCs produced by *Trichoderma* spp. belonged to alcohols, ketones, alkanes, furanes, pyrones, mono – and sesquiterpenes. This procedure detected 13 compounds in the *T. atroviride* cultures that were not associated with other *Trichoderma* spp., indicating the possibility of detection and identification of *T. atroviride* (Stoppacher et al. 2010).

The yeast *Torulaspora globosa* strain IS 112 isolated from sugarcane rhizosphere was found to suppress the development of sorghum anthracnose disease caused by *Colletotrichum sublineolum*. This strain was characterized by its yeast killer activity. Suspensions of the sensitive strain *Saccharomyces cerevisiae* NCY1006 and *Torulopsis glabrata* ATCC 15126 were prepared after cultivation in yeast broth medium containing methylene blue. Aliquots of 100 µl of the suspensions were spread over the culture medium in petridishes and dried. Then this BCA *T. globosa* was inoculated. The petridishes were incubated for 5 days at 30 °C. Production of the killer toxin was inferred from the formation of an inhibition halo and a blue zone (dead cells) surrounding the BCA colony (Fig. 2.1). The results revealed that *T. globosa* strain IS 112 possessed killer activity against the two sensitive yeast species tested (Rosa et al. 2010).

2.1.3 Immunoassays

Interactions between BCAs and their hosts could not be studied, because of the absence of methods that allow unambiguous identification of individual genera in complex environments, when certain mixed populations of fungi interact in soil or compost. Immunoassays have been demonstrated to be useful for the detection,

identification, differentiation and quantification of fungus-like and fungal organisms that are capable of causing plant diseases and also that are capable of inhibiting development of fungal pathogens present in various habitats. Antisera containing antibodies specifically induced against the fungal species have been produced. Polyclonal antisera are produced in the laboratory animals like rabbits, fowls and horses, whereas monoclonal antisera are produced by immunizing mouse. Polyclonal antisera contain polyclonal antibodies (PAbs) that react with many antigenic areas (epitopes) present on the surface of antigen that induce the production of antibodies in the immunized animals. PAbs are less specific, as they cross-react with closely related species of fungi that share common antigenic determinants (epitopes). On the other hand, monoclonal antibodies (MAbs) produced by hybridoma that are formed by the fusion of β-lymphocytes (capable of producing antibodies) and myeloma cells (capable of independent and indefinite multiplication). Thus, a hybridoma produces antibodies that react only with a single epitope on the antigen. Hence, the reaction will be more specific and sensitive, when MAbs are employed, compared with that using PAbs (Narayanasamy 2001, 2011; Appendix 2.2).

Immunoassays based on the precipitin reaction resulting in the formation of visible precipitate following interaction between the antigen and PAbs, require large volumes of antisera. Further, the results will be available only after several days. Hence, in order to enhance the specificity, sensitivity and rapidity of the immunoassays, labeled antibody techniques have been developed. By attaching a label to either antigen or antibody, the sensitivity of detection of antigen-antibody reactions can be significantly increased. The antibodies specific for a microorganism may be labeled with three types of markers (labels) such as enzymes, fluorescent dyes, radioactive or non-radioactive materials. The choice of immunoassays depends on the level of sensitivity, specificity, accuracy and rapidity required for the study. In addition, adaptability to field conditions, relatively inexpensive nature and amenability to testing of large number of samples within short periods are the other desirable attributes considered for selecting the immunoassay concerned (Narayanasamy 2005).

Among the immunoassays, enzyme-linked immunosorbent assay (ELISA) has been very widely employed for the detection and differentiation of fungal biocontrol agents (BCAs) and plant pathogens (Appendix 2.3). Monoclonal antibodies (MAbs) that could react and specifically detect *Ulocladium atrum* effective against the gray mold pathogen *Botrytis cinerea* has been developed. Colonization of dead strawberry leaflets by *Ulocladium atrum* effective against *Botrytis cinerea* was quantified in the presence and absence of the pathogen by employing plate-trapped antibodies (PTA)-ELISA format using specific MAbs. The assessment of *B. cinerea* colonization by PTA-ELISA procedure revealed that amount of *B. cinerea* mycelium was significantly lower (5-folds) in the presence of strain 385 of *U. atrum* after 6 days of incubation. When *U. atrum* strain 385 and *B. cinerea* colonized the leaves simultaneously, colonization of *U. atrum* strain 385, was better in comparison with other *U. atrum* strains. Biocontrol efficacy of the strain 385 was the highest. It appeared that there might be a close relationship between biocontrol efficacy and

level of colonization by both the BCA and the pathogen (Berto et al. 2001). Five murine hybridoma cell lines secreting MAbs with similar binding specificities were raised for detecting *U. atrum* in cyclamen leaves. The MAb UA-PC3 was employed for quantitative estimation of the biomass of *U. atrum* in cyclamen leaves colonized by this BCA. The antigen secreted by *U. atrum* was water soluble and it was present on the surface of conidia and hyphae and secreted into culture fluids. The antigen was produced in greater concentration in vivo than in in vitro conditions (Karpovich-Tate and Dewey 2001).

An elaborate study was carried out, employing different immunoassays for the detection, identification and differentiation of *Trichoderma* spp. with biocontrol potential against several fungal plant pathogens. The enzyme β-1,3-glucanase produced by *Trichoderma* spp. was found to be an ideal candidate for production of MAbs specific to the genus *Trichoderma*. This enzyme is extracellular and produced constitutively or semiconstitutively. Chromatographically purified β-1,3-glucanase from *Trichoderma* spp. was employed for production of MAb MF_2 by a hybridoma cell line. The MAb MF_2 recognized a protein epitope specific to the genus *Trichoderma*. Enzyme-linked immunosorbent assay (ELISA) showed that MF_2 antigen produced by actively growing mycelium of *T. koningii* was constitutive and occurred in the absence of laminarin, a substrate shown to induce production of β-1,3-glucanase. Western blotting analysis showed that MF_2 bound to proteins had molecular masses similar to that of *Trichoderma* glucoamylases and β-1,3-glucanases (Appendix 2.4). Localization of the MF_2 antigen(s) by immunogold electron microscopy indicated that it was expressed in the hyphal cell walls and septa and in the cell walls of phialoconidia. Immunofluorescence (IF) observations on dual cultures of *Trichoderma* and *Rhizoctonia solani* revealed that only the cell walls of *Trichoderma* coiling around the pathogen were stained by MF_2. The specificity of MF_2 resulted in the development of combined baiting-ELISA technique for the detection of *Trichoderma* spp. in naturally infested composts. The phylogenetic analysis based on the sequences of the ITS1-5.8 S-ITS2 rRNA-encoding regions of the isolates confirmed the results of ELISA test using MF_2 developed in this study (Thornton et al. 2002; Appendix 2.5).

2.1.4 Nucleic Acid-Based Techniques

Nucleic acid (NA)-based techniques have been applied for rapid detection and precise identification, differentiation and quantification of microorganisms including microbial plant pathogens and the microorganisms with biocontrol potential against plant pathogens. Culture-dependent methods require long time and the results obtained may be inconclusive. Techniques depending on the hybridization of probes with complementary sequences of target DNA sequences or amplification of sequences of the target DNA fragment of the test organism have been employed in the recent years. Molecular methods of identification and differentiation of BCAs or their strains have higher levels of accuracy, sensitivity, specificity and rapidity.

Sequence information of the internal transcribed spacers (ITS) and ribosomal genes has been useful to develop rapid methods of detecting and differentiating fungus-like and fungal BCAs. The principles and procedures followed for detection, identification, differentiation and quantification of microbial plant pathogens which are applicable to biotic biocontrol agents are discussed in detail in the earlier publication (Narayanasamy 2011).

Precise identification of the BCA and its strains is essentially required as emphasized by the studies on *Trichoderma harzianum (Th)* which has been registered as a biocontrol agent for the management of several crop diseases. However, *Th* is also reported as the incitant of mushroom green mold disease (Ospina-Giraldo et al. 1999). Further, a mycotoxin belonging trichothecene class capable of causing serious human and animal ailments is also produced by some isolates of *Th* (Sivasithamparam and Ghisalberti 1998). A putative trichodiene synthase (*tri5*) gene was isolated for the first time in *Trichoderma.* This gene is required for the biosynthesis of trichothecene. It is possible to identify the isolates of *Th* that have *tri5* gene in their genomes, by employing specific *tri5* primers for a polymerase chain reaction (PCR) procedure. The isolates of *Th* lacking *tri5* gene could be selected and developed as biocontrol agent for commercial use (Gallo et al. 2004). *Trichoderma hamatum* exhibited mycoparasitic relationship with *Sclerotinia sclerotiorum* in the dual plates. Northern blot analysis revealed the presence of *T. hamatum prb1* transcripts in RNA isolated from the overlap region between *T. hamatum* and *S. sclerotiorum*, whereas no *prb1* expression was detected in *T. hamatum* controls. *Prb1* expression was evident in mycoparasitism in samples harvested at 3 and 4 days, after inoculation of plants with the BCA and pathogen (Carpenter et al. 2005).

For the development of a fungal species or strain for commercial purpose, selection of the most effective isolate against the designated pathogen, is the most important requirement. For example, the pycnidial hyperparasites of powdery mildew pathogen are considered to belong to a single species, *Ampelomyces quisqualis*, although differences in morphological and cultural characteristics have been observed. Application of restriction fragment length polymorphism (RFLP) and sequence analysis of the ribosomal DNA (rDNA) ITS showed that high genetic diversity existed among isolates of *A. quisqualis* and it would not be appropriate to group all isolates into one species. These techniques provided a sound basis for the selection of the most effective isolate for developing AQ-10 Biofungicide (Ecogen Inc) for commercial application (Kiss 1997; Kiss and Nakasone 1998). An isolate-specific RFLP marker was identified for *Trichoderma atroviride* (formerly *T. harzianum*) isolate C65 effective against the kiwifruit stem-end rot pathogen *Botrytis cinerea*. A dot blot assay was developed by incorporating the identification marker to facilitate screening of large number of leaf and flower/fruit samples for the presence of the strain C65. In order to enhance the sensitivity of detection, the dot blot assay was employed in conjunction with a *Trichoderma* semi-selective medium. This modified diagnostic assay was useful to track the spread and survival of the strain C65 on kiwifruit leaves in shadehouse and flower/fruit in the orchard over two consecutive growing seasons in New Zealand. The BCA strain was able to survive on both leaves and flower/fruit over the entire growing season (Dodd et al. 2004a, b).

Reliable identification and evaluation of the selected BCA for its biocontrol potential are essential to provide authenticated source of fungal biocontrol agents. The genetic assessment of BCAs is not only necessary, but also desirable to gain required insight into the safe and intended application of the biocontrol agents. The relationship between functional group within *Trichoderma* spp. and their biocontrol activity was determined, based on a combination of physiological, biochemical (enzyme production) and molecular (ITS sequences) criteria. The study indicated that the efficacy of different strains of *T. harzianum* depended on the target pathogen and needed functions for biological control. In addition, the importance of selection of the most efficient strains for control of targeted pathogen was also highlighted (Grondona et al. 1997). *Trichoderma* SKT-1, an effective biocontrol agent of seed-borne pathogens of rice, could not be unequivocally classified, based on the morphological characteristics alone, because of the overlapping features with other *Trichoderma* spp. Hence, the sequences of 5.8S rDNA with ITS region (about 514-bp) of the putative BCA were compared with those of known *Trichoderma* spp. to determine its phylogenetic relationship. Complete identity of the length and sequences of the ITS regions of *Trichoderma* SKT-1 with *T. asperellum* NRRL 5242 was observed. Based on the similarity in the sequences of ITS region, the BCA was identified as *T. asperellum,* indicating the reliability of nucleic acid-based techniques for identification of the efficient strains within the same morphologic species of the BCAs (Watanabe et al. 2005). In another study, *Trichoderma* SKT-1 very effective against the powdery mildew pathogen *Oidium* sp. infecting parsley was identified as *T. asperellum,* based on the sequence similarity of the 5.8S rDNA with ITS region (about 514-bp) of the BCA DNA. The length and sequence of the regions from SKT-1 were entirely identical to those of an isolate of *T. asperellum* NRRL 5242. Thus, the sequence similarity of the ITS region of the rDNA may be a reliable basis for the precise identification of the isolates/strains of biocontrol agents (Koitabashi 2005).

Isolation, based on conventional methods require longer periods and the results may be inconclusive for identification and differentiation of fungal BCAs. A polymerase chain reaction (PCR)-based technique was developed for the detection, identification and quantification of *Trichoderma* spp. in soil samples. A new species *Trichoderma martiale* isolated from sapwood in trunks in cocoa (*Theobroma cacao*) was identified, based on the sequences of translation-elongation factor 1-α (*tef1*) and RNA polymerase II subunit (rpb2). *T. martiale* was a close relative of, and morphologically similar to *T. viride*. This BCA was able to restrict cocoa black pod rot caused by *Phytophthora palmivora* in the in situ field trail (Hanada et al. 2008). The isolates of *Trichoderma* spp. were analysed, based on the ITS1 region of the nuclear rDNA cluster and *tef1*. The isolates GL12, GL13 and Th 23 provided effective protection to bell pepper against *Phytophthora capsici*. The isolates GL12 and GL13 belonged to the same clade as indicated by phylogenetic analysis. The results showed that identification of genetically distinct isolates that have potential for biocontrol activity was more reliable (Roberts et al. 2010).

Penicillium oxalicum is a potential BCA effective against tomato pathogens like *Sclerotinia sclerotiorum, Botrytis cinerea, Phytophthora parasitica, P. infestans* and

Verticillium spp. *P. oxalicum* strain Py-12 effective against *S. sclerotiorum* was identified by employing the PCR assay. The ITS region of rDNA was amplified by the PCR with two universal primers specific to ITS1 and ITS4. The PCR product was ligated into the plasmid vector pUCm-T and cloned in *Escherichia coli* and sequenced. The sequences of the amplified fragment showed 100 % homology with the ITS sequence of *P. oxalicum.* Thus, the strain PY-1 was conclusively identified as *P. oxalicum* (Yang et al. 2008). *Pythium paroecandrum* isolated from wheat field suppressed the growth of *Botrytis cinerea* causing gray mold disease of grapevine. Using specific primers, the ITS region of rDNA of *P. paroecandrum* was amplified in a PCR assay and the PCR product was sequenced. The ITS region had more than 900 bases and it showed similarity to *P. cylindrosporum* (95.6 %), *P. regulare* (95.6 %) and *P. sylvaticum* (94.8 %). The morphological characteristics and molecular analysis showed that *P. cylindrosporum* was the closest relative to *P. paroecandrum* (Abdelghani et al. 2004). *Pythium lycopersicum* isolated from field soil samples showed morphological similarity to *P. ornamentatum*. The ITS region of the nuclear rRNA of *P. lycopersicum* resembled many of the mycoparasites belonging to the genus *Pythium* such as *P. oligandrum*. The sequences of the ITS regions of *P. lycopersicum* and *P. oligandrum* exhibited homogeneity of 97.8 %. The ITS region of *P. lycopersicum* was probably the smallest with 761 bases and showed 11 major differences with *P. acanthicum,* the most closely related species (98.3 % homogeneity). Variations in morphological and molecular characteristics are considered to justify the creation of species status to this BCA. *P. lycopersicum* acted on *Botrytis cinerea* as mycoparasite (Karaca et al. 2008).

Suppressive soil sites are known to prevent the development of Fusarium wilt disease of banana, although the pathogen *Fusarium oxysporum* f.sp. *cubense* (FOC) is present, suggesting the presence of deleterious biotic or abiotic factors. In order to find out the role of antagonistic fungi in disease suppression, endophytic *Fusarium oxysporum* isolates obtained from suppressive soils were genetically characterized and compared with the characteristics of an isolate of *F. oxysporum* earlier identified as being antagonistic to FOC infecting banana. One isolate (BRIP 29089) showed significant antagonistic activity and reduced the disease severity of wilt disease in cvs. Lady finger and Cavendish. Another isolate (BRIP4592) increased the wilt disease severity. The results revealed the possibility of nonpathogenic isolates of a pathogenic species aggravating the disease symptoms instead of being ameliorative in nature. It is essential that the identity of the isolates of BCAs has to be established unequivocally for use in disease control (Forsyth et al. 2006). *Pythium oligandrum* colonizes roots without damaging host plant cells and survives in the rhizosphere, where it functions as a biocontrol agent through different mechanisms like mycoparasitism, antibiosis and competition for nutrients and space and promotion of plant growth. Real-time PCR assay was employed to detect the presence and persistence of *P. oligandrum* in the rhizosphere of tomato plants grown in soilless culture. *P. oligandrum* was detected after its inoculation and its population increased throughout the growing season. The amount of *P. oligandrum* was significantly higher than on control plants which were virtually free of the BCA (Vallance et al. 2009).

An endophytic fungus *Piriformospora indica* has been reported to increase the biomass of several plant species colonized in the roots by the fungus. The effect of *P. indica* on wheat was assessed in greenhouse and field conditions. The presence and persistence of *P. indica* in wheat roots was determined by a sensitive PCR procedure. The annotated sequence (AJ459235) of the β-tubulin gene of *P. indica* was used to design the specific primers and to develop the PCR protocol for the detection of the fungal endophyte. In the root DNA samples from inoculated wheat, *P. indica* could be detected at 7 days after inoculation and it persisted in the root tissues for 3 months. DNA isolated from wheat roots in sand yielded more intense bands than those from soil, suggesting more intensive colonization in sand substrate. Disease severity due to stem base disease caused by *Pseudocercosporella herpotrichoides* was significantly reduced by *P. indica* (Serfling et al. 2007). In another study, primers were designed to amplify a fragment of approximately 540-bp, comprising the internal transcribed spacer region 1 (ITS2), 5.8S rDNA and ITS2 from all taxonomic clades of the genus *Trichoderma*. The ITS regions of strains tested, were amplified, but none of the non-*Trichoderma* origin was positive in the tests. PCR with community DNA from soil also yielded products of expected size. All amplified sequences originated exclusively from *Trichoderma* spp. mainly being the representatives belonging to Hamatum, Harzianum and Pachybasioides clades. Most of these species are known to have biocontrol potential against microbial plant pathogens (Hagn et al. 2007). *Gaeumannomyces* spp. and *Phialophora*-like anamorphs constitute the G-P complex associated with cereal roots and they are involved in the incidence of take-all disease infecting wheat, barley, oat and corn. Molecular techniques have been applied for detection and differentiation of the components of the G-P complex. A complete sequence of the ITS1-5.8S-ITS2 rDNA region of rDNA from *G. graminis* var. *tritici* (*Ggt*) and unknown G-P complex isolates were amplified with primers psn DNA2 p and pITS4, yielding a single fragment of about 650-bp. An alignment of ten DNA sequences, six from the unknown G-P complex isolates and four from *Ggt* showed 99 % sequence similarity. Based on the sequence similarity, the unknown G-P complex isolates were identified as *Phialophora* spp. The *Philaophora* spp. isolates were less sensitive to 2,4-diacetylphloroglucinol (2,4-DAPG) than *Ggt* causative agent of wheat take-all disease. The results suggested that *Phialophora* spp. might work in concert with 2,4-DAPG producers like *Pseudomonas* spp. to suppress the wheat take-all disease (Kwak et al. 2010).

A reliable source of authenticated fungal BCA strains has to be maintained to ensure the consistency of the performance of the BCA both in vitro and in vivo. Specific DNA markers that allow authentication of strains and permit monitoring of contamination likely to occur over the years due to mutation or lack of stringent quality control process have been identified. Universally primed polymerase chain reaction (UP-PCR) technique has been employed to differentiate *Trichoderma* strains. Visual inspection of aligned PCR banding profiles may be used as the basis of species identification. If the profiles differ significantly from relevant reference or type strains, UP-PCR products may be tested in hybridization experiments. Cross-hybridization of UP-PCR products resembles traditional DNA/DNA hybridization

by facilitating investigation of sequence complementarity. The hybridization signals of UP-PCR products may indicate the genetic relatedness of the isolates to the reference isolate. As DNA/DNA hybridization between even closely related fungal species is low, this technique may be a useful tool for species designation and establishing extent of genetic relatedness. BCA-specific primers that amplify diagnostic sequences of the BCA concerned are employed in the universally primed polymerase chain reaction (UP-PCR) format. Single 15-20-base pairs (bp) long primers that produce multiple amplification products are employed. As this test does not require DNA sequence data of the test organism, it can be used to resolve more conclusively similarity of *Trichoderma* strains. The UP-PCR assay was found to be a powerful tool useful for monitoring strain of BCAs of interest, in addition to identification of commercially important *Trichoderma* strains (Lübeck et al. 1999).

Strains of *Trichoderma* spp. that form the active component of commercial products applied for biocontrol of microbial plant pathogens, could be characterized by employing universally-primed (UP)-PCR analysis. Distinct and reproducible fingerprints for various strains of *Trichoderma* spp. were generated by applying appropriate UP primers. The fingerprints permitted differentiation of a collection of other strains of *Trichoderma* spp. as well. UP-PCR analysis was combined with dilution plating method and a semi-selective medium was used to recover *Trichoderma* spp. strains after application in commercial greenhouses. The strains recovered were subjected to UP-PCR analysis with one selected primer. The procedure developed in this study was useful to identify the isolates from the biocontrol products applied in different greenhouses. In addition, the presence of a *Trichoderma* strain in the untreated bench in a greenhouse was also detected, indicating the possible spread of the BCA to untreated plants. The UP-PCR assay has the potential for verification of the active component concentration in commercial sample and also for monitoring the establishment and spread of *Trichoderma* strains (Lübeck and Jensen 2002).

Trichoderma isolates (42) collected from the rice fields in the Philippines were characterized by using rDNA-ITS 1 analysis and universally primed (UP)-PCR assay. Two groups of *Trichoderma* were differentiated based on the length and restriction pattern of the ITS region of the rDNA and UP-PCR banding profiles, using UP primer L45. Large majority of the isolates (40) were assigned to *T. harzianum,* because of the similarity in their UP-PCR banding profiles and morphological characteristics. Two isolates were identified as *T. viride* as indicated by rDNA-ITS 1 analysis and distinct UP-PCR banding profiles. One of the isolates exhibited good cellulolytic and competitive saprophytic abilities which are desirable attributes for a BCA. This isolate was distinguished from other isolates by applying single and pair-wise combinations of UP primers. Further, a distinctive diagnostic marker was identified so that it could be applied as a valuable tool for monitoring the isolate in field tests (Cumagun et al. 2000). The endophytes isolated from leaves of Norway maples were found to be antagonistic to *Rhizocotnia solani,* causing black scurf disease of potato. They were identified by sequencing the ITS regions of rDNA and comparing with the sequences of known species of fungi. These endophytes were identified as *T. atroviride*, *Epicoccum nigrum, Alternaria longipes* and *Phomopsis* sp.

Among the endophytic fungi, *T. atroviride* exhibited highest rate of inhibition of *R. solani* in dual culture plates (Lahlali and Hijri 2010). In the further studies, using UP-PCR cross-blot hybridization procedure, delineation of species within the *Trichoderma viride/atroviride/koningii* complex was attempted. Sequences from the ribosomal DNA ITS regions showed limited variation among the species with the *Trichoderma* species complex also known as *Hypocrea rufa* (perfect stage) complex. Infraspecies variation detected, was sometimes greater than intraspecies variation. Strains of *T. viride, T. atroviride, T. koningii, T. asperellum* and their respective teleomorphs (perfect stages) could be defined morphologically and molecularly, suggesting that the species of *Trichoderma* are more separated than indicated by ITS sequence phylogeny. The results suggested that a revision of the species is needed, because of the heterogeneity of *T. viride.* For routine identification of strains of *T. viride,* a macrooarray (DNA chip) containing membrane bound UP-PCR products for all reference strains may be useful (Lübeck et al. 2004).

The biocontrol agents may be introduced into the soil or other substrates at different concentrations and formulations. It is essential to detect the presence and prolification of the introduced BCA in order to relate the presence of BCA to the effectiveness of control of the targeted disease(s). The rhizosphere competence (ability to persist and proliferate) of *T. atroviride* isolate C52 on onion roots was assessed both in the glasshouse and field. The BCA was introduced into the soil as solid-substrate, seed-coating and pellet formulations. The UP-PCR band profile was generated using the primer L45 specific for isolate C52 for identification of the BCA in the samples recovered from the soil. When isolate C52 was introduced into *Sclerotium cepivorum*-infested soil as both pellet and solid-substrate formulations, the effectiveness of disease control was found to be similar. However, the pellet treatment doubled the percentage of healthy plants, compared with control treatment. Pellet formulation maintained *T. atroviride* at a higher concentration than the solid-substrate formulation and onion white rot disease incidence was reduced by 50 %. Proliferation of *T. atroviride* in the rhizosphere appeared to be formulation-dependent. The UP-PCR technique provided conclusive identification of the BCA present in the soil samples (McLean et al. 2005). The dispersal and survival of *Trichoderma atroviride* SC1 in the soils of vineyards in northern Italy were studied after introducing the BCA into the soil in 2 consecutive years. Isolation-dependent method of counting colony-forming units (CFU) and a specific quantitative real-time PCR assay were employed. High concentrations of *T. atroviride* were detected even at 18 weeks after inoculation. A vertical migration up to a depth of 0.4 M was recorded during the first week after inoculation. Horizontal spread of the BCA from the point of inoculation was seen up to 4 M and the population progressively decreased with increasing distance (both vertical and horizontal). *T. atroviride* could colonize the rhizosphere and the grapevine leaves were also colonized by this BCA strain. *T. atroviride* survived for 1 year and dispersed becoming an integral part of the local microbial community under the conditions prevailing in the test location. The BCA strain possessed the attributes of persistence and ability to spread rapidly representing desirable qualities for development as commercial product (Longa et al. 2009).

Population of biocontrol agents introduced into different substrates or soil has to be monitored in order to relate the BCA population to the level of protection obtained by using the BCA concerned. In order to quantify *Trichoderma harzianum* in different growing media, a quantitative (q) real-time (RT)-PCR format was developed. The results of qRT-PCR procedure were compared with the colony counting method. Quantification patterns of an initial rapid increase in the BCA population followed by decrease over time were similar in both methods. However, data from qRT-PCR revealed a population curve of active *T. harzianum* with a delayed onset of initial growth which subsequently increased throughout the experiment. *T. harzianum* was able to develop rapidly in both peat-and compost-based media. Compost amended with *T. harzianum* was more effective in reducing infection rate of *Fusarium oxysporum* and loss of fresh weight of plants, compared with peat amended with the BCA (Beaulieu et al. 2011).

Studies on population dynamics of biocontrol agents in different habitats and in plants have provided useful data on different aspects of plant-pathogen-biocontrol agent interactions. Rhizosphere competence of strains of *Trichoderma atroviride* was studied by employing molecular techniques. Determining ecological fitness of BCAs and quantitative monitoring of populations of *Trichoderma* complex could be achieved by combination of different molecular tools (Hermosa et al. 2001; Dodd et al. 2004a, b; Rubio et al. 2005). Molecular markers like sequence characterized amplified regions (SCARs) allow to differentiate the wild (parent) biocontrol strain from other fungal populations belonging to the same morphological species or genus. *Stachybotrys elegans* effectively suppressed the development of *Rhizoctonia solani* as a mycoparasite. A specific and sensitive polymerase chain reaction (PCR) assay was developed for the detection of *S. elegans* by employing a pair of primers (SE-13F and SE-13R) with sequence characterized amplification regions (SCAR). Both conventional and real-time PCR formats were applied to detect *S. elegans* in pure cultures as well as in field soil samples. A product of 880-bp was amplified by conventional PCR from all isolates of *S. elegans,* but not from other species of *Stachybotrys*, soil fungi or bacteria and plant DNA, indicating the specificity of the assay. *S. elegans* was detected and quantified in field soils at 2 days after its inoculation using real-time PCR with conjugated fluorescent SYBR Green I dye. This assay could detect reliably as little as 150 ng of DNA per g of natural soil. This procedure developed in this study has the potential for studying the distribution of *S. elegans* in the soil under field conditions to assess the effectiveness of biocontrol of *R. solani* by this BCA (Taylor et al. 2003).

Accurate identification of a BCA strain, proposed to be launched on market, at species and strain level is essential. A sequence characterized amplified region (SCAR) specific to T1 strain of *T. atroviride* (Ta) was successfully identified to facilitate monitoring population dynamics of this strain in two soils by applying real-time PCR assay. A primer pair (PF74/PR88) targeting a 141-bp fragment was specific to T1 strain and detected it without any cross-detection of other *Trichoderma* spp. or strains of *Ta* in several nonsterile field soils. This fragment PF741/PR88 had no significant sequence homology with any other known database sequence, further confirming its specificity to T1 strain. The primer set developed in this

study could detect T1 strain both in pure cultures and two different soils artificially infested with T1 strain. It was possible to detect T1 strain, even when the concentration was as low as 1×10^3 CFU/g of nonsterile soil containing other fungal populations. The results of SCAR based tests were similar to those obtained using soil plate dilution method. This molecular assay can be advantageously employed for environmental monitoring, since it is less expensive and more rapid in providing results than time-consuming and labor-intensive conventional isolation-based methods (Cordier et al. 2007).

Gliocladium catenulatum has been employed as a biocontrol agent against soilborne pathogens like *Sclerotinia sclerotiorum.* Randomly amplified polymorphic DNA (RAPD) technique was useful to develop strain-specific primers for *G. catenulatum* strain J1446. This strain could be differentiated from 16 strains of *G. catenulatum* as well as from *Trichoderma virens* and isolates of *Nectria* spp. and *Fusarium* spp. Expected amplification of the target DNA occurred only from DNA samples from treated cucumber leaves and potato tubers. The limit of detection was 5 pg or more of the DNA of strain J1446. Some variations between *Gliocladium* strains were observed from the results of random amplified microsatellites (RAMS) procedure and the universally-primed (UP)-polymerase chain reaction (PCR) assay. But these techniques did not amplify fragment specific to strain J1446. Cross-blot hybridization of UP-PCR products differentiated strain J1446 from *T. virens,* but not from other *Gliocladium* isolates (Paavanen-Huhtala et al. 2000).

Colletotrichum coccodes has been reported to be effective against the weed *Abutilon theopharasti*. A technique using diagnostic molecular markers generated from random amplified polymorphic DNA (RAPD) in PCR assay was developed to detect the strain 183088 of *C. coccodes* in plant and soil samples. The strain-specific marker was converted into a sequence-characterized amplified region (SCAR) and the specific primer sets (N5F/N5R, N5Ri/N5Ri) were designed for use in PCR detection assays. These primer sets amplified a single product of 617-bp and 38-bp respectively with DNA isolated from the target strain *C. coccodes* 183088. Absence of amplified products from DNA from other isolates of *C. coccodes,* other species of *Colletotrichum* and 11 other organisms confirmed the specificity of the primer sets. The specificity of detection could be substantially increased (1,000 folds) by employing nested PCR format using the primer set N5F/N5R for the first PCR amplification and the primer set N5Fi/N5Ri for second PCR amplification. The presence of the BCA could be detected in plants, greenhouse and field soils in which the BCA strain was deliberately released. This technique allowed accurate detection of *C. coccodes* strain 183088 among a background of soil microorganisms and it has the potential for monitoring field soils (Dauch et al. 2003). *Phomopsis* sp. is a natural pathogen of *Carthamus lanatus* ssp. *lanatus* (saffron thistle) an introduced widespread weed in crops and pastures in Australia. Many species of *Phomopsis* have been named primarily after the host plant species, resulting in a proliferation of species name. In order to develop *Phomopsis* sp. as a mycoherbicide, host affiliation cannot be used as a sole basis of identification. Hence, molecular analysis of the genome of *Phomopsis* sp. was taken up. Combined analysis of the repetitive DNA fingerprinting and sequencing of the ITS and TEF1-α analysis indicated that the

majority of isolates of *Phomopsis* sp. from saffron thistle were distinct from other species, available at GenBank and described as pathogenic to crop plants. The majority of isolates of *Phomopsis* sp. from saffron thistle form a lineage related to *Diaporthe angelicae*. Identification based on molecular characteristics is emphasized, since the genus *Phomopsis* includes many crop pathogens with wide host range (Ash et al. 2010).

Determination of molecular diversity among the species of fungal BCAs, will help in characterizing the isolates for different modes of biocontrol activity and their development for effective crop disease management. The genetic diversity of 17 *Trichoderma* isolates belonging to five species aggregates *viride, hamatum, harzianum, atroviride* and *longibrachiatum* was studied using random amplified polymorphic DNA (RAPD) analysis. The results showed that all *T. harzianum* isolates were clustered forming group I and all others formed group II. There were minor variation that could be related to the source of origin from different climatic zones. The presence of chitinase gene was revealed by the polymerase chain reaction (PCR) assay using primers designed, based on the sequences of the conserved regions of the gene that contributed to the biocontrol activity. Some of the tested *Trichoderma* isolates were effective not only in reducing the seed and peg infection of groundnut by *Aspergillus flavus,* but also reduced the *A. flavus* populations in the groundnut rhizosphere. Strain typing by RAPD finger-printing provides a rapid and convenient tool for screening large member of BCA isolates for their biocontrol efficacy (Anjaiah et al. 2001). Molecular characterization of genomes of the BCAs may enable to have an insight into the extent of variations in different species/strains/isolates of the BCAs. Using appropriate markers, the most efficient strain(s) may be identified. *Coniothyrium minitans* strains from 17 countries world-wide were examined to assess the intraspecific diversity. Sequences of the ITS1 and ITS2 regions and the 5.8S gene of rRNA genes were identical in all 24 strains of *C. minitans* studied, irrespective of colony type and origin. The results indicated that *C. minitans* was not genetically variable, despite phenotypic difference (Muthumeenakshi et al. 2001). Three different approaches were followed to assess the intraspecific variations in eight strains of the mycoparasite *Coniothryrium minitans*. The genomic DNA of the BCA was analysed by RAPD and amplified fragment length polymorphism (AFLP), in addition to morpho-physiological characteristics of *C. minitans* strains. All individual strains could be differentiated using the three types of analysis. However, there was no close correspondence between these types of analyses. The results suggested that AFLP analysis might be a more efficient and reliable tool than the other two techniques to detect intra-specific variability in *C. minitans* and to monitor its presence and persistence after application on the field (Grendene et al. 2002).

Random amplified polymorphic DNA (RAPD) procedure was applied to determine the genetic variability among ten isolates of *Trichoderma* spp. effective against chickpea wilt pathogen *Fusarium oxysporum* f.sp *ciceris*. The primer OPA13 could assign all the isolates of *Trichoderma* into different species. Using the primer OPA10, species specific banding pattern were obtained (Dubey and Suresh 2006). In another study, the genetic variability of among eight isolates of *T. harzianum* was

assessed, in order to find the relationship between RAPD profiles and ability to antagonize *Sclerotium rolfsii*. The technique showed the existence of high intraspecific variation among the isolates of *T. harzianum.* The level of antagonism exhibited by the isolates was not correlated with the RAPD banding patterns (Sharma et al. 2009). The strawberry rhizosphere-associated fungi antagonistic to *Verticillium dahliae* were characterized on the genotypic level using BOX-PCR technique. *Trichoderma* strains displayed higher diversity in all soils, but a high degree of plant specificity was shown by BOX-PCR fingerprints. In general, *Trichoderma* strains with antagonistic activity were highly abundant. Fungal fingerprints of the same samples obtained by denaturing gradient gel electrophoresis (DGGE) of the 18S rRNA gene fragments also indicated a plant-dependent composition of the fungal community. The results suggested that some antagonistic fungi might be specifically enriched in different rhizosphere (Berg et al. 2005).

Effectiveness of fungal biocontrol agents for the management of postharvest diseases of fruits and vegetables has been demonstrated. Colonization of wounds in fruits and dispersal of the BCAs in the environment could be assessed by molecular methods. A specific fragment of DNA (L4) of *Aureobasidium pullulans* effective against postharvest rots of sweet cherries and table grapes was cloned, sequenced and employed to design two SCAR primers and a 242-bp riboprobe. Both SCAR primers and the 242-bp digoxigenin (DIG)-labeled riboprobe were highly specific for the L-47 strain of *A. pullulans*. The limit of detection was 10^5 cells of *A. pullulans*/ml which was 10 times lower than the limit of the isolation-based method (Schena et al. 2002). The conventional method was unable to differentiate strain K of the yeast BCA *Pichia anomala* from other isolates/strains. Random amplified polymorphic DNA (RAPD) amplification with primer OPN 13 produced a fragment of about 2,000-bp which was specific to strain K. The SCAR marker (262-bp) designed from the above mentioned fragment was amplified with K1 and K2 primers for strain K as confirmed by Southern blot assay. By combining a plating technique on a semi-selective medium, followed by a direct strain K-SCAR amplification without DNA extraction, the colonies of *P. anomala* on apples were quantified at 24 h after treatment. Decrease in population densities was observed from 1 week after application in cold storage conditions (De Clercq et al. 2003).

A monitoring system was developed for the identification and quantification of two strains of Ach 1-1 and 1113-5 of *Aureobasidium pullulans* effective against the postharvest pathogens *Botrytis cinera* and *Penicillium expansum,* infecting apples in storage. The RAPD technique was applied to identify five specific RAPD fragments for strain Ach 1-1 and three other fragments for strain 1113-5. The fragment of 528-bp specific to strain Ach 1-1 and another fragment of 431-bp specific to 1113-5 were selected, cloned, sequenced and used to design sequence-characterized amplified region (SCAR) primers. The SCAR markers could be employed to identify strains Ach 1-1 (with 189-bp and 389-bp SCAR primers) and 1113-5 (with 431-bp SCAR primer). These markers could be used to differentiate Ach 1-1 and 1113-5 strains from 14 strains of *A. pullulans* and eight yeast species commonly associated with apple fruit surfaces. In addition, a semi-selective medium was also developed for growing the BCA strains. The combination of the selective medium and SCAR

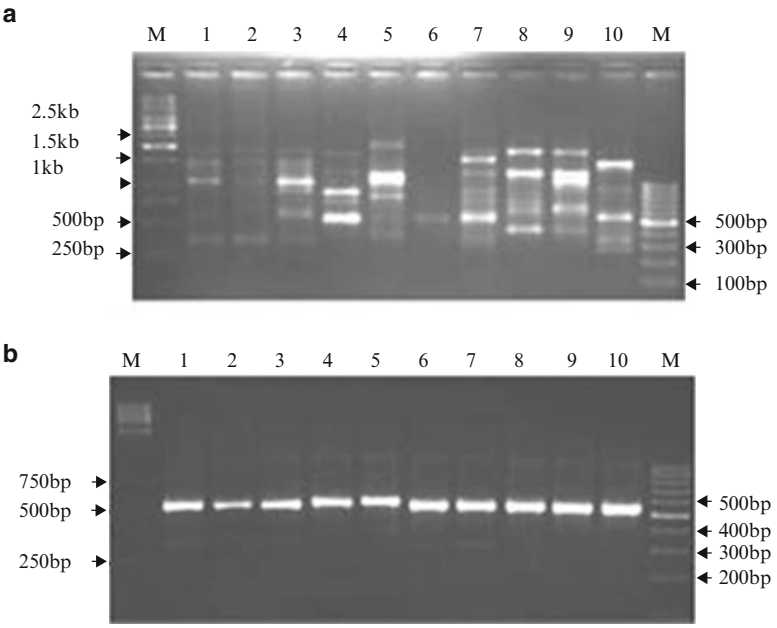

Fig. 2.2 Genetic diversity of *Fusarium oxysporum* f.sp. *lycopersici* (FOL) as determined by RAPD banding polymorphism Internal transcribed spacer (ITS) banding pattern of 10 isolates of FOL sensitive to *Trichoderma harzianum*; ITS region was amplified using the universal primers ITS1 as a forward and ITS4 as a reverse primers. *Lanes* marked by *M* represent 1-kb molecular size marker positioned at *right* and *left extremes*; note the presence of a single band of about 550-bp amplified from the rRNA of all isolates of FOL (Courtesy of Mishra et al. 2010 and with kind permission of Springer Science + Business B. V., Heidelberg, Germany)

markers constitutes an effective tool to specifically identify and quantify the selected strains of the BCA to target the fungal pathogens (El-Hamouchi et al. 2008).

Plant pathogens like *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) show wide genetic diversity in their pathogenicity as well their sensitivity to the biocontrol agents. The biocontrol potential of *Trichoderma harzianum* in suppressing the isolates of *Fol* was assessed. *T. harzianum* inhibited all isolates of *Fol* effectively in dual culture assay. The culture filtrates were also effective against *Fol* isolates. Genetic diversity among the *Fol* isolates, causal agent of tomato Fusarium wilt, was determined by employing different RAPD primers. The consensus primers ITS1 and ITS4 were employed to amplify a region of the rRNA gene repeat unit. All *Fol* isolates amplified a single band of about 550-bp, except for two isolates *Fol4* and *Fol5*. These two isolates showed variation in length in this region in which *Fol4* showed a single band of higher molecular weight of around 560 (Fig. 2.2). The variations detected, did not appear to have relationship to the biocontrol potential of *T. harzianum* (Mishra et al. 2010).

It is essential to develop monitoring systems to detect and quantify the BCAs introduced into the soil or applied on the plant. This information is required for the

registration of the biocontrol agents for commercial purposes. Molecular analysis based on arbitrarily primed (AP)-PCR and RAPD techniques was applied for the precise identification of 19 isolates of yeasts obtained from the surface of several fruits and vegetables in Southern Italy. Closely related genetically different strains which had same morphological characteristics were differentiated. The isolates (6) characterized by molecular analysis were found to be effective against *Penicillium digitatum,* infecting grapefruit, *Botrytis cinerea, Rhizopus stolonifer* and *Aspergillus niger,* infecting table grapes and *B. cinerea* and *R. stolonifer,* infecting cherry tomato, when tested both on unwounded and wounded fruits. The most efficient strain LS15, when applied as preharvest spray on table grape, resulted in significant reduction in gray mold disease. RAPD-PCR procedure was shown to be useful for establishing the identity of the BCA and for monitoring the survival of the antagonist in the field conditions (Schena et al. 2000). *Trichoderma asperellum* isolates 659-7, PR10, PR11 and PR12 were able to suppress the cocoa black pod disease caused by *Phytophthora megakarya* through their mycoparasitic activity. The identity the *T. asperellum* isolates was established by applying sequence analysis of the gene for translation elongation factor 1 (*tef1*). Molecular fingerprinting using RAPD and UP-PCR demonstrated high genetic similarity between isolates 659-7, PR11 and PR12 and high dissimilarity between PR10 and other three isolates (Tondje et al. 2007). *Epicoccum nigrum* strain 282 (EN282) was found to significantly reduce brown rot disease of peaches caused by *Monilinia laxa*. The RAPD procedure was employed and a 600-bp RAPD-PCR product was identified as specific for EN282 strain. Two sets of primer pairs were designed for use in conventional and real-time PCR which could specifically detect EN282 in stone fruit. A semi-selective medium (ENSM) was also developed for preliminary screening and identification, as this medium differentially favored the growth of the BCA. Real-time PCR assay was more sensitive than conventional PCR for quantification of EN282 DNA, but less specific for the detection of the BCA present in the fruit samples. Both conventional and real-time PCR were useful in detecting the EN282 DNA extracted from viable *E. nigrum* colonies or from peach fruit (Larena and Melgarejo 2009a, b).

The yeast *Pichia membranefaciens* was shown to be antagonistic to the gray mold pathogen *Botrytis cinerea* capable of infecting several fruit crops such as grapevine, apple and peaches. Yeasts are taxonomically diverse and they have morphological characteristics with affinities to Ascomycetes and Basidiomycetes. Identification of these putative BCAs using nucleic acid-based techniques has provided reliable conclusions. Polymerase chain reaction (PCR) coupled with restriction fragment length polymorphism (RFLP) analysis of PCR products has been useful for identification of fungus-like and fungal BCAs. PCR was applied for the amplification of the complete ITS region of its nuclear ribosomal DNA of *P. membranifaciens* using universal primers ITS-1 and ITS-4. ITS sequences of this BCA were compared with those of related species and the identity of this isolate was established conclusively (Masih et al. 2001). *Pichia anomala* strain K an epiphytic yeast isolated from apple provided high level of protection against *Botrytis cinerea* causing gray mold and *Penicillium expansum* causing blue mold disease in apple fruits. Monitoring the presence of strain K requires a specific method capable

of quantifying the BCA population and differentiating it from the native microflora. The classical method for quantification of the BCA involves counting colony-forming units (CFUs) on a selective or semi selective medium. This method provides the advantage of assessing the population of living cells. But this method cannot differentiate strain K from other species or strains of BCAs naturally occurring on apple fruits. Identification of *P. anomala* strain K based on the PCR amplification of the SCAR marker of 262-bp (derived from a RAPD fragment), using primers K1 and K2 (De Clercq et al. 2003) was time-consuming and not suitable for routine use. Hence, a quantitative-competitive PCR (QC-PCR) method with enzyme-linked oligosorbent assay (ELOSA) based on the 262-bp SCAR marker was developed. For quantification, an internal standard (IS) was included and the IS was also amplified by the primers K1 and K2. The optimized ELOSA detection system for the target and the IS sequences was able to detect 0.5 ng of amplified product. Quantification of strain K cells by QC-PCR-ELOSA provided an acceptable level of accuracy within the range of 10^3–10^6 yeast cells per apple. The sensitivity threshold of QC-PCR-ELOSA applied under practical conditions permitted detection of less than 10^3 yeast cells per apple. The technique was found to be very specific and sufficiently accurate for reliable monitoring of strain K populations (Pujol et al. 2004).

Rhodotorula mucilaginosa strain S-33 is an effective BCA against *Botrytis cinerea* causing tomato stem canker disease and cucumber stem blight caused by *Didymella bryoniae.* Dot blot hybridization technique has been shown to be useful for screening many samples of an organism. Prior amplification of a fragment of the genome of the target organism enhances the sensitivity of the dot blot tests. Yeast species could be detected and differentiated by dot blot hybridization. Monitoring population dynamics of pathogen-BCA is essentially required to assess the biocontrol potential of the BCA in the location concerned. Specific probe RD5 was employed to amplify the target fragment of the genome of *Rhodotorula mucilaginosa* in the PCR assay, followed by probing of the PCR amplicon with digoxigenin (DIG) in the dot blot hybridization. A dot blot assay can be used to analyze many samples of a single test organism, as the PCR products from different samples are blotted on the membrane and hybridized to the selected probe specific for the target organism. The probe RD5 effectively detected *R. mucilaginosa* in cucumber plants infected with gummy stem blight pathogen *D. bryoniae* and sprayed with *R. mucilaginosa*. This test could detect the BCA in pure culture and also plants sprayed with BCA in commercial greenhouses. This procedure could be employed for monitoring the BCA population in diseased cucumber plants for effective management of the disease (Utkhade and Cao 2005; Appendix 2.6). A specific and sensitive detection and identification technique was developed for two strains of *Aureobasidium pullulans* CF10 and CF40. The procedure was based on a sequence characterized amplified region (SCAR) derived from RAPD-and multiplex-RAPD-PCR analysis. Genetic variability among 200 isolates of *A. pullulans* was initially determined. Ten DNA fragments present only in the target strains CF10 and CF40 were selected, cloned and sequenced. Two SCAR primers were designed using the sequences of the selected fragments. Two primer pairs obtained from SCH3 RAPD fragment of CF40 and RAPD of CF10 were found to be highly specific and

sensitive. These two strains of *A. pullulans* could be detected simultaneously in a single PCR assay. The procedure developed in this study could be used for specific and sensitive detection and identification of strains of fungal BCAs. In addition, it has the potential for application in studies on the efficacy and persistence of introduced strains of *A. pullulans* for the biocontrol of the fire blight disease caused by *Erwinia amylovora* (Loncaric et al. 2008).

Soil management practices such as application of organic amendments and other agricultural practices, not only influence plant growth, but can also modify the microbial community composition composed of both beneficial and harmful microorganisms. Monitoring the structure and dynamics of fungal communities in soils under agricultural and environmental perturbations has been found to be a challenge. A terminal restriction fragment length polymorphism (T-RFLP) technique was applied to monitor fungal community structure of three different soils with contrasting physiochemical properties. This technique involves the use of PCR primers targeting different variable regions of the SSU rDNA designed to specifically amplify fungal rRNA genes from soil samples. PCR amplification of the 3′ end of the SSU rRNA gene with the primer nu-SSU-0817-5′ and the fluorescently labeled primer nu-SSU-1536-3′ and digestion of the amplicons with restriction enzymes *Alu*I and *Mbo*I were found to be optimum. The impact of compost or manure on the fungal community structure was assessed by T-RFLP procedure. This technique was shown to be sensitive and reproducible. The shift in fungal community structure differed with the organic amendment applied and also the fungal community structures of the soils were affected in a different way by the same organic amendment. Thus each soil was characterized by a specific fingerprint, indicating the effectiveness of T-RFLP technique for discriminating the structures of fungal communities existing in soils. The sensitivity of the method was indicated, when the impact of two different organic amendments was assessed. This technique revealed different responses of the fungal community depending on the soil and the type of organic amendment. The results suggest that the fingerprinting method has the potential for use as rapid tool to investigate the effects of various disturbances in soils due to agricultural practices (Edel-Hermann et al. 2004).

Relationships between biotic changes and local decrease in soil conduciveness in disease patches towards the disease induced by *Rhizoctonia solani* AG 2-2 in sugar beet field were examined. The genetic structure of microbial communities in these soil samples was studied by employing T-RFLP of 16S and 18S rRNA genes for fungi and bacteria. Fluorescently-labeled terminal restriction fragments (TRFs) were separated by a capillary electrophoresis sequencer. The total numbers of TRFs detected were 245 and 260 for fungi and bacteria respectively. Modifications were found in the genetic structure as well as in the expression of genes involved in the metabolic process. Significant differences were observed in the balances among the populations of the fungal communities in the inside and outside of the diseased patches. T-RFLP analysis revealed that the peaks corresponding to *R. solani* and *Trichoderma* spp. were higher inside the diseased patches than in the healthy areas. The pathogen appeared to induce changes in genetic and physiological structure of microbial populations and development of antagonists. T-RFLP

technique might be useful to select isolates of BCAs with greater biocontrol efficacy (Anees et al. 2010).

Attempts have been made to overcome problems associated with cultivation-dependent methods for detection and identification of fungal BCAs. It is important that the persistence and spread of the BCAs released into the environment are precisely monitored. Real-time PCR methods for the identification of genus/species/strains of the BCAs have been developed to precisely establish the identity of the BCAs like *Trichoderma atroviride*. A primer and Taq-Man probe set were constructed based on mutations in an endochitinase gene. This procedure was highly specific for the detection and quantification of *T. atroviride* SC1 strain. The detection limit and quantification calculated from the relative standard deviation were 6,000 and 20,000 haploid genome copies per g of soil. This technique can analyze many samples within short time and it could be effectively applied to trace the fate of *T. atroviride* SC1 released as an open-field biocontrol agent (Savazzini et al. 2008). In a later study, a PCR-based technique in order to detect and determine the diversity of *Trichoderma* (perfect stage *Hypocrea*) was developed. Specific *Trichoderma* primers (ITSTrF/ITS*Tr*R) that comprise an approximate 650-bp fragment of the ITS region from all taxonomic clades of the genus *Trichoderma* were designed. The amplicon (PCR product) was found to be suitable for the identification of all isolates using *Trichokey* and *Tricho* BLAST programs. Furthermore, the *Trichoderma* communities in the rhizosphere of different potato genotypes grown under field conditions in Germany could be studied by using the primers designed in this study. The *Trichoderma* composition was site-dependent as revealed by cloning and sequencing of BCA DNAs. Denaturing gradient gel electrophoresis (DGGE) analysis showed the existence of high heterogeneity of *Trichoderma* communities. Quantitative PCR (QPCR) assay indicated that copy number of *Trichoderma* significantly differed, depending on the sites of sample collection (Meincke et al. 2010).

Banana wilt disease development could be suppressed by soilborne *Trichoderma* spp. (Zum Felde et al. 2005). The distribution of various species of endophytic and epiphytic *Trichoderma* associated with banana roots was investigated. The genetic structures of endophytes and epiphytes were compared using amplified fragment length polymorphism (AFLP) technique. A modified TSB medium was used for selective isolation of *Trichoderma* spp. The restriction enzymes *Eco*RI and *Mse*I were employed for DNA digestion. Six species of *Trichoderma* were isolated from banana roots. Of these, *T. asperellum, T. lixii* and *T. virens* were found as endo-and epiphytes. In addition to these, *T. brevicompactum* was also detected as an endophyte, while *T. atroviride* and *T. koningiopsis* were also found as epiphytes. Endophytic and epiphytic isolates of *Trichoderma* from banana roots differed significantly as number and species. The AFLP analysis indicated that the genetic structure of epiphytes was more diverse than endophytes. The endophytic *T. asperellum* and *T. virens* showed higher genetic identity (diversity indices) than their endophytic counterparts. The endophytic *Trichoderma* spp. appear to have a higher genetic conservation and were compatible with relatively stable microenvironments inside roots (Xia et al. 2011). *Pythium oligandrum* capable of colonizing

rhizosphere of tomato plants in the soilless culture and providing protection against fungal pathogens was studied by applying inter-simple-sequence-repeat (ISSR) analysis. Isolates of *P. oligandrum* (90) were obtained from the inoculated roots of tomato. The primer [GACA] $_4$ was used to amplify the DNA from the *P. oligandrum* isolates followed by SDS-PAGE technique. Among the 90 isolates collected at the end of cropping season, 84 were identified as strain CBS 530.74, four as LMSA 1.01.63 and two as CBS 109981. The ISSR analysis could be successfully applied to differentiate the strains of fungal BCA isolated from the rhizosphere at the end of the cropping season (Vallance et al. 2009).

Mycotoxins produced by fungal pathogens contaminate grains, legumes, fruits and other farm produce cause serious ailments in humans and animals, when the contaminated materials are consumed. Aflatoxin contamination in groundnut is an important problem to be tackled. *Trichoderma* spp. have been shown to have the potential for suppression of the fungi, like *Aspergillus* spp. present in groundnut. In order to identify and differentiate species/strains of *Trichoderma,* single nucleotide polymorphism (SNP) procedure was followed to provide a comprehensive diagnostic solution. Amplified fragment length polymorphism (AFLP) analysis could distinguish all the *Trichoderma* isolates based on six primer pair combinations which generated 234 polymorphic bands. In addition, individual AFLP bands were identified and it was possible to differentiate closely related species. Further, AFLP bands that correlated with different types of antagonism to *A. flavus* were also identified in this study, facilitating selection of the most efficient strain for the management of aflatoxin problem in groundnut (Bukhariwalla et al. 2005).

The soilborne pathogen *Rhizoctonia solani* infects a wide range of crops causing damping-off and root rot diseases. Combinations of in vitro and in vivo assays were applied to evaluate a broad spectrum of fungal antagonists for their potential to suppress the development of *R. solani.* Based on the ability to parasitize pathogen mycelium and to inhibit germination of sclerotia of *R. solani,* six antagonists were selected. These antagonists were genotypically characterized by their BOX-PCR fingerprints and they were identified as *Trichoderma reesei* and *T. viride* by partial 18S rDNA sequencing. When the potato sprouts were treated with *Trichoderma* isolates, they significantly suppressed the symptoms induced by *R. solani*. The DNA-dependent single-strand conformation polymorphism (SSCP) analysis of 16S rDNA/ITS sequence was employed to analyze the effect of *Trichoderma* treatment on indigenous root-associated microbial communities. The results indicated that the pathogen and the vegetation time have much more influence on the composition of the microbiota than the BCA treatment (Grosch et al. 2006). Single-strand conformation polymorphism (SSCP) technique was applied to assess the effects of *Pythium oligandrum* on the fungal populations, colonizing the tomato rhizosphere in the soilless system and the fungal dynamics throughout the cropping season. The SSCP fingerprinting may reveal rapid changes in microbial communities even if their composition is not known. This technique could be employed for following the changes in genetic structure of the fungal populations in the rhizosphere. SSCP analyses of three different DNA regions indicated increases in the number and areas of peaks as the cropping seasons advanced. The 28S rDNA gene was used as the

basis to assess the genetic structure of the rhizosphere fungal communities in the presence of *P. oligandrum*. No differences in the SSCP profiles were detected between inoculated and control plants at any date of sampling. The complexity of the fungal profiles was greater at the end of the cropping season. Apparently the genetic structure of the fungal community changed with time (Vallance et al. 2009).

Ampelomyces quisqualis isolates (52) obtained from parasitized apple powdery mildew pathogen *Podosphaera leucotricha* and 13 more isolates from other species of Erysiphaceae in four European countries were screened for their genetic diversity using single-strand conformation polymorphism (SSCP) analysis of the ITS region of the rDNA. Based on the ITS-SSCP patterns, the isolates were assigned to eight groups. The isolates showed types of growth viz., slower-and faster-growing types which were included in different SSCP groups. A phylogenetic analysis of the ITS sequence of representatives of these groups confirmed the results obtained with the SSCP procedures. All the isolates from *P. leucotricha* belonged to a distinct SSCP of genetically homogenous isolates. The results suggested that *Ampelomyces* mycoparasites occurring on apple powdery mildew pathogen were slightly different from other *Ampelomyces* groups which contain mycoparasites from various powdery mildew species (Szentivańyi et al. 2005). In order to understand the tritrophic relationships between plants, powdery mildew fungi and the mycoparasite strains of *Ampelomyces,* the nuclear ribosomal DNA ITS and partial actin gene (*act1*) sequences of 55 *Ampelomyces* strains from *Erysiphe necator,* causative agent of grapevine powdery mildew disease, were analyzed together with those of 47 strains isolated from other powdery mildew species. The phylogenetic analyses distinguished five major clades and strains from *E. necator* that were present in all but one clade. In addition, nine inter-simple sequence repeat (ISSR) markers were also employed for strain-specific identification of *Ampelomyces* mycoparasites to monitor the environmental fate of strains applied as BCAs. The genetic distances among strains calculated, based on ISSR patterns revealed the genetic diversity of *Ampelomyces* strains naturally occurring in grapevine powdery mildew. The results indicated that the strains of *Ampelomyces* isolated from *E. necator* were genetically diverse and no strict mycohost association could be recognized among these strains (Pintye et al. 2012).

2.2 Assessment of Biocontrol Potential of Fungi

Fungi are associated with plant environment and their interactions with microbial plant pathogens may be of different kinds. These interactions can appreciably affect plant health in various ways and they may have some form of direct or indirect contact. Interactions between two populations have been differentiated into mutualism, protocooperation, commensalism, neutralism, competition, amensalism, parasitism and predation (Odum 1953). These types of interactions may be observed at both macroscopic and microscopic levels in nature. Biological control may be considered as a net positive result derived from a variety of specific and non-specific

interactions. Mutualism refers to the association between two or more organisms deriving benefits from such association. A form of mutualism called as protocooperation indicates that organisms interacting with others do not depend entirely on each other for their survival. Many of the biocontrol agents can be considered facultative mutualists, since survival rarely depends on any specific host. Depending on the environmental conditions, degree of disease suppression may vary. A symbiotic association in which one organism is benefited and the other is neither benefited or harmed, is designated commensalism. When there is no positive or negative effect on the organisms interacting with each other, the association is termed as neutralism. If the interaction results in adverse effects exerted by one organism on the other, the interaction is called antagonism. Organisms exhibit competition to obtain nutrients or occupy available niche for their survival. Such competition may lead to decreased growth, activity or sporulation of the poor competitor. Many biocontrol agents are able to outgrow the slow-growing microbial plant pathogens which are starved out or prevented from gaining access to the specific host tissues that favor their development. Parasitism indicates the ability of one organism to develop by obtaining required nutrition from another organism. Predation is generally seen in the interaction between animals resulting in killing one organism by another for consumption. Protection of the plants may be achieved by exploiting suitable forms of interactions between biocontrol agents and the microbial plant pathogens.

Biocontrol of potential of fungus-like and fungal species is assessed by screening their isolates/strains under in vitro and in vivo conditions by performing different tests. The adverse effects of the BCAs directly or indirectly on the pathogen development are measured by the tests.

2.2.1 Laboratory Tests

2.2.1.1 Dual Culture Method

This test is performed, as a preliminary method to select fungi with antagonistic activity against plant pathogens. Various isolates of the fungal species are grown and maintained on suitable medium. Likewise, the pathogens are also maintained on the media that favor their development. The mycelial plugs or disks of the isolates and fungal pathogen are placed in the same plate with mycelia in direct contact with agar 4 cm apart from other, so that they can grow towards each other. Observations are also made at periodic intervals on the colony diameter, pigmentation, rates of growth, malformation in the hyphae of pathogen at positions nearest to the putative biocontrol agent and sporulation of the pathogen in the presence of the putative BCA (Weller et al. 1985; Benhamou et al. 2002). The zone of inhibition of the growth and sporulation of the pathogen are calculated relative to the parameters recorded for the controls (Appendix 2.7). This method may indicate the mechanism of biocontrol activity of the test isolates through antagonism. The results are subjected to statistical analysis to know the level of significance of the biocontrol activity.

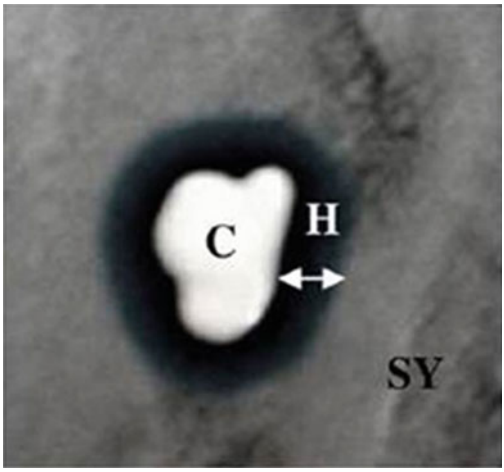

Fig. 2.3 Blue halo of cell death of the sensitive strain *Saccharomyces cerevisiae* NCYC1006 induced by *Torulaspora globosa* strain IS112, capable of producing killer toxin (Courtesy of Rosa et al. 2010 and with kind permission of Springer Science+Business Media B.V., Heidelberg, Germany)

Antagonism between the yeast BCA *Pichia membranifaciens* and the gray mold pathogen *Botrytis cinerea* infecting grapevine was demonstrated by placing both organisms in PDA plates and also in PDB broth liquid culture and incubating them at 25 °C. A small zone of inhibition appeared around the yeast. The hyphae of *B. cinerea* developing in the vicinity of inhibition failed to sporulate and hyphal cells showed different types of malformation and coagulation of portoplasts (Masih et al. 2001). The antagonism between *Torulaspora globosa* against *Colletotrichum sublineolum* infecting sorghum was demonstrated using dual culture technique. *T. globosa* strain IS112 inhibited the mycelial growth of *C. sublineolum* in PDA medium. Hyphal deformities were observed when mycelial samples were examined under the microscope. A blue zone surrounding the inhibition halo revealed that the BCA strain killed sensitive strains of *C. sublineolum* (Fig. 2.3; Rosa et al. 2010). In another study, biocontrol activity was determined after 10 days, using a scale from 1 to 5 as follows : 1–test fungus entirely over grown by the pathogen; 2–test fungus growing over the pathogens; 3–mycelia intergrowing; 4–mycelia grown up to each other and arrested and 5–visible inhibition zone. Control plates contained two mycelial plugs of the pathogen alone. Five replicates were maintained for each isolate of the test fungus and the pathogen. This procedure was followed to screen the isolates of fungi effective against *Fusarium oxysporum* f. sp. *cubense,* causing Panama wilt disease of banana (Nel et al. 2006). Dual culture method was applied to determine the biocontrol potential of eight isolates of *Trichoderma harzianum.* Two isolates each of *T. aureoviride* and *T. koningii* were evaluated for the suppression of growth of *Pyrenophora tritici-repentis* causing tan spot disease of wheat. Inhibition percentage of mycelial growth of the pathogen caused by *Trichoderma* spp. varied between 50 and 74 %. The inhibition of growth might be, due to plasmolysis of conidia and hyphal cell of the pathogen, as revealed by microscopic observations (Perelló et al. 2003). Isolates of *Aspergillus, Penicillium* and *Trichoderma* from soils cropped to onion were evaluated for their antagonistic

potential against *A. niger* causing black mold disease of onion using dual culture method (Özer 2011).

The fungal endophytes were isolated from the leaves of Norway maples and screened for their efficacy in inhibiting the development of *Rhizoctonia solani* infecting potatoes. The radial mycelial growth of the pathogen towards the antagonistic fungus (Ri) and that on a control plate (Rc) were measured and the mycelial growth inhibition was calculated according to the formula:

$$\left(\text{Rc-Ri}\right) / \text{Rc} \times 100$$

The fungus *Trichoderma viride* inhibited *R. solani* at a faster rate than *Phomopsis* sp., *Alternaria longipes* and *Epicoccum nigrum*. The culture filtrates of these fungi were also tested for growth inhibition. The inhibition varied according to the type of antagonistic fungal isolates that might produce various kinds of antifungal compounds. The inhibition increased proportionally with the concentration of the culture filtrate. *T. viride* culture filtrates provided the greatest inhibition of *R. solani* (Lahlali and Hijri 2010). The dual culture method may be useful to select the BCA isolates that act through antagonism and parasitism. Other tests have to be employed to identify the fungal species that act on the plant pathogen through other mechanisms such as competition for nutrition and space and induction of resistance in host plant species against the target pathogens. Dual culture method has been employed to screen fungal and bacterial biocontrol agents that were found to be effective against microbial plant pathogens (Soytong et al. 2005; Anand and Reddy 2009; Živković et al. 2010).

2.2.1.2 Glass Slide/Leaf Tests

Biocontrol efficacy for the control of obligate fungal pathogens has been determined in vitro on solid and liquid media and leaf disks under various conditions. Sterilized glass slide is covered with a thin film of 2 % water agar followed by inoculation with aeciospores of the rust pathogens and aliquots of conidial suspension of efficient isolates of *Cladosporium tenuissimum.* The culture filtrates of antagonistic isolates grown in liquid cultures were also tested for the biocontrol activity against the aeciospores of the rust pathogens. Light and scanning electron microscopes were used to observe the interaction between the rust pathogens *Cronartium flaccidum* and *Peridermium pini,* infecting needle pine (*Pinus* spp.). The rust aeciospores were directly parasitized by *C. tenuissimum.* The hyperparasite exerted a mechanical force to penetrate the spores and subsequently proliferated within the spores. Dissolution of spore walls indicated the possibility of enzymatic action by the BCA. Culture filtrates of *C. tenuissimum* inhibited germination of rust spores. Cladosporal purified from the culture filtrates was identified as β-1,3-glucan biosynthesis inhibitor (Moricca et al. 2001). Antagonistic activity of *Cladosporium tenuissimum* on strawberry powdery mildew pathogen *Podosphaera aphanis* was assessed by leaf and glass-slide bioassays. Conidial suspensions of the BCA were sprayed on detached leaves/slides followed by inoculation by shaking leaves with

heavy sporulating lesions and incubated for 48 h at 20 ± 1 °C under 12:12 h light/dark conditions. Effect on germination of *P. aphanis* conidia was studied by removing the conidia and germlings from the slide/leaf surface using 2 × 4 cm piece of transparent adhesive tape and examining under a light microscope after staining with cotton blue. The germ tube length was measured and the hyphal biomass was calculated by multiplying the germination percentage by the average germ tube length for each replicate. Three replicates were maintained for each treatment and the data were analyzed statistically (Pertot et al. 2007).

The interaction between *Rhizoctonia solani* and the mycoparasitic endophyte *Chaetomium spirale* ND35 was studied, using light and electron microscopes. Coiling of *C. spirale* around *R. solani* and its intracellular growth in its host (*R. solani*) was observed frequently. Later *C. spirale* was found to be associated with drastic morphological changes of the host cell, characterized by refraction of plasma membrane and cytoplasm disorganization. Application of immunochemistry technique in transmission electron microscope (TEM) revealed that contact between the antagonist and the pathogen was mediated by an amorphous β-1,3-glucan enriched matrix originated from cell wall of the antagonist and sticking to its host surface. Simultaneously the host cell reacted by forming hemispherical wall appositions which were intensely labeled by the antibodies specific to β-1,3-glucan. These appositions were formed at sites of potential antagonist entry. However, *C. spirale* could penetrate this barrier successfully, indicating that the antagonist might produce β-1,3-glucanases capable of dissolving the barriers (Gao et al. 2005).

Confocal microscopy technique was employed to visualize in situ the interaction between the fungal endophytic BCAs and *R. solani* infecting potatoes causing black scurf disease. The BCAs showing significant antagonistic activity in the dual culture method were studied using the confocal microscope. Agar plugs containing mycelia of the fungi were placed on opposite sides of a plate containing PDA medium. Microscope coverslips were placed on the top of agar between antagonistic strains. When the hyphae were observed on the surface of coverslips, they were removed and immediately stained with SytoGreen 13 dye for 30 min at room temperature. Coverslips were mounted in glycerol solution on a microscope slide and observed under the confocal microscope. *Trichoderma viride* could establish close contact with the hyphae of *R. solani* by coiling. The coils were very dense and appeared to tightly encircle the pathogen hyphae. Penetration of the pathogen hyphae by *T. viride* was seen at 7 days after contact, followed by loss of turgor of hyphal cells. *Phomopsis* sp. and *Epicoccum nigrum* did not penetrate pathogen cells, but induced abnormal cell morphology and lysis of cells, possibly due to production of extracellular chitinolytic enzymes produced by these BCA species (Lahlali and Hijri 2010).

2.2.1.3 Agar Drop Test

As a preliminary screen, this test may be useful to select potential BCAs rapidly from a large number of samples. Five drops of potato dextrose agar (PDA) (0.7 ml/petridish) are placed. Mycelial macerate (10 µl) of the 1 week old culture

of the pathogen (*Sclerotium cepivorum*) was used to inoculate the agar drops in the petridishes. An agar plug (2 mm^2) from the edges of the culture of each test fungus was added to each of the precolonized agar drops. The plates were then incubated for 5 weeks at 20 °C. Sclerotia (100) from each agar drop were tested for degradation (soft or collapsed) by squeezing them with a pair of forceps (Clarkson et al. 2002).

2.2.1.4 Leaf Disk Assay

Apple leaves naturally infected by scab pathogen *Venturia inaequalis* were inoculated with antagonistic fungi. Colonization of the leaf disks by *Microsphaeropsis* sp. and *Trichoderma* sp. was evaluated by visual observation and reisolation. The leaf disks were placed on petridishes containing water agar amended with tetracycline (100 mg/l) and incubated at room temperature for a period of 1–6 weeks. Observations were made at an interval of 3 days for the presence of fungal structures. *Trichoderma* sp. was identified, based on spore morphology and typical pigmentation, while *Microsphaeropsis* sp. could be identified by the presence of pycnidia and pale brown conidia. The BCAs were reisolated from a portion of leaf disk to determine colonization of the leaf tissues (Carisse et al. 2000). The biocontrol potential of entomopathogenic species of *Lecanicillium longisporum* and *L. attenuatum* effective against aphids, was assessed against *Sphaerotheca fuliginea* causing cucumber powdery mildew disease by detached leaf disc bioassays. Suspension of conidia and blastospores of *Lecanicillium* spp. were applied onto leaf discs (15 mm) punched out from cucumber plants inoculated with *S. fuliginea.* Powdery mildew symptoms did not develop, when the BCA isolates were applied at 1 and 8 days after pathogen inoculation. When the BCA isolates were applied on leaf discs showing severe disease symptoms (at 11 and 15 days after pathogen inoculation), further production of conidia by the pathogen was suppressed, compared to untreated control leaf discs. This investigation showed the potential of *Lecanicillium* spp. for the control of fungal pathogen and three species of aphids (Kim et al. 2007). Six strains of *Trichoderma harzianum* (*Th*) of which five strains were isolated from commercial preparations were evaluated for the biocontrol efficacy against bean rust pathogen *Uromyces appendiculatus* using leaf disc assays. Conidial spore suspensions and culture filtrates of the BCA strains were tested. Wide variations were observed in the efficacy of *Th* strains in reducing the number of uredial pustules formed on bean leaves, the range of efficacy values being 1–50 %. Increasing the conidial culture filtrate concentrations showed positive relationship with biocontrol efficacy. The culture filtrates seemed to have protective but not curative effect (Burmeister and Hau 2009).

2.2.1.5 Detached Florets Assay

Biocontrol efficacy of *Coniothyrium minitans* and *Trichoderma atroviride* for the control of alfalfa blossom blight disease caused by *Sclerotinia sclerotiorum* was evaluated by this assay. Newly opened florets from racemes of alfalfa plants raised

in the greenhouse were inserted at pedicel end into moist, autoclaved vermiculite kept in petridishes at the rate 12 florets/dish. Spore suspension (4.5×10^6 conidia/ml) of *C. minitants* or *T. atroviride* (12 µl) were applied on individual florets in each dish using a pipette. The dishes were placed in a laminar flowhood for 2 h for the evaporation of moisture. They were inoculated with an ascospore suspension (6×10^3 ascopores/floret) of the pathogen. Necessary controls inoculated either with BCAs and pathogen only were maintained. *C. minitants* showed greater biocontrol efficacy compared with *T. atroviride* based on the results of both in vitro and field evaluation (Li et al. 2005).

2.2.1.6 Assay Using Petridish-Grown Plants

Biocontrol potential of the fungal endophytes against Chinese cabbage Verticillium yellows disease induced by *Verticillium longisporum* was assessed. The pathogen was grown on oat meal agar (OMA) for 2 weeks. Chinese cabbage seedlings (1 day old) were transplanted into each pathogen colony on the medium. Seedlings grown for 1 week with and without individual endophyte were challenged with the pathogen. The seedlings with the agar medium were overlaid directly onto the fungal colony of the pathogen kept in plastic trays and incubated in a growth chamber. Observations on the symptom appearance were made at 3 weeks after transplanting (Narisawa et al. 2004).

2.2.1.7 Visualization Using Different Kinds of Microscopes

Interaction between the biocontrol agents (BCAs) and fungal pathogen has been observed at different stages using light, fluorescence and electron microscopes.

A. **Light microscopy**
   A cube of soil agar containing germinated sclerotium of *Sclerotium cepivorum* was placed in a sterile petridish and onion seed was so positioned that the root tip was in close proximity to the sclerotium for 48–72 h at 20 °C. Another cube of soil agar containing active *T. koningi* (Tr5) mycelium was placed adjacent to the seedling root, between the developing pathogen and onion bulb. The plates were incubated in darkness for 72–96 h. Free-hand sections of a portion of the roots were prepared and stained with 0.05 % Ruthenium Red (Sigma) for 2 min. The sections were destained with distilled water to remove excess stain. Root tissues colonized by *T. koningii* showed detachment of hyphal cells at septa, dissolution of cell walls and bursting of hyphal apices of *S. cepivorum*. Lysis of *S. cepivorum* hyphal tips with release of protoplasm could be observed more frequently. In most cases, the pathogen could not develop further (Metcalf and Wilson 2001). The interactions of the endophyte *Phialophora fortinii* and another dark septate endophytic (DSE) fungus with virulent strains of *Verticillium longisporum* were studied. Root segments of Chinese cabbage were made using a razor blade and stained with 0.05 % cotton blue in 50 % acetic

acid. Colonization patterns of the endophytes in host roots and host responses were observed using a compound microscope. Intracellular pigmented hyphae of *P. fortinii* extending into the inner cortical cells and forming irregularly lobed, rounded thick walled cells were observed. On the other hand, hyphae of DSE taxon colonized epidermal cells intracellularly. Localized hypersensitive defense reactions in host roots were seen in some of the root cortical cells colonized by *P. fortinii.* These reactions might be involved in the restriction of the ingress of the pathogen into the adjacent root cells of host plant (Narisawa et al. 2004). Mycoparasitism of *Trichoderma harzianum* on *Colletotrichum acutatum* and *C. gloeosporioides* causing anthracnose diseases of several crops was evidenced by microscopic observations of mycelial growth taken from the points of contact between the BCA and the pathogens. The hyphae of *T. harzianum* grew initially alongside and formed compact coils around hyphae of *C. acutatum* and *C. gloeosporioides. Gliocladium roseum* caused abnormally stunted, highly branched hyphal tips and swollen hyphae at the points of direct contact with the pathogens (Živković et al. 2010).

B. **Fluorescence microscopy**
Biocontrol agent *T. harzianum* was transformed with genes encoding green fluorescent protein (GFP). The suspensions of soil samples (0.01 g) were filtered through nonfluorescent membrane filters and observed under epifluorescence microscope at ×100 magnification. Typical fluorescent hyphae and chlamydospores of the GFP-transformed strain (Thz IDI-M3) of *T. harzianum* were visualized in soil samples containing the transformed strain, but not in samples collected from natural soil. Use of GFP along with epifluorescence microscopy was shown to be a useful tool to distinguish active hyphal biomass of the fungus that is functional for biological control, from inactive propagules like conidia or chlamydospores that are usually enumerated by plate counts. The biocontrol potential of the transformed strain may depend on the extent of hyphal growth through soil and contact with the target propagules of the pathogen like sclerotia of *Sclerotinia sclerotiorum* (Orr and Knudsen 2004).

C. **Electron microscopy**
Both scanning electron microscope (SEM) and transmission electron microscope (TEM) have been employed for observing the process of antagonism of the BCAs against microbial plant pathogens. *Trichoderma harzianum* isolate 1051 effectively suppressed the development of *Crinipellis perniciosa* causing the cocoa witches' broom disease. The BCA produced several hydrolytic enzymes that could adversely act on the cell walls of the pathogen. The effect of a chitinase secreted by *T. harzianum* on *C. perniciosa* was assessed using scanning microscope. The pathogen mycelium was incubated with partially purified chitinase of the BCA. Disruption of pathogen cell wall could be observed clearly within 24 h. Heat-denatured enzyme had no adverse effect on the cell walls of the pathogen (Fig. 2.4; De Marco et al. 2000). Colonization of aerial plant parts by *Trichoderma* spp. in cocoa plants was demonstrated using scanning electron microscopy (SEM). *T. harzianum* and *T. hamatum* and two other *Trichoderma*

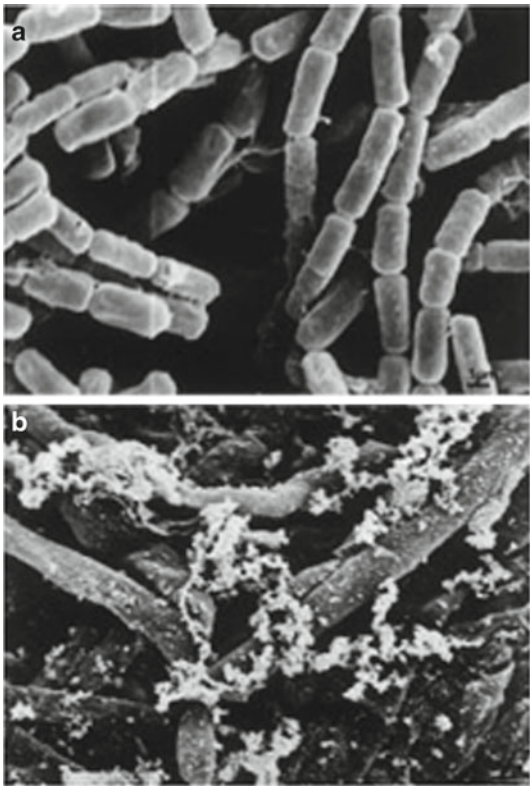

Fig. 2.4 Scanning microscopy (SEM) of hyphae of *Crinipellis perniciosa*, (causative agent of cocoa witches' broom disease) treated with partially purified chitinase produced by *Trichoderma harzianum* 1051 (**a**) intact hyphae of the pathogen; (**b**) pathogen hyphae incubated with chitinase produced by *T. harzianum*, showing disruption of pathogen cells (Courtesy of De Marco et al. 2000 and with kind permission of Springer Science + Business Media B.V., Heidelberg, Germany)

spp. could be isolated from surface-sterilized cocoa stem tissue including the bark and xylem, apical meristem and less frequently from leaves. Under the SEM, emerging fungal hyphae from the trichomes could be observed at 6 h after inoculation of the cocoa seedlings. Repeated single trichome/hyphae isolations confirmed the identity of the *Trichoderma* spp. Points of penetration of cocoa trichomes might be the portals of entry for *Trichoderma* spp. becoming systemic later (Bailey et al. 2009).

Scanning electron microscopic observations showed that aeciospores of the two needle pine stem rust pathogens *Cronartium flaccidum* and *Peridermium pini* were parasitized by the antagonistic fungus *Cladosporium tenuissimum.* Aeciospores were penetrated with or without formation of appressoria from *C. tenuissimum* and the BCA proliferated within the aeciospores (Moricca et al. 2001). TEM technique was employed to study the interaction between non-pathogenic *Fusarium oxysporum* strain Fo47 and *Pythium ultimum,* causing damping-off disease of cucumber. Samples of main roots of cucumber seedlings inoculated with Fo47 and later challenged with the pathogens were examined by observing ultrathin sections treated with β-1,4-exoglucanase-gold complex. The specific labeling pattern obtained with exoglucanase-gold complex confirmed

that Fo47 could successfully penetrate cells of *P. ultimum* both in the rhizosphere and inside the root tissues. *Pythium* cells that could evade the first defensive line in the rhizosphere could penetrate the root epidermis, but their growth was restricted to the outermost tissues. The results indicated that Fo47 exerted direct inhibitory effect on *P. ultimum,* a combination of antibiosis and mycoparasitism (Benhamou et al. 2002).

2.2.1.8 Biochemical Assays

Biocontrol activities of fungal species employed for the management of crop diseases have been shown to be associated with production of hydrolytic enzymes such as chitinases, endoglucanases, proteases and amylases that act on the mycelia of phytopathogens. The isolates that produce these enzymes in greater quantities are more efficient as BCAs. Hence, assessing the capacity of the putative BCAs to produce hydrolytic enzymes may be useful in selecting the appropriate isolate or strain of the fungal species. The amylase dextrinizing and saccharifying activities of *Trichoderma harzianum* were evaluated in a reaction system containing potato soluble starch solution in sodium acetate buffer. The tests were performed at pH 4.0 and 60 °C. The purified amylase showed discrete hydrolytic effect on *Crinipellis perniciosa*, the causative agent of cocoa witches' broom disease (de Azevedo et al. 2000). *Trichoderma koningii* was investigated for its ability to adversely affect the development of *Sclerotium cepivorum* causing *Allium* white rot disease of onions. Production of chitinolytic enzymes that might act on the pathogen was determined by electrophoresis technique. Chitin agar containing growing hyphal tips of *T. koningii* was added to 2.0 ml of chitinase medium with crab shell chitin in mixer salt solution (MSS). Sclerotia of *S. cepivorum* were also added to the liquid culture. Chitinolytic enzymes produced by *T. koningii* were detected by polyacrylamide gel electrophoresis (PAGE). Using citric acid buffer system, four isozymes with Rf values of 0.15, 0.24, 0.46 and 0.62 were detected. These enzymes were also produced in the presence of *S. cepivorum* sclerotia. These enzymes were identified as two endochitinases (Rf 0.15 and 0.24) and two exochitinases (Rf 0.46 and 0.62). Contact between hyphae of the BCA and pathogen was not necessary for lysis of pathogen cells to occur. The proteins (Rf 0.46 and 0.62) could be detected, when *T. koningii* colonized *S. cepivorum*-infected roots and they might be involved in the antagonistic activity of the BCA (Metcalf and Wilson 2001).

The importance of chitinolytic enzymes for the biocontrol efficacy of fungal and bacterial BCAs has been demonstrated. Chitinolytic activities of strains of *Trichoderma harzianum* isolated from the sugarcane rhizosphere regions were determined in order to relate this characteristic to the biocontrol efficacy of this BCA against the sugarcane red rot pathogen *Colletotrichum falcatum*. Inhibition of mycelial growth of *C. falcatum* by the extracellular proteins produced by *T. harzianum* strain T5 was assessed in 96-well microtiter plates. There was an increase in the antifungal activity in the medium amended with chitin. *T. harzianum* -T5 had higher levels of *N*-acetyl-glucosaminidase and β-1,3-glucanase activities, when it

Table 2.1 Effect of mycofumigation on Verticillium wilt disease severity and populations of *Verticillium dahliae* (Stinson et al. 2003)

Treatment	Pathogen population (CFU/g)[a]	DI (4 weeks)[a]	DI (5 weeks)[a]
Autoclaved, non-infected control	0 b	0.0 b	0.0 c
V. dahliae	66,840 a	55.0 a	60.0 a
V. dahliae + *M. roseus*	21,438 ab	17.5 b	32.5 b
V. dahliae + *M. albus*	1,257 b	10.0 b	20.0 bc

DI: Disease index based on a scale 0–3, where 0 = symptoms absent; 1 = slight symptoms with small chlorotic areas on leaves and plants not stunted; 2 = symptoms moderate, leaves showing yellowing, partial wilting and slightly stunted; 3 = complete wilting, severely stunted and all leaves are affected

DI: $\sum$(number of plants in each class of severity × class number) × 100/(mean number of plants grown in autoclaved soil × number of diseased plants)

[a]Means followed by the same letter are not significantly different at $P < 0.05$

was cultivated on minimal medium incorporated with chitin or pathogen cell wall fragments. Treatment of mycelial discs obtained from actively growing *C. falcatum* culture with chitinolytic enzymes secreted by *T. harzianum* T5 resulted in leakage of electrolytes from the pathogen cells, indicating the possible relationship between the production of chitinolytic enzymes and biocontrol efficiency of the BCA concerned (Viswanathan et al. 2003). The microorganisms collected from wheat anthers were tested for their ability to utilize tartaric acid which is poorly utilized by the Fusarium head blight (FHB) pathogen *Gibberella zeae*. Tartaric acid-utilizing *Cryptococcus* strain OH 71.4 and OH 182.9 reduced the disease severity, regardless of the sequence, timing and concentration of inoculum applied. Tartaric acid has the potential for use in formulation of tartaric-utilizing antagonists, since tartaric acid is a relatively inexpensive byproduct from the production of grape and other fruit juices. Testing candidate microorganisms capable of utilizing tartaric acid may be useful for preliminary screen for shortlisting the putative BCAs to be taken for further testing (Khan et al. 2001).

Chemical fumigants have been used to manage soilborne pathogens. As an alternative to the chemical use, the efficacy of natural volatiles from fungi like *Muscodor* spp. has been evaluated. Population of soilborne pathogens *Rhizoctonia solani, Pythium ultimum* or *Aphanomyces cochlioides* was decreased by mycofumigation with *Muscodor albus* and *M. roseus.* Consequently sugarbeet stand establishment was increased, while disease severity was reduced. The mycofumigants were applied as colonized agar strips, ground pesta and alginate formulations. The Stabileze formulation containing a mixture of water-absorbent starch, corn oil, sucrose and fumed silica was also applied to reduce incidence and severity of eggplant (brinjal) Verticillium wilt caused by *V. dahliae*. Both *M. roseus* and *M. albus* reduced the disease severity. *M. albus* mycofumigation reduced the populations of the pathogens significantly (Table 2.1; Stinson et al. 2003). An unidentified filamentous fungus strain Kyu-W63 produced antifungal volatiles. This putative BCA was tested for its biocontrol potential under greenhouse conditions against parsley powdery mildew (*Oidum* sp.) in sterile polycarbonate pots. Disease severity was

significantly reduced due to the volatile compounds produced from the BCA placed in the soil in the pots (Koitabashi 2005). The biocontrol efficacy of *Fusarium oxysporum* strain By 125, *Nectria haematococca* Bx247, *Phomopsis* sp. By 231 against Verticillium wilt of cotton and their plant growth-promoting effect on cotton plants were assessed under greenhouse conditions. The biocontrol efficacy of the isolates varied from 63.63 to 69.78 % and the plant biomass was increased by 18.54 to 62.63 % by these BCA isolates. In vitro assays revealed the potential of these isolates to fix nitrogen leading to increase in plant biomass (Zheng et al. 2011).

2.2.1.9 Immuno-Detection of Chitinolytic Enzymes of Biocontrol Agents

Trichoderma harzianum isolate 1051 was able to suppress the development of *Crinipellis perniciosa,* causative agent of witches' broom disease of cocoa. The biocontrol potential appeared to be associated with the production of a chitinase with a molecular mass of about 37-kDa. The chitinase secreted by the BCA was analyzed by the sodium dodecyl-sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) technique. The proteins separated by SDS-PAGE technique were transferred to nitrocellulose membranes and detected by immunoblotting, employing polyclonal antibodies specific for the chitinase produced by isolate 1051. The partially purified chitinase enzyme drastically disrupted the cell walls of *C. perniciosa* within 24 h, after treatment with the chitinase. The results indicated that antagonistic activity of *T. harzianum* isolate 1051 against the pathogen was due to the action of its chitinase, targeting the cell walls of pathogen hyphae (De Marco et al. 2000).

2.2.2 Greenhouse/Growth Chamber Evaluation of Biocontrol Activity

Different kinds of tests are performed to determine the biocontrol potential of the BCAs that are found to be more efficient under laboratory conditions. Laboratory tests may not be suitable for selection of BCAs that have mechanisms of actions other than antagonism or mycoparasitism. It is essential to detect and identify the BCAs with one or more mechanisms of action on the phytopathogens by assessing their biocontrol potential using live plants.

2.2.2.1 Bioassays Using Seeds/Planting Materials

Protection of plants against diseases may be achieved by applying the BCAs into the soil or by treating seeds with BCA preparations. The latter option is economical and easier and it requires smaller quantities of BCAs. Cotton seeds were coated with latex sticker and dry, granular preparations of *Trichoderma virens* strain G-6, *T. koningii* strain TK-7 and *T. harzianum* strain TH-23 and planted in sterile

vermiculite seedling trays. After 3 days of incubation at 27 °C, cavities with treated seed were drenched with 10 ml aliquots of *Rhizoctonia solani* mycelial fragment suspension (0.012 %). The seedlings, harvested after 10-day-incubation, were rinsed free of vermiculite and hypocotyls with roots were excised. Presence of symptoms of infection in the seedlings, was recorded. Controls with BCA or pathogen alone were maintained. Counts of surviving seedlings were made for each treatment. Biocontrol efficacy was calculated as 0–100 % damping-off disease incidence (Howell et al. 2000).

Six *Trichoderma* spp. and two bacterial species (*Stenotrophomonas maltophilia* and *Bacillus cereus*) were evaluated for their efficacy against *Fusarium graminearum* causing cereal damping-off in the greenhouse at mean temperatures of 18 °C and 80 % RH. The extent of inhibition of pathogen mycelial growth by the BCAs was not related to the biocontrol efficacy of in vivo assays. All isolates were initially assessed for their effect in reducing disease in wheat cultivar Klein Centauro (moderately resistant) and ProINTA Oasis (susceptible) planted in sterilized soil infested with *F. graminearum*. Six fungal isolates that were more effective, were tested using naturally infested field soil and eight wheat cultivars with differing resistance levels. The parameters, plant stand, percentage of diseased emerging seedlings, plant height and dry weight were recorded for each treatment. One isolate *Trichoderma harzianum* provided significant protection to some of the cultivars (Dal Bello et al. 2002). *T. virens* GL 21 applied as a granular formulation, in combination with *Burkholderia cepacia* BC-1 or *B. ambifaria* BC-F applied as seed treatment, significantly improved suppression of damping-off disease of cucumber caused by *Rhizoctonia solani* over individual application of these BCAs. *B. ambifaria* BC-F combined with *T. virens* GL21 as seed treatment provided higher level of suppression of damping-off due to *Pythium ultimum* (Roberts et al. 2005).

The efficacy of *Trichoderma harzianum* T22 was evaluated along with the conventional fungicide using them for seed treatment against soilborne damping-off pathogens *Fusarium oxysporum* f.sp. *spinaciae, Pythium ultimum* and *Rhizoctonia solani* infecting spinach. *T. harzianum* was applied as proprietary organic disinfectant (GTGII) and another product (GTGI). The number of emerged seedlings and the number of damped-off or wilted seedlings were counted weekly for 4–7 weeks. Post-emergence damping-off or wilt was calculated as the percentage of emerged seedlings. Total above ground dry biomass per pot was determined. Further, the area under preemergence disease progress curve (AUDPC pre), area under post-emergence disease progress curve (AUDPC post) and area under total disease progress curve (AUDPC total) were also calculated. Both products provided equivalent control to the conventional fungicide mefenoxam against *P. ultimum* in one trial and significant reduction of damping-off in the second trial. Three proprietary products also enhanced seed germination compared to untreated controls. No treatment was found to reduce incidences of diseases caused by all three pathogens (Cummings et al. 2009).

Fungal BCA *Clonostachys rosea* and commercial products containing *Trichoderma harzianum* and *T. koningii* were evaluated for their biocontrol potential against seedborne pathogens *Alternaria dauci* and *A. radicina* in carrot seeds.

Table 2.2 Effects of different priming treatments on total seedling emergence and final stand of healthy carrot seedlings after different periods of priming (Jensen et al. 2004)

		Treatments				
Seedling emergence (%)		Hydro-primed 40 % MC	Bioprimed A[a]	Bioprimed B[a]	Bioprimed C[a]	P values
Non-primed	Total	43.0	43.0	43.0	43.0	
	Healthy	37.7	37.7	37.7	37.7	
Priming 1 day	Total	44.5 a	62.6 a	58.5 a	59.5 a	0.0847
	Healthy	32.5 a	58.7 b	53.5 b	56.0 b	0.0029
Priming 8 days	Total	22.0 a	62.0 b	62.0 b	56.0 b	0.0001
	Healthy	0.5 a	59.0 b	59.0 b	52.0 b	0.0002
Priming 14 days	Total	30.0 a	54.0 bc	59.0 c	46.0 b	0.0002
	Healthy	6.7 a	52.0 bc	56.0 c	43.5 b	0.0001

A – *Clonostachys rosea* IK 726 pre-40 % MC
B – *C. rosea* IK 726 pre – 38 % MC
C – *C. rosea* IK 726 post – 40 % MC
In each column, means of four replicates followed by the same letter are not different significantly at P-values indicated
[a]Bioprimed

The spores of *C. rosea* were scraped from PDA cultures in water and filtered through nylon mesh. Seeds were coated with 4 ml of conidial suspension on shaker for 10 min and air-dried in a laminar flowhood. Hydropriming increased the incidence of seedborne infection. Biopriming with addition of *C. rosea* reduced the incidence of *A. radicina* and *A. dauci* to <2.3 and <4.8 % respectively in seeds with initial infection levels of 29 and 11 % respectively by *A. radicina* and *A. dauci*. In the case of low infection level (4.7 %), biopriming of seeds reduced the incidence to less than 0.5 %. Further, biopriming enhanced seedling stand, compared with hydroprimed or nonprimed seeds (Table 2.2). The bioprimed seeds with *C. rosea* IK726 transformed with a green fluorescent protein (GFP) reporter gene revealed the presence of the BCA covering the seeds with a fine web of sporulating mycelium of the BCA, when viewed under the microscope. Biopriming with *C. rosea* was found to provide protection to healthy and infected seeds without any risk of adverse effects on seedling establishment (Jensen et al. 2004; Appendix 2.8).

2.2.2.2 Bioassay on Whole Plants

Sclerotia of pathogens in soil samples are retrieved by flotation and sieving and the biocontrol activity is assessed by sclerotial degradation assay. Extent of sclerotial degradation (soft or collapsed) was determined by sequeezing them with forceps under a low-power binocular microscope. The BCAs causing greater sclerotial degradation are advanced to further testing stage in glasshouse pot trial. Soil amended with medium-grade vermiculite and sclerotia of the pathogen are mixed and the BCA as wheat bran cultures (1 g/100 g) or spore suspension (to give 1×10^7 spores/100 g) is incorporated. The soil mix is transferred to pots and onion seed is

Table 2.3 Biocontrol efficacy of *Penicillium oxalicum* strain PY-1 in suppressing development of symptoms induced by *Sclerotinia sclerotiorum* (Yang et al. 2008)

Treatments	Number of lesions induced per pot	Diameter of lesions (cm)
Control (check)	6.5 ± 0.50	2.80 ± 0.50
Concentration of BCA spores		
10^7/ml	ND	–
10^6 ml	2.0 ± 0.71	1.47 ± 0.20
Undiluted culture filtrate (CF)	ND	–
Ten-fold dilution of CF	4.0 ± 0.71	1.39 ± 0.11
LSD (P = 0.05)	1.01	0.88

ND Not visually detectable

planted at one seed/pot. Control without BCA is maintained. Appropriate statistical design has to be adopted with required number of replicates for each treatment. Observation on the emergence of onion plants and symptoms of white rot are recorded once in a week up to 14 weeks (Clarkson et al. 2002).

In order to assess the efficacy of the BCAs in reducing the infection of alfalfa petals or seeds by *Sclerotinia sclerotiorum,* selected racemes of alfalfa plants grown in the greenhouse were inoculated with pathogen alone, BCAs *Coniothyrium mimitans* or *Trichoderma atroviride* alone or combination of individual BCA and pathogen separately. Controls sprayed with water were also included. Three replications for each treatment were maintained. The treated racemes were individually covered with clear plastic bags to prevent cross-contamination. The alfalfa plants were transferred to a growth chamber and maintained at 20 °C under fluorescent light for 12 h/day. Infection by the pathogen was counted in each treatment after 7–12 days. *C. mimitans* was more effective than *T. atroviride* in suppressing Sclerotinia pod rot and seed rot due to *S. sclerotiorum* (Li et al. 2005). *S. sclerotiorum* also infects oilseed rape (*Brassica napus*) causing stem rot disease. *Penicillium oxalicum* strain PY-1 was evaluated for its biocontrol potential using a hyphae-mediated infection technique. Seedlings of oilseed rape were planted at the rate of two seedlings/pot. The pathogen was grown in potato dextrose broth. The mycelial mass, after rinsing with sterile water was homogenized in sterile blender with sterile water. Spores, culture filtrate of PY-1 strain or sterile water were sprayed on rape plants and air-dried. Then the suspension of hyphal fragments of the pathogen was sprayed on the plants treated as above. The inoculated plants were placed at 20° ± 2 °C and 100 % RH for 1 week. The number and size of lesions induced by *S. sclerotiorum* were recorded for each treatment. Spores (10^6 or 10^7/ml) and the culture filtrate (10-fold dilutions) suppressed the infection by *S. sclerotiorum* (Table 2.3; Yang et al. 2008).

The sensitivity of assay for determining the biocontrol efficiency of microorganism may be compared by using different plant species that may be sensitive to the pathogen. The potential of *Trichoderma hamatum* 382 for the control of Rhizoctonia damping- off of radish and crown and root rot of *Poinsettia* was assessed by employing radish bioassay and poinsettia bioassay (Krause et al. 2001). Isolates of *Fusarium oxysporum* including the BCA Fo47 and nonpathogenic isolates from the banana rhizosphere were multiplied in PDA and transferred later to Erlenmeyer flasks

containing Armstrong Fusarium medium (Booth 1977). In addition, two field strains *Trichoderma* (T22 and T5) were also tested. Banana plantlets were inoculated by applying aliquots of 40–50 ml of each fungal spore suspension to the steam-pasteurized soil as a drench. After 7 days, the plants were replanted in larger pots. The commercially available *Trichoderma* products (TS1 and B-rus) were dissolved in distilled water and applied as described above. A pathogenic isolate of *F. oxysporum* f.sp. *cubense* (*Foc*) race 4 was multiplied in PDA (half strength). Mycelial plugs taken from the cultures were used to inoculate sterilized millet seeds kept in Erlenmeyer flasks. Colonized millet seeds were transferred to steam sterilized soil at the rate of 15 ml seeds/500 ml of soil. The infested soil was transferred to plastic pots in which banana plants treated with BCAs were planted. Prior to planting, the roots of banana plants were slightly bruised by manually squeezing the root system to ensure infection by the pathogen. Appropriate controls were maintained. Disease development was recorded by cutting the rhizome open using scalpel, based on the disease severity rating as per Inihab's Technical Guidelines (Nel et al. 2006). *Calonectria pauciramosa* causes collar and root rot disease of red clover (*Trifolium pretense*). *Trichoderma harzianum* strain T22 was evaluated for its biocontrol potential against *C. pauciramosa*. The degree of virulence and T22 effects in controlling infections were highly variable among the isolates tested. In the nursery trial, the effectiveness of T22 in controlling infection was inversely related to the degree of virulence of *C. pauciramosa.* Overall, strain T22 exhibited good antagonistic activity in reducing microsclerotia production on carnation leaf and incidence and severity of collar and root rot disease (Vitale et al. 2012).

Efficacy of endophytic fungus *Phialocephala fortinii* and a dark septate endophytic (DSE) fungus as BCAs against *Verticillium longisporum,* causing Verticillium wilt of Chinese cabbage was assessed. The BCA isolates were grown on oatmeal agar for 2 weeks and 1 day-old Chinese cabbage seedlings raised axenically were transplanted into each fungal colony on the medium. Seedlings were incubated in growth chamber 20–25 °C under 16-h photoperiod for 1 week. These seedlings with agar medium were overlaid directly into the pathogen colony (*V. longisporum*) kept in plastic trays and incubated in the growth chamber as mentioned above. Disease severity ratings were assigned to each treatment based on a 0–3 scale. The DSE fungus LtVB3 was more effective in suppressing disease development as reflected by the percentages of external and internal disease symptoms of 84 and 88 % respectively (Narisawa et al. 2004). The hypovirulent binucleate *Rhizoctonia* (HBNR) isolates (3) were evaluated for their efficacy in protecting tomato plants against Fusarium crown and root rot (FCRR) disease caused by *F. oxysporum* f.sp. *radicis-lycopersici* (FORL). The HBNR-colonized barley grains were pulverized in a blender and mixed with potting medium (soilless peat-based). Pots were filled with the BCA-potting medium mix and one surface-sterilized tomato seed was sown in each pot. After a period of 21 days, the HBNR-amended soil was transferred to plastic pots containing FORL-infested potting medium (500 g) at a concentration of 1×10^5 spores/g of soil. The plants were maintained in the greenhouse for 7 days at 16–22 °C. Seedlings not treated with HBNR and challenged or not challenged with FORL were maintained as controls. Foliar symptom severity was

Table 2.4 Efficacy of fungal endophytes in reducing development of disease caused by *Rhizoctonia solani* in potato (Lahlali and Hijri 2010)

	Parameters evaluated		
Treatments	Disease index[a]	Yield (g/plant)	Disease severity
Control without BCA and pathogen	1.00 a	199.05 d	0.20
Control [*R. solani* (Rs)]	4.46 d	39.26 a	0.89
Rs + *Epicoccum nigrum*			
Isolate 1	1.86 b	132.07 bc	0.36
Isolate 8	1.13 a	176.34 d	0.22
Isolate 18	1.80 b	143.20 c	0.36
Rs + *Phomopsis* sp.	1.86 b	121.45 bc	0.37
Rs + *Alternaria longipes*	2.86 c	113.40 b	0.57
Rs + *Trichoderma viride*	1.00 a	211.25 d	0.20

Disease index calculated using the scale 1–5, where 1 = no lesions; 2 = small lesions on lower part of stem; 3 = moderately severe lesions; 4 = complete girdling of stem by lesions; 5 = death of plant
Disease severity of stem black scurf disease of emerged potato plantlets for each treatment
[a]Mean of 15 replicates

recorded for each treatment using a 0–4 disease rating scale. The HBNR isolates significantly reduced vascular discoloration and discoloration of total root systems (Muslim et al. 2003). In a later study, the biocontrol potential of nine isolates of binucleate *Rhizoctonia* (BNR) for reducing soybean root rot/seedling disease complex due to *R. solani* was assessed. *R. solani* anastomosis groups AG-4 and AG2-2 were tested using different combination of BNR isolates. Eight of the nine BNR isolates, when combined with AG-4 or AG 2-2, significantly increased emergence and survival of soybean and reduced disease severity, compared with controls containing pathogen alone (Khan et al. 2005). Fungal endophytes isolated from leaves of Norway maples were evaluated in the greenhouse for their efficacy in protecting potatoes from infection by *Rhizoctonia solani.* The BCAs and the pathogen were grown on rye seeds kept in bags for 30 days at room temperature. Sterilized Pro-Mix (pot mixture) was infested with *R. solani* (50:50). After 2 weeks, the infested and non-infested Pro-Mix were inoculated separately with test antagonists. After 1 week, potato seed tubers disinfested with sodium hypochlorite were planted at the rate of 1 tuber/pot. Observations on disease development were recorded up to 10 weeks after planting. *Trichoderma viride* was the most effective with 0.20 disease index which was equal to that in untreated control. The next best was *Epicoccum nigrum* in decreasing the effects of the pathogen (Table 2.4; Lahlali and Hijri 2010).

The biocontrol agents may be able to promote better growth of plants in addition to protecting them against fungal pathogens. Isolates of nonpathogenic, plant growth-promoting fungus (PGPF) *Fusarium oxysproum* was tested against *F. oxysporum* f.sp. *radicis-lycopersici* (FORL) causing Fusarium crown and root rot (FCRR) in tomato. The PGPF *F. equisetti* was found to be the most effective in controlling FCRR in the hydroponic rockwool systems. The disease reduction rate provided by *F. equisetti* was consistently high and significant in four experiments. The numbers of CFU of FORL per gram of fresh weight of stems that could be isolated were significantly reduced in plants treated with *F. equisetti.* The stem

extracts from *F. equisetti*-treated and pathogen-challenged plants significantly inhibited the germination and germ tube length of FORL microconidia, indicating the secretion of anti-fungal compounds by the BCA in the treated plants (Horinouchi et al. 2007). The efficacy of a plant growth promoting fungus *Penicillium* sp. EU0013 was assessed for suppressing the development of *Fusarium oxysporum* f.sp. *lycopersici* causing Fusarium wilt of tomato. The strain EU 0013 and its benomyl-resistant mutant EU 0013-90S inhibited the growth rate to the same level in dual culture tests. Two weeks old tomato seedlings were dipped in four different concentrations of conidial suspensions of the mutant (1×10^3–1×10^6/ml). Seedlings were planted in soils infested with either *F. oxysporum* f.sp. *lycopersici* 1CU1 or race 2. The disease suppression attained the maximum level in tomato seedlings treated with conidial suspension (at a concentration of 1×10^6/ml) of the mutant EU0013-90S. Higher root colonization with EU0013-90S showed significant reduction in *Fusarium* wilt disease. The results suggested that root colonization by the fungal BCA would be an important factor affecting its biocontrol efficiency against soilborne fungal pathogens (Alam et al. 2011).

Fusarium solani causes root rot disease of bean and the efficacy of *T. harzianum* and *T. asperellum* in protecting the bean plants against this pathogen, was assessed in the greenhouse. The pathogen and the BCAs were multiplied separately in wheat bran-corn mill medium. The roots of bean plants were dipped in fungal BCA biomass suspension (1.8×10^7 CFU) and planted in pots containing greenhouse soil infested with the pathogen grown on moistened wheat bran-corn mill. Development of symptoms (yellowing of leaves, vascular discoloration and wilting) on plants in different treatments was recorded till 6–8 weeks after planting. Stem sections were examined for the presence of pathogen under light microscope and isolations from the stem sections were made after surface sterilization. The BCAs either alone or in combination provided significant protection to bean plants against *F. solani* (Akrami et al. 2009). Soil conduciveness for *Rhizoctonia solani* AG-2-2 causing sugar beet root rot and damping-off disease was investigated. The in vivo antagonistic potential of *Trichoderma* isolates from disease patches and healthy areas was assessed by carrot bioassay. Carrot seeds (20 seeds/pot) were sown in rectangular plastic pots. *Trichoderma* isolates were cultivated on malt extract agar. Conidial suspensions of each isolate (4×10^6/ml) were added to the pots at the rate of 15 ml of conidial suspensions. *R. solani* grown on millet seeds was added at 4 seeds/pot one at each corner of the pot and the pots were kept in the greenhouse at 20–25 °C. Number of diseased seedlings in each treatment was counted. The area under disease progress curve (AUDPC) was calculated for comparing the disease development in different treatments. *Trichoderma* isolates from disease patches were found to be more efficient than the isolates from healthy areas (Anees et al. 2010).

The fungal biocontrol agent (BCA) *Meira geulakonigii* was evaluated for its efficacy in suppressing the development of powdery mildew pathogen *Podosphaera* (*Sphaerotheca*) *fusca* and phytophagous mite species *Tetranychus cinnabarinus* under greenhouse conditions. Application of *M. geulakonigii* reduced significantly the coverage of cucumber leaves by *P. fusca* on detached cotyledons as well as on growing plant foliage. Cucumber fruit yield was also substantially increased,

probably because of the disease suppression, following application of the BCA. Further, the mite population on the treated leaves was considerably reduced. The usefulness of the BCA for suppression of the powdery mildew disease and mite population could be a desirable attribute favoring its advancement for further evaluation for commercialization (Sztejnberg et al. 2004). Biocontrol agents differ in their biocontrol efficacy against different microbial plant pathogens. They may be specific against certain pathogen or may have wide spectrum activity showing antagonistic activity against several pathogens. The fungal endophyte *Piriformospora indica* was tested for its efficacy against the pathogens of winter wheat such as powdery mildew pathogen *Blumeria graminis* f.sp. *tritici*, stem base disease pathogen *Pseudocercosporella herpotrichoides* and root-infecting *Fusarium culmorum.* The effect of *P. indica* on germination rate of wheat, was assessed by pouring 100 ml of conidial suspension on the soil substrate immediately before sowing wheat seeds. Percentage of germination of wheat seeds was scored at 7 days of incubation in the greenhouse. The wheat plantlets were spray-inoculated with spore suspension of the BCA and the treated plants were challenge-inoculated with the pathogens individually after 7 days. For infection assays involving *F. culmorum*, fresh weights of roots and shoots of plants harvested after 30, 60, 90 and 120 days of growth were recorded. In the case of *P. herpotrichoides,* symptoms were scored by a predetermined scale, based on the number of brownish tissue layers at the stem base and expressed as fraction in percentage of the total layers developed. Effect on *B. graminis* f.sp. *tritici* was determined based on the number of mildew colonies per square centimeter present on selected leaves at 3, 6 and 9 days after inoculation. The symptom severity induced by these pathogens was significantly reduced by the endophytic *P. indica* (Serfling et al. 2007).

The antagonistic activity of phyllosphere yeasts present on leaves of greenhouse-grown tomatoes against *Botrytis cinerea* was evaluated using a detached leaf assay. Nine isolates reduced the disease index by >90 % relative to the controls. The yeast *Rhodotorula glutinis* Y-44 isolate was as effective as the fungicide in controlling the disease in the greenhouse. Population dynamics were studied over 8 weeks after application of Y-44 isolate. The BCA could colonize the plant surface successfully and there was a gradual reduction in the yeast isolate population. The BCA population decreased by 10-folds at 8 weeks after application. Y-44 isolate was effective against the gray mold disease affecting tomatoes under storage reducing the disease incidence by 50 % in wound inoculated tomatoes (Kalogiannis et al. 2006). Powdery mildew disease caused by *Podosphaera fusca* causes serious damages in greenhouses-grown melon crops. Mycoparasite-based products AQ10® containing *Ampelomyces quisqualis* and Mycotal® containing *Lecanicillium lecanii* and three strains of *Bacillus subtilis* were evaluated for their efficacy in reducing disease incidence. Melon plants routinely watered with complex nutrients and protected with pesticides, were planted at 8-leaf stage in experimental plots consisting of six rows each with 20 plants. All treatments were arranged in completely randomized block design (RBD), providing at least three replicates of 4 plants for each treatment. Mycoparasite-based products were applied with or without mineral oil. First application of the BCAs was made at the appearance of small mildew colonies (3–4 days

after pathogen inoculation). The second application was done at 10 days after the first round. Observations on the severity and number of leaves infected were recorded. Disease severity was expressed as percentage of leaf area covered by powdery mildew growth. Leaf disks (1 cm diam) were taken from infected areas and submerged in Tween 20 (0.2 ml/l) solution. The conidia were counted using a hemocytometer and expressed as number of conidia produced/cm^2 of leaf tissue. Mycoparasites were able to effectively reduce incidence and severity of powdery mildew disease of melon in the greenhouses (Romero et al. 2007).

The biocontrol potential of *Simplicillium lanosoniveum* for control of soybean rust disease caused by *Phakopsora pachyrhizi* was assessed. The fungal BCA did not grow or become established until uredinia were burst open. *S. lanosoniveum* colonized leaves within 3 days and sporulated within 4 days. The BCA suppressed the development of new uredinia by about 4-fold compared with control. In the presence of *S. lanosoniveum,* uredinia turned darkened (red-brown) and germination of urediniospores was markedly reduced. Assays using quantitative real-time PCR assay showed that the BCA colonized leaf surfaces, when plants were infected with *P. pachyrhizi*, either in a latent stage of infection or when symptoms appeared. However, when plants were inoculated prior to infection by *P. pachyrhizi,* no increase in BCA DNA could be recorded, suggesting that the presence of the rust pathogen was essential for the establishment of the BCA on soybean leaf surfaces. The amounts of DNA of *P. pachyrhizi* and rust disease severity were reduced in soybean leaves colonized by *S. lanosoniveum*, indicating the mycophilic and disease-suppressive nature of the BCA (Ward et al. 2012).

2.2.3 Evaluation of Biocontrol Activity Against Postharvest Pathogens

Postharvest pathogens mostly invade tissues of fruit and vegetable wounds occurring at different stages after harvest, during transit and in storage. Wound competence is a critical factor for the BCAs to successfully compete for space and nutrients against pathogens causing postharvest diseases. Wounding of plant tissue results in increased lypolitic acylhydrolase activity, activation of phospholipase and lipoxygenase and formation of free radicals. The yeast species *Cryptococcus laurentii* (LS-28) and *Rhodotorula glutinis* (LS-11) were multiplied in Lilly-Barnett (LB) medium in shake cultures at 28 °C for 16–18 h. Yeast suspension in sterile distilled water (1×10^8 CFU/ml) were prepared. Wounds were made using cork borer to remove the tissues (5 mm × 2 mm) from the apple fruits. Yeast cell suspension (30 µl/wound) was placed in the wounds immediately after wounding. Wounds were inoculated with 15 µl of freshly prepared conidial suspensions of *Botrytis cinerea* (2×10^4 spores/ml) or *Penicillium expansum* (1×10^4 spores/ml). Three replications were maintained for each treatment with suitable controls. Apples were

incubated at 20 °C in the dark at 90 % RH for 5–7 days. Numbers of wounds infected by the two pathogens were recorded. *C. laurentii* was faster and colonized more number of wounds than *R. glutinis*. Combined application of both yeast species enhanced colonization of wounds and antagonistic activity of both biocontrol agents against both pathogens (Castoria et al. 2003). Similar assessments were made to determine the biocontrol potential of *R. glutinis* for the control of *P. expansum* infecting stored apples (Castoria et al. 2005).

Biocontrol activity of *Cryptococcus laurentii* against *Alternaria alternata* and *Monilinia fructicola* infecting jujube fruit was evaluated under different storage conditions along with fungicides. Jujube fruits were wounded (3 mm deep and 3 mm wide) with a sterile nail. Yeast cell suspensions (10^8 CFU/ml) were applied at 25 µl/wound as challenge inoculation into the wounds. The fruits were placed in plastic boxes and kept at 20 °C for 5 days. Disease incidence (number of fruits infected) and lesion diameter in each treatment were recorded. *C. laurentii* was effective in reducing the diseases caused by both pathogens in jujube (Qin and Tian 2004). In a later study, *C. laurentii* was evaluated for its ability to control post harvest decay of peach due to *Botrytis cinerea* (gray mold), *Penicillium expansum* (blue mold) and *Rhizopus stolonifer* (Rhizopus rot). The BCA was multiplied in nutrient yeast dextrose broth (NYDB) liquid medium and yeast cells were gathered by centrifuging. The pellet containing yeast cells were resuspended in sterile water and the concentration was adjusted to 2×10^9–5×10^9 CFU/ml. Wounds (5 mm × 3 mm) were made using the tip of a sterile dissecting needle at the equatorial region of peaches. Aliquots (30 µl) at different yeast cell concentration were pipetted into each wound followed by challenge inoculation with the conidial suspensions of the pathogens at 15 µl/wound at 3 h after BCA placement. The fruits were kept at 25 °C for 4–5 days and infection rates by the different pathogens were recorded. Required replications and suitable statistical designs were used. Development of decay of peach was significantly reduced by *C. laurentii* without impairing any of the quality parameters (Zhang et al. 2007).

The yeast strain WY-1 was evaluated for its efficacy for suppressing postharvest decay of radish. The strain WY-1 was identified using 18S and internal transcribed spacer (ITS) 1-5.8S-ITS2 rDNA region sequences. The 18S and ITS1-5.8S-ITS2 rDNA sequences of the strain WY-1 were amplified and sequenced using universal primer pair NS1/NS8 and ITS4/ITS5. The ITS1-5.8S-ITS2 rDNA sequence and 18S neighbor-joining tree showed that WY-1 was a strain of *Cryptococcus albidus*. WY-1 strain inhibited the mycelial growth of *Alternaria* spp. and *Fusarium* spp. causing the decay of radish by 45.3 and 59.6 % respectively. Application of WY-1 strain at 10^8 CFU/ml reduced the incidence of decay and lesion development. When incubated for 6 days at 20 °C or 24 days at 4 °C, disease incidences were 2.8 and 1.4 % respectively, as against 98.6 % infection in untreated control treatment. *C. albidus* WY-1 was as effective as the fungicide thiabendazole. The population of *C. albidus* was high around inoculation sites at 4 °C even after 32 days after inoculation (6.7×10^5 CFU/cm^2), indicating the effectiveness of the WY-1 strain in suppressing the postharvest decay of radish (Chen et al. 2012).

2.2.4 Field Evaluation of Biocontrol Efficacy

Innumerable fungi have been isolated from soil, phyllosphere, spermosphere and different plant organs in the various habitats where microbial plant pathogens also exist, for assessing their biocontrol potential. Several fungal species that exhibited effectiveness against target pathogen(s) in vitro were found to be ineffective under field conditions/storage conditions, where they are expected to protect the intended crops or postharvest produce. Many factors in different combinations influence their competence and capacity to inhibit the development of phytopathogens and disease incidence/severity. Results obtained under field conditions are variable, because of several diverse factors. The availability of suitable ecological stages in the life cycle of the pathogens, favorable environmental conditions for rapid buildup of BCA populations and persistence for long period, in the absence of hosts by producing resting spores that can resist adverse conditions, are important. The fungal species have to prove their effectiveness under field conditions and such species/strains/isolates are considered for development of commercial products for large scale application. Some factors that have significant influence on the successful control of diseases under field condition are discussed below:

The nature of the pathogens, crop requirements for obtaining maximum yield, macroclimate of crop canopy, agricultural inputs including irrigation, fertilizers and fungicides and interactions among other organisms present in the plant environment have to be considered, while the efficacy of biocontrol agents is assessed. Field trials are conducted to assess the efficacy of the selected species/strains/isolates of the BCA that are found to be effective in in vitro assays. Experiments are carried out under field conditions for two or more seasons/years at as many locations as possible. Statistical designs such as random block design (RBD) and split-plot design are adopted, depending on number of treatments that are to be tested and compared. In the case of soilborne pathogens, sites that have past history of target disease incidence at high levels are selected. Alternatively high populations of the target pathogens are incorporated by using pathogen biomass raised on artificial media or large quantities of plant tissues infected by the pathogens. In the case of pathogens infecting aerial plant parts, the field experiments have to be conducted in seasons, when disease pressure can be expected to be high. The BCAs effective against seedborne pathogens may be tested by treating the seeds. Application of BCA may be taken up after sowing or transplanting the seedlings, depending on the disease intensity and buildup of BCA populations. The efficacy of the BCAs may be significantly affected by the form of BCA preparations such as spore suspensions, mycelial cultures with media, pellet, granules, powders etc.

2.2.4.1 Pathogen Factors

Availability and source of inoculum for initiation of disease early in the season is a critical factor. In the case of apple scab disease caused by *Venturia inaequalis,* the infected leaves shed on the soil form the important source of inoculum, since the

ascospores discharged from the fallen leaves cause infection in the young leaves and apple buds which begin to open in the spring. Hence, in order to reduce the inoculum produced from fallen leaves, water suspension of mycelia of *Athelia bombacinia* was applied either alone or in combination with urea. Formation of pseudothecia in the infected plant tissues was retarded by 14–21 days and ascospore production was delayed by 7–22 days, following treatment with BCA, compared with untreated control (Zvára et al. 1994). However, complete pseudothecia inhibition could be achieved only when very high antagonist concentration was applied. The BCA *Microsphaeropsis* sp. was applied as postharvest spray or as a ground application at 90 % leaf fall. This BCA was equally effective as *A. bombacina* in reducing ascospore production. Postharvest application of *Microsphaeropsis* sp. reduced total amount of airborne ascospores trapped by 70.7 and 79.8 %, as compared with non-treated plots respectively in 1997 and 1998 (Carisse et al. 2000). Fungal colonizers of sporulating colonies of *V. inaequalis* were tested for their efficacy in reducing the production of conidia of *V. inaequalis* on apple seedlings under controlled conditions. Four most effective isolates of the 63 screened isolates were evaluated under orchard conditions. Application of conidial suspensions of *Cladosporium cladosporioides H39* reduced the conidial production of *V. inaequalis* by 69 %, but there was no effect at 2 weeks after BCA application. Epiphytic and endophytic colonization of treated apple leaves by the BCA was significantly higher at 6 weeks after the last application (Köhl et al. 2009).

2.2.4.2 Biological Agent Factors

Most of the microorganisms, although showed effectiveness under laboratory conditions, proved to be ineffective in the greenhouse or field tests, since they were unable to establish themselves in environments prevailing in the test locations. This situation may probably be due to poor competitiveness and lack of growth conditions conducive for their development. The putative BCAs should be fast-growing and aggressive against target pathogens. In addition, the putative BCA should have wide spectrum of activity. *Trichoderma koningii* is effective against wheat take-all and root rot diseases (Worastit et al. 1994). If the microorganism has potential to act through two or more mechanisms on the pathogen, it may be able to provide better protection to the crops. The fungal BCA *Talaromyces falvus* (anamorph : *Penicillium dangeardii*) could suppress *Verticillium dahliae,* causing wilt diseases of tomato, potato and egg- plant (brinjal) and also parasitize *Sclerotinia sclerotiorum, Rhizoctonia solani* and *Sclerotium rolfsii.* Chitinase produced by *T. flavus* effectively arrested the development of *S. rolfsii* and *V. dahliae*. Spore germination, hyphal growth and melanization of newly formed sclerotia of *V. dahliae* were significantly retarded by the antifungal compounds produced by *T. flavus.* Microsclerotia were killed, when treated with culture filtrate of *T. flavus* and this toxicity was considered to be due to the glucose oxidase activity of the BCA (Madi et al. 1997). Rapid colonization of tissues by the BCA making the plant tissues unavailable for infection by the pathogenic fungus is one of the effective mechanisms of action by the BCA.

Development of epidemics of *Botrytis cinerea* on grapevine and *Septoria nodorum* and *S. tritici* on wheat crops was prevented by the BCAs *Ulocladium atrum* and *Chaetomium cochlioides* (Lennartz et al. 1998). The biocontrol potential of *Talaromyces flavus* against *Verticillium albo-atrum* causing potato wilt disease was assessed in vitro and in the greenhouse conditions. *T. flavus* was applied as seed treatment and soil application and the treatments were randomized in complete block design with four replications. The isolate Tf-Po-V-52 was the most effective in suppressing the disease development, when applied as seed treatment, with minimum infection index. Under field conditions the *T. flavus* isolate-treated plots had an infection index of 0.15 as against 3.5 in the untreated control plots. The results showed that *T. flavus* could be useful for the management of potato wilt disease caused by *V. albo-atrum* (Naraghi et al. 2010).

Sclerotinia sclerotiorum has wide host range that includes many crops (total of 383 plant species). The pathogen produces sclerotia externally on affected plant parts and also internally in the stem pith cavities. Sclerotia from infected plant tissues reach the soil, where they overwinter and produce apothecia or mycelium during the next season. Pathogens of this nature have to be controlled by biological destruction at sites, where the inoculum for new infections are likely to be produced by establishing the BCAs at the source of inoculum. Sclerotia of *S. sclerotiorum* could be killed in root surface, inside roots and stem of sunflower plants by applying *Coniothyrium minitans* (Huang 1977). Application of *C. minitans* not only reduced apothecial production from sclerotia buried in the soil, but also increased parasitism on sclerotia produced on bean plants (McLaren et al. 1996). Exogenous nutrients from senescent petals or plant tissues and pollen grains are required for ascospore germination and elongation of germ tubes or mycelia of *S. sclerotiorum* to infect other plant tissues of alfalfa (Li et al. 2005). Senescent petals of alfalfa usually remain attached to pods during pod development facilitating infection of alfalfa pods and seeds by *S. sclerotiorum.* Field experiments with *C. minitans* and *Trichoderma atroviride* demonstrated that *C. minitans* effectively suppressed Sclerotinia seed rot in all 3 years of testing, whereas *T. atroviride* was not effective in reducing seed rot in any of the field trials. Application of *C. minitans* was equally effective as the fungicide benomyl. This study clearly indicated the potential of *C. minitans* to be an alternative to fungicides (Table 2.5; Li et al. 2005).

Sclerotium cepivorum causes onion white rot disease accounting for substantial losses. The efficacy of three strains of *T. viride* (L4, S17A, 99-27) was assessed in a three-stage screening system to degrade sclerotia of the pathogen on agar and in soil and to reduce white rot disease on onion seedlings. Two strains (L4 and S17A) were selected for field evaluation based on the results of the screening procedures followed. These two strains of *T. viride* consistently reduced white rot symptoms, when they were found drilled in guar gum with bulb onion seed in 2000 and 2001. The BCA application was as effective as tebuconazole. The phytotoxic effects of the fungicide observed in the field experiments may be a favorable basis for opting for BCA use against *S. cepivorum*. However, the formulation of BCA bran cultures and the precise placement in furrows using a special drilling equipment may limit the extent of BCA application (Clarkson et al. 2002).

Table 2.5 Biocontrol potential of BCAs and fungicide against *Sclerotinia sclerotiorum* causing blossom blight of alfalfa (Li et al. 2005)

	% diseased pods			% diseased seeds		
Treatments	2000	2001	2002	2000	2001	2002
S. sclerotiorum (Ss)	64.6 a[a]	41.8 a	72.2 a	8.7 a	21.5 a	24.6 a
T. atroviride (Ta) + Ss	50.9 b	39.8 a	42.2 b	7.7 a	19.0 a	19.5 a
C. minitans (Cm) + Ss	38.3 bc	30.1 ab	29.7 c	4.0 b	12.5 b	8.0 b
Benomyl + Ss	33.3 cd	22.0 bc	23.1 c	4.3 b	9.2 b	5.6 b
Water	20.3 d	11.9 c	22.0 c	4.6 b	8.2 b	6.3 b
Standard error	0.3	0.3	0.4	0.1	0.1	0.2

[a]Means followed by different letters are significantly different at P = 0.05 level according Duncan's multiple range test (DMRT)

Bottle palm *(Hyophorbe lagenicaulis)* is seriously affected by *Thielaviopsis* bud rot disease caused by *Thielaviopsis paradoxa.* The biocontrol efficacy of formulated product containing *Chaetomium cupreum* CC1-10 and *C. globosum* CG1-12 was tested along with a crude methanolic extract of these two BCAs under field conditions. The pelleted formulation was applied into the rhizosphere soil at the rate of 20 g/tree and the extract was sprayed (50 ml/20 l of water) at 7 days intervals. Complete randomized block design with six replications was adopted for placement of treatments. Bottle palms completely recovered from disease, following application of the *Chaetomium* formulation. The treated trees produced fresh healthy leaves indicating that the infected trees were rescued by treatment with the BCA formulation (Soytong et al. 2005). Impact of the application of the BCA *Piriformospora indica* on the incidence wheat diseases under field conditions was assessed. Symptoms induced by powdery mildew pathogen *Blumeria graminis* f.sp. *tritici* did not show any difference compared with untreated control. In contrast, severity of stem base disease caused by *Pseudocercosporella herpotrichoides* was significantly reduced, following application of the endophytic BCA. Differential effects of the BCA on the diseases, affecting leaves and stem bases of wheat plants were observed, although the BCA was able to reduce all the diseases under greenhouse conditions (Serfling et al. 2007).

Different kinds of fungicides and other plant protection chemicals are applied on the crops and soils to protect the plants against biotic and abiotic stresses. It may be desirable to have biocontrol agents that may be able to function effectively against target pathogen, when the fungicide is also applied against the same pathogen or other pathogens. Thiram has been shown to alter the expression of several genes of the BCA *Fusarium oxysporum* CS-20. Based on nucleic acid sequences homology, one band (BM1 24-1) showed homology to an ABC transporter. Quantitative real-time PCR technique was used to evaluate gene expression after exposing the mycelia of CS-20 to 25 µg active ingredient of thiram in liquid culture. The ABC transporter was upregulated by 15-fold and 10-fold at 1 and 2 h respectively after treatment with thiram. At 8 h after treatment no difference in ABC transporter expression could be discernible. Transcription of the gene encoding ESTBM 24-1 was induced in CS-20, in response to treatment with thiram and it might provide

resistance in *F. oxysporum* isolate C-20 to fungicide and toxins. The ability to tolerate the toxins produced by the pathogens and other chemicals may be critical to the successful biocontrol of the target pathogen (Fravel et al. 2007).

2.3 Identification and Differentiation of Mycorrhizal Biological Control Agents

2.3.1 Methods of Detection and Identification

Symbiotic associations of mycorrhizal fungi with plant roots are extremely common in nature. Several plant species have co-evolved with these symbionts indicating their dependence on these associations. Plants provide the fungi with photosynthates and they in return obtain mineral nutrients from the fungi. In contrast, pathogenic fungi develop at the expense of host plants which may ultimately succumb to the damages inflicted by the pathogenic fungi. However, in some cases, the mycorrhizal association might be less mutualistic or even parasitic to the plant (Klironomos 2003). Two principal types of mycorrhizal associations have been recognized (i) arbuscular mycorrhizal (AM) symbiosis and (ii) ectomycorrhizal (ECM) symbiosis.

The arbuscular mycorrhizal (AM) symbiosis is formed between the roots of the most higher plants and Zygomycete fungi belonging to the order Glomales. It has been estimated that more than 80 % of the land plants may form this type of association, including many agriculturally and horticulturally important crop species (Smith and Read 1997). It appears that this mutalistic association is very ancient dating back to more than 400 million years, when colonization of land by plants is considered to have occurred and it remains a crucial factor for sustainability of most terrestrial ecosystem (Parniske 2008). Possibly because of long periods of coevolution with plants, AM fungi have been highly interdependent at both ecological and physiological levels. The AM fungi are obligate biotrophs that depend on their ability to colonize a host plant species for completion of their life cycle. The mycelia of AM fungi have an extraradical phase that grows out into the soil and an intraradical phase that proliferates inside the root. These two phases grow in very different environments and clearly differ in morphology and physiology (Dodd et al. 2000). AM fungi transfer inorganic nutrients and water to the plant and receive carbohydrates in exchange. By driving this bidirectional nutrient transport between soil and plants, they are highly relevant for global phosphorus (P), nitrogen (N) and CO_2 cycles. Furthermore, they affect directly and indirectly the diversity and productivity of land-plant communities by their central role at the soil-plant interface. More importantly the role of AM fungi in protecting plants against microbial plant pathogens such as *Phytophthora parasitica* (Cordier et al. 1998), *Aphanomyces euteiches* (Rosendahl 1985) and *Sclerotium rolfsii* (Krishna and Bagyaraj 1983) has been investigated.

Ectomycorrhizal (ECM) symbiosis is observed between fungi belonging to Basidiomycetes and Ascomycetes and plants belonging to the taxa of Gymnosperms and Angiosperms. Large trees, shrubs and occasionally herbs develop ECM symbiosis. Ectomycorrhizal fungi form, frequently an extensive, fan-like network of hyphal structures that grow from the ECM root. The ECM fungi can be grown in pure cultures. These fungi, when colonize plant roots, form a fungal sheath known as mantle which covers the rootlets. The hyphae can penetrate between the cells of the root and form a network of intercellular hyphae. But the hyphae do not penetrate into the plant cells. Extraradical mycelium produced from the mantle spreads on the soils and also absorbs nutrients from the soil. The extraradical mycelial hyphae become aggregated forming root-like structures called rhizomorphs. Hyphae of the mantle may penetrate between cells of the root cap behind the apex and form the characteristic Hartig net between epidermal cells. The Hartig net forms the interface between the plant and the ECM fungus and it is involved in the bidirectional nutrient transfer. The mycorrhizal mycelium surrounds the plant roots and the quantity and quality of the exudates from the roots significantly differ from the non-mycorrhizal roots. As the mycorrhizal mycelium forms a physical barrier, entry of root-infecting pathogens is prevented as in the case of *Phytophthora cinnamomi* which is excluded from infection of roots of eucalyptus by the mycorrhizal fungus *Pisolithus tinctorius*. On the other hand, the mycorrhizal fungi, like *Leucopaxillus cerealis, Laccaria laccata, Lactarius deliciosus* and *Suillus luteus* were able to produce antibiotics that inhibited *P. cinnamomi* in agar plate tests. Likewise, protection offered by the mycorrhizal fungi *P. tinctorius* or *Thelephora terrestris* led to reduction in infection of pine trees by *P. cinnamomi* to 10 % of that in unprotected pine trees (Campbell 1989).

Application of mycorrhizae to agricultural systems is highly promising, because they not only possess potential for the control of crop diseases, but they also provide other benefits such as enhanced phosphorus nutrition and tolerance to metal toxicity and drought. Inspite of the several advantages, adequate efforts have not been taken to develop precise methods for identifying effective mycorrhizal fungal species/isolates and to understand the mechanisms by which biocontrol activities of these agents lead to reduction in disease incidence and/or intensity. Available information on these aspects is discussed to indicate the useful lines of research.

2.3.1.1 Taxonomy of Mycorrhizal Fungi

The mycorrhizal fungi have been classified based on the spore morphology and mycelial characteristics. As the arbuscular mycorrhizal fungi (AMF) are biotrophs, identification of these fungi is very difficult due to the lack of cultural characteristics. Further, these fungi do produce asexual stages only, but not sexual stages, the characteristics of which are required for taxonomic classification of fungi. This situation led to many AMF species being placed in one genus *Glomus*, although they belong phylogenetically to different orders. The individual species forming different spore forms have been described as members of different orders. Further, many

species cannot be reliably identified, while examining heterogenous field samples. However, certain morphological characteristics useful for the tentative identification of the fungi that have biocontrol potential are described briefly.

Arbuscular mycorrhizal fungi (AMF) have been classified into six genera under order Glomales and class Zygomycetes as detailed below (Morton and Benny 1990).

Class	:	Zygomycetes
Order	:	Glomales
Suborder	:	Glomineae
Family	:	Glomaceae
Genus	:	1. *Glomus*
		2. *Sclerocystis*
Family	:	Acaulosporaceae
Genus	:	1. *Acaulospora*
		2. *Entrophospora*
Suborder	:	Gigasporineae
Family	:	Gigasporaceae
Genus	:	1. *Gigaspora*
		2. *Scutellospora*

The taxa included in Glomales were primarily identified based on spore morphology. The morphological characteristics considered, do not reveal the diversity that occurs among strains of a morphologic species. Identification of AMF based on morphological characteristics is further complicated, since one root sample contains more than one species of AMF. Hence, the search for more reliable techniques has to be intensified.

A. *Glomus mosseae*

Class: Zygomycetes; Order : Glomaromycota (Mucorales); Family : Endogonaceae

Chlamydospores (thick-walled hyphal cells) borne on undifferentiated nonseptate hyphae, may be intercalary or terminal in position. Chalmydospores may be formed in loose sporocarp in the root tissues or found free in soil. These spores may be yellow to brown, globose to ovoid or irregular in shape. The chlamydospores may germinate in agar, but further development is not seen (Mosse 1962).

The ectomycorrhizal (ECM) fungi establish association commonly with trees like *Pinus* spp. growing in forest ecosystems. These fungi form puff balls with moderately thick hard peridium. They belong to the Basidiomycetes and Ascomycetes and a few of the members of Zygomycetes and Hyphomycetes also form ectomycorrhizae. It is estimated that as many as 5,000–6,000 fungal species may be involved in ectomycorrhizal association (Molina et al. 1992). All ECM fungi are included in the Kingdom Eumycota, class Zygomycetes, Hyphomycetes, Ascomycetes and Basidiomycetes. Ascomycetes forming ectomycorrhizal association with higher plants are placed in orders Pezizales, Elaphomycetales and Leotiales. Pezizales exist as saprophytes on wood, soil and other organic materials. They produce conspicuous fruiting bodies called as cups, morels and truffles. Majority of the ECM fungi belong to Basidiomycetes.

Morphological characteristics have been used for the identification of the ECM fungi. The base and flesh are examined for the classification of epigeous fungi that form mushroom-like and truffle-like fruiting bodies. The characteristics of cap, hymenium, stipe (stem) and universal veil are considered. In the case of hypogeous fungi, characteristics of the peridium, gleba, locules and columella are taken into consideration (Krishna 2005).

B. *Pisolithus tinctorius*

Class: Basidiomycetes; Order : Lycoperdales

The fruiting bodies are oval or club shaped with well-developed rooting base. Powdery mass of spores are released, when the peridium breaks. Basidia produce globose, brown spores with warts arranged irregularly on the surface. The ectomycorrhizal (ECM) fungi can be grown in artificial media and hence, the morphological characteristics can be determined using light and/or electron microscopes. The ECM fungi are commonly found in soils of boreal and temperate forests and have greater saprophytic capacity to survive on organic matter. Ectendomycorrhizal (EEM) fungi form another group of fungi capable of establishing symbiotic associations with conifer and deciduous tree nurseries. The EEM fungi can be differentiated from ECM fungi by the presence of a thin or fragmented mantle and intracellular penetration into root cortical cells. They belong to the class Ascomycetes, order Helotiales and Pezizales. *Wilcoxnia* spp. are frequently recovered from seedling roots (Yu et al. 2001).

When the mycorrhizal fungus penetrates the root, following formation of the appressorium, the intraradical mycelium develops. The AMF enters the epidermis and exodermal cells by coiling hyphae which branch and reach the root cortex. Two principal types of colonization of root tissues by AMF are recognized. Arun type of colonization is characterized by rapid hyphal growth through intercellular spaces. These hyphae are involved in the translocation of nutrients from and to the extraradical mycelium. Short side-branches may penetrate cortical cells from which branched, thin-walled and short-lived arbuscules are formed. The arbuscules are enclosed in membranes of plant origin. Paris type of colonization is common in large trees and it is typified by an extensive network of intracellular coiled hyphae spreading within the root cortex. Arbuscules are less frequently formed and the growth of the fungi is much slower compared with Arun type of colonization (van Arale 2009).

2.3.1.2 Biochemical Techniques

Microscopy-based methods have been used for visualizing root colonization of mycorrhizal fungi. Specific stains such as acid fuchsin or trypan blue are employed to stain fungal structures of *Glomus mosseae* in roots of leek (*Allium porum*) (Saito et al. 1993). Biochemical methods have been employed to characterize some of the mycorrhizal fungi. Development of mycorrhizal fungi may be monitored by

determining the contents of ergosterol and phospholipid fatty acids. Ergosterol has been demonstrated to be a suitable marker for assessing the biomass of extraradical mycelium of ECM fungi (Wallander et al. 2001). On the other hand, fatty acids could be used as biochemical markers for both AM fungi and ECM fungi, as they are present at high concentration in these fungi (Wallander et al. 2001; Olsson et al. 2003), Fatty acid analysis can facilitate generation of specific fatty acid signatures with which AM and ECM fungi could be distinguished. It can also form the basis for differentiating AM fungi from saprophytic and parasitic fungi and bacteria (Olsson 1999). Furthermore, fatty acid analysis might indicate the extent of AM fungal root colonization within a species (van Aarle and Olsson 2003).

Identification of mycorrhizae based on protein or isozyme patterns has been shown to be useful to differentiate certain species. Isozyme polymorphism (variations in forms of an enzyme) has been used as the basis for resolving the identity of isolates of a morphologic species of AM/ECM fungi. Isozyme profiles were used for the identification and also quantification of Morels. It was also possible to characterize the intraspecific crosses among monosporal strains of *Morchella esculenta* (Wipf et al. 1996). Differentiation of *G. mosseae* and *G. caledonium* was achieved based on the polymorphism in MDH and esterases (ESTs). Separation of *Gigaspora* at the subgeneric level was possible utilizing the MDH profiles as the criterion for differentiation (Giovannetti et al. 2003). One-dimensional sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) technique was applied to separate spore proteins. The banding patterns could be the basis for differentiating inter-and intra-specific variations among isolates of *Glomus mosseae* and *G. coronatum* (Dodd et al. 1996). In a later study, the consistency of spore protein patterns was assessed by analyzing the samples from different locations, hosts and generations of fungi using SDS-PAGE technique. Profiles of soluble proteins from spores were consistent and remained unaltered due to location, host or genera of the mycorrhizal fungi tested. The similarity index was 98 % for two isolates of *G. coronatum* obtained from different locations. In the case of *G. mosseae,* similarity index was 100 % for isolates from different plant species (Avio and Giovannetti 1998). The reliability of using the electrophoretic patterns of soluble proteins of spores of different *Glomus* spp. was tested. The AM fungi *G. mosseae, G. clarum, G. etunicatum* and *G. fasciculatum* could be discriminated by the protein profiles generated by SDS-PAGE procedure. Each fungal species possessed specific signature protein bands that were consistent and reproducible and they can be used as marker for ecological investigations (Xavier et al. 2000).

Different isoforms of an enzyme synthesized by a fungal species are involved in specific physiological functions. The electrophoretic patterns of enzymes have been shown to be useful for the identification and differentiation of mycorrhizal fungi. PAGE separation and specific staining of the proteins (enzymes) were employed to identify and quantify the mycorrhizal fungi. The diagnostic enzyme(s) represented by the unique band present only in the samples of a particular species/strain is identified. Then a large number of samples can be run. The study by

Kjoller and Rosendahl (2002) indicated that generally the roots of a plant species are dominated by a single species of AM fungi, as in the case of *Glomus geosporum* and the AM flora did not change significantly during one crop season. Pea plants are infected by the fungal pathogen *Aphanomyces euteiches* causing root rot disease. Infection by *A. euteiches* was significantly reduced by bioprotection offered by the AM fungus *Glomus fasciculatum* (Rosendahl 1985). Extent of bioprotection was determined by measuring the root rot disease index and quantifying the pathogen in roots by ELISA technique (Slezack et al. 1999). Bioprotection by *G. mosseae* to pea plants against *A. euteiches* was found to be dependent on a fully established symbiosis with the presence of arbuscules. Upon mycorrhization of wild type pea cv. Frission, synthesis of one basic and four acidic chitinase isoforms and one chitosanase isoform was induced. After infection by *A. euteiches* in bioprotected plants, mycorrhiza-related chitinase and chitosanase isoforms were maintained at higher levels. The results provide evidence for the involvement of mycorrhizae-related chitinolytic enzymes in the bioprotection of pea plants against the root rot pathogen (Slezack et al. 2000).

2.3.1.3 Immunological Techniques

Immunological techniques have been demonstrated to be more sensitive, rapid and reliable for the detection, differentiation and quantification of fungal pathogens as well as fungal biocontrol agents, when compared to conventional isolation-based methods. The effectiveness of the immunoassays for the identification of some mycorrhizal fungi has been reported. Among the immunoassays, fluorescent antibody technique and enzyme-linked immunosorbent assay (ELISA) have been successfully applied to identify and differentiate AM and ECM fungi. Polyclonal antibodies (PAbs) labeled with fluorescent dye were produced, using sporewall antigens to induce PAbs. These antibodies reacted specifically to reveal the differences in AM fungi. Polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) specific to beta glucans and their variants in AMF were produced. By using the MAbs specific to β-1, 3-glucans, the genera *Glomus* and *Acaulospora* in the *Glomineae* were investigated for the distribution of β-1, 3-glucans in these two genera. The results suggested that *Glomus* and *Acaulospora* might represent an outlying group in the Zygomycetes, whereas *Gigaspora* would remain firmly placed in this class (Aldwell and Hall 1987). The cross-reacting PAbs related to non-specific glycoconjugates were successfully employed to differentiate *Acaulospora laevis* and *Gigaspora margarita* (Sanders et al. 1992). An unidentified ECM fungal species associated with *Quercus pubescens* was detected and differentiated from three species of the genus *Tuber* by employing ELISA technique (Zambonelli et al. 1993). Both PAbs and MAbs were generated using the hyphae as immunogens. The dot immunoblot assay (DIBA) was successfully applied for the detection of mycorrhizal fungi belonging to the family Acaulosporaceae (Gobel et al. 1995).

2.3.1.4 Nucleic Acid-Based Techniques

The genomic region of fungi encoding RNA contains genes and spacers that have evolved at different rates. Hence, it may be easier to design nucleic acid markers representing the taxa at different taxonomic levels. Analysis of the rDNA reveals a high degree of conservation in coding regions. But considerable differences in sequences of the spacers in ITS and intergeneric spacer (IGS) have been detected. Several target regions with varying levels of genetic resolution can be identified within families. Different nucleic acid-based techniques have been applied for the detection and differentiation of AM and ECM fungi. Among the molecular techniques applied for the detection and identification of mycorrhizal fungi, polymerase chain reaction (PCR) has been the most commonly applied procedure. Molecular characterization of AM fungi has been achieved by PCR on DNA from roots of host plants, spores or soil samples. The primers targeting rDNA regions as molecular markers have been constructed for specific identification of mycorrhizal fungi. The ribosomal genes contain both conserved and variable regions and desired sequences of these regions are used for designing universal primers and amplify the target DNA sequences, employing the polymerase enzyme at appropriate conditions. As these genes occur in high copy numbers, it is sufficient to have small quantities of DNA to run PCR amplification. The rRNA gene of *Glomus mosseae* was probably the earliest to be utilized as an indicator of the phylogenetic status of AM fungus (Simon et al. 1992). Later, a randomly amplified fragment of *G. mosseae* was employed to generate specific primers for detecting and quantifying this AM fungus in the root system (Lanfranco et al. 1995; Edwards et al. 1997).

Identification of taxa in the order Glomales was earlier based extensively on the morphology of the spores. The inadequacy of classical taxonomic approach was eliminated by molecular studies that helped resolve certain controversies arising out of morphological observations. Two spore types named 'synanomorphs' were produced from the same hypha and these spores were similar to those of *Glomus leptotrichum* and *Acaulospora gerdemanii*. However, their rDNA sequences showed that this dimorphic fungus belonged neither to *Glomus* nor to *Acaulospora* nor to any of the established families. But it belonged to one of the several ancestral, highly divergent lineages within Glomales (Redecker et al. 2000). The molecular investigations suggest that a new classification of AM fungi has to be attempted, since the genus *Glomus* is inadequately defined and represents a conglomerate of fungi that are morphologically hard to differentiate. Based on the rDNA ITS sequences, a high degree of variations could be detected in the natural populations of *G. mosseae* suggesting the need for splitting this AM fungal species (Antoniolli et al. 2000).

Inhibitors of PCR amplification may be present in plants, fungi and organic matter present in the soil. The nested-PCR format may be utilized to circumvent the problem of PCR inhibitors. Primers specific for different species of fungi were employed to assess the populations of AM fungi in the greenhouse and also in field-collected samples. The presence of more than one fungus even in a single root piece (1 cm) could be detected by the nested-PCR procedure. A synergistic interaction between AM fungi might be possible in such mycorrhizal associations (Kjoller

and Rosendahl 2000). By combining nested-PCR with single-strand conformation polymorphism (SSCP) technique, isolates of *Glomus mosseae, G. caledonium* and *G. geosporum* present side by side in colonized roots could be differentiated. This was possible, because of the polymorphism existing in the gene coding for a large subunit (LSU) of ribosome. The SSCP analysis has the potential to detect single-base substitutions. Both known and unknown AM fungi present within roots could be detected and differentiated by the SSCP analysis (Kjoller and Rosendahl 2000). The SSCP technique was used to screen the nested PCR products for the presence of multiple sequence types. Nested PCR products were sequenced, if necessary, after reamplification of single SSCP types. The root-derived sequences were aligned with sequences from spores of 17 cultural *Glomus* isolates. Five of these isolates were from the same field cropped with peas. Some of the root-derived sequences were lineages other than those species isolated from the field (Kjoller and Rosendahl 2000).

A PCR assay was developed for the identification of ectomycorrhizal fungi in root samples collected from the field and mycelia and basidiocarps. Primers designed based on the sequences of ITS1 and ITS4 and their derivatives were employed in conjunction with random amplified polymorphic DNA (RAPD) procedure. However, coamplification of contaminating DNA material also occurred. In the present study, primers were developed from the sequences of ITS8. Amplification of DNA isolated from mycelia and basidiocarps resulted in single PCR products of variable sizes (300–560-bp). After electrophoresis, the PCR products were directly characterized by sequencing using ITS8 as the sequencing primer. This procedure resulted in clearly distinguishable PCR fragments (Fig. 2.5). The protocol developed in this study is applicable to a broad range of species within Basidiomycetes. In all analyses, single PCR amplicon was obtained giving no chance for ambiguity. The procedure could be completed in a single working day. The DNA isolation did not require any organic solvents and very small rootlets (3–5 mm) were sufficient, indicating the efficiency and simplicity of the procedure developed (Kulmann et al. 2003; Appendix 2.9).

Quantitative real-time PCR (qRT-PCR) assay was applied to quantify rapidly the colonization of the AM fungus *Glomus intraradices* in planta. This technique utilized fluorigenic probes with specialized instrumentation to detect the PCR product during the exponential phase of the amplification cycle. The *G. intraradices-specific* primer GiAM was designed, using the sequences of ITS 28S rDNA region. Primers for host tissue were developed, using chitinase and chalcone synthase gene sequences. The respective specificity of the primers was confirmed using appropriate targets. The GiAM primer showed amplification only in the presence of the *G. intraradices,* but not when the template was substituted by plant or water controls. The GiAM primer amplified as low as 1 pg of its target DNA which allowed detection of a single spore of the fungus. The consistent amplification of the diagnostic 64-bp fragment from a single spore of *G. intraradices* with the specific primer GiAM strongly indicated the level of sensitivity of the qRT-PCR assay developed in this study (Alkan et al. 2004). Differences in AM fungi at the species level may be reliably assessed by adopting a combination of DNA amplification by PCR followed by cleaving the PCR products

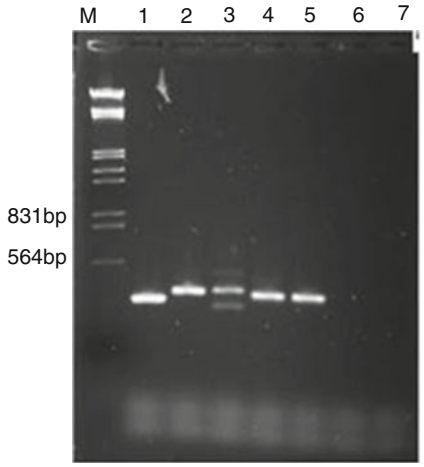

Fig. 2.5 Identification of ectotrophic mycorrhizal fungi using a specific PCR assay based on the amplification of the template DNA obtained from mycelia associated with roots. Pattern of ethidium bromide-stained fragment after gel electrophoresis in a TAE agarose gel: *Lane M*: molecular size marker; *Lane 1: Amanita muscaria; Lane 2*: root sample P1, *Hebeloma helodes; Lane 3*: root sample P2, *Hebeloma mesopheum; Lane 4*: root sample; Pt, *Rhizopogan rubexens; Lane 5*: root sample IM, *R. rubescens; Lane 6*: extraction without sample and *Lane 7*: negative control (Courtesy of Kulmann et al. 2003 and with kind permission of Korean Society of Mycology)

with restriction enzymes. The PCR-restriction fragment length polymorphism (RFLP) procedure was employed to identify the Basidiomycetes forming mycorrhizal association with birch trees. The fungal DNAs were isolated from axenic cultures of fungi collected from the forests. The ITS regions of the fungi were amplified using specific primers and the PCR products were digested with restriction enzymes *Alu*I, *Bsur*I, *Hinf*I, *Hpa*II and *Taq*I. The PCR-RFLP analysis was useful for the identification of fungi belonging to Basidiomycetes (Krupa 1999).

Ectomycorrhizal (ECM) communities colonizing the roots of loblolly pine (*Pinus taeda* L.) were assessed by applying terminal restriction fragment length polymorphism (T-RFLP) procedure. DNA was extracted from root tips of pine trees separated from soil cores after classifying the root tips based on the morphological characteristics (morphotypes). Labeled primers were employed to generate terminal restriction fragments (TRFs) for molecular fingerprinting of root colonizing fungi. The morphotypes generally correlated well with specific TRFs and sequence analysis showed that TRFs could be employed to discriminate among fungal types. Sequence analysis indicated that the ECM fungi belonging to Russulaceae, Thelephorales and Tricholomataceae could be fingerprinted with T-RFLP facilitating their identification and differentiation. As some morphotypes shared TRFs, dual analysis ITS1 and ITS2 might be required for accurate fingerprinting of fungal types. This study showed that T-RFLP approach could be used to analyze the mycorrhizal communities using the root tips collected from soil cores (Burke et al. 2005).

Molecular methods based on fungus-specific amplification of the nuclear ribosomal internal transcribed spacer (ITS) regions have provided reliable characterization of mycorrhizal fungal species/strains. PCR-based techniques are used frequently now to overcome the problems associated with limited morphological variations and culture biases in orchid mycorrhizal investigations (McCormick et al. 2004; Suarez et al. 2006). Fungus-selective primers which minimize amplification of plant sequences, while allowing robust amplification of all tested Basidiomycetes were developed to study fungal diversity in mycorrhizas associated with orchids. The primer pair ITS4-Tul was very effective for screening environmental samples for an array of orchid-associated *Tulasnella* spp. (Taylor and McCormick 2008). Inclusion of the ITS region and the large subunit (LSU) rDNA region has been shown to allow robust species level resolution and phylogenetic analyses. The ITS region and the 5′ part of LSU rDNA of a set of well-characterized, but phylogenetically diverse AM fungi, were sequenced for designing primers. These primers were found to be suitable to amplify DNA from members of all known lineages of the order Glomales. These primers targeted specific binding sites in the small subunit (SSU) or LSU rDNA. Mixture of primer sets were employed to cover the defined sequence variability. The DNAs of all tested AM fungi were successfully amplified with the primers developed in this study. This procedure may allow molecular ecological studies covering all AM fungi lineages (Krüger et al. 2009).

White spruce (*Picea glauca*) roots are colonized by ectendomycorrhizal fungi *Wilcoxina* spp. The *ech42* endochitinase gene from *Trichoderma harzianum* has been inserted into plant genomes to enhance their resistance to fungal pathogens. Transgenic black spruce (*P. mariana*) expressing *ech42* was shown to be more resistant to the root rot pathogen *Cylindrocladium floridanum* (Nöel et al. 2005). In order to assess the impact of endo-chitinase gene present in the transformed spruce plants on the colonization of root tips by *Wilcoxina* spp. and the dark septate endophyte *Phialocephala fortinii,* a real-time PCR assay was developed. The ITS rRNA region was amplified and the amplicons were sequenced. Colonization of root tips by *Wilcoxina* spp. was monitored by real-time PCR to quantify the fungus present during the development of ectendo-mycorrhizal symbiosis in *ech42*-transformed and control lines. Although there was higher levels of expression of endochitinase in the transformed plants, no significant differences in the numbers of *Wilcoxina* molecules could be found in transformed and control lines. The results indicated that transformation of spruce with *ech42* did not alter the soil fungal biomass or the development of ectendomycorrhizal symbiosis involving *Wilcoxina* spp. (Stefani et al. 2010).

Several basidiomycetous fungi have been investigated for mycorrhizal association with orchids. A DNA array was developed from extensive clone library sequence data sets. Based on the ITS spacer regions, oligonucleotides were designed for seven groups of sequences sharing at least 97 % sequence similarity termed as operational taxonomic units (OTUs), corresponding to the members of the Tulasnellaceae family. Oligonucleotides were also constructed for two subsets of closely related OTUs in order to cover a broader spectrum of tulasnelloid fungi. Multiple primer pairs were employed for the evaluation of the array system. It is

possible to widen the DNA arrays for including specific detector oligonucleotides for other and number of microorganisms. The DNA array is a powerful, rapid and cost-effective tool for simultaneous detection and identification of wide range of orchid mycorrhizal fungi (Lievens et al. 2010).

2.3.2 Assessment of Biological Control Potential of Mycorrhizal Fungi

Mycorrhizal fungi form an economically and ecologically important group of symbiotic fungi that are able to colonize the roots of over 80 % of plant species. They are ubiquitous in most of the natural and agricultural terrestrial ecosystems. Several studies have highlighted the importance of arbuscular mycorrhizal fungi (AMF) in controlling microbial plant pathogens and their potential for biocontrol of pathogens in agriculture either directly suppressing the pathogens or by improving health of plants that become resistant to pathogenic infection. Methods of assessing the biocontrol potential of mycorrhizal fungi are discussed in this section.

2.3.2.1 Dual Culture Method

The organ and cell cultures of a host tree may be challenged with compatible ECM fungi or specific pathogens. This system is useful to detect modifications in morphological, physiological and molecular aspects of the mycorrhizal fungi/pathogen. The spruce (*Picea abies*) callus cells were multiplied in dual cultures with ECM fungi such as *Amanita muscaria*, *Lactarius determinus, Hebeloma crustuliniforme, Sullius variegatus* or the pathogen. The growth pattern of ECM fungi was similar to the hyphal mantles found under natural condition. In contrast, *H. annosum* overgrew the callus rapidly and induced dissolution of callus cells (Sirrenberger et al. 1995).

2.3.2.2 Biochemical Analysis

Biochemical changes in response to AMF colonization and pathogenic infection in tomato roots were determined. Polyacrylamide gel electrophoresis (PAGE) technique was applied to assess the protective effects of *Glomus mosseae* (*Gm*) and *G. intraradices* (*Gi*) against *Phytophthora parasitica* causing tomato root rot disease. *P. parasitica* was inoculated on non-mycorrhizal and mycorrhizal-tomato plants precolonized for 4 weeks with either of the AM species. In non-mycorrhizal plants, two acid β-1,3-glucanase isoforms were constitutively expressed and their activity was higher in mycorrhizal roots. Two additional acidic forms were detected in extracts from *Gm*-colonized tomato roots, but not in *Gi*-colonized roots. Roots infected by *P. parasitica* exhibited greater enzyme activities. However, the pathogen did not induce the isoforms related to *Gm* colonization. When tomato plants

colonized by *Gm* were infected by *P. parasitica,* two additional basic isoforms were distinctly recognizable. Differences in the expression of isoforms of β-1,3-glucanases in AMF-colonized and non-mycorrhizal plants, when infected by the pathogen, may have a role in the disease development (Pozo et al. 1999).

2.3.2.3 Immunological Tests

The cellular bases of the bioprotective effect of *Glomus mossea* (*Gm*) on tomato root infection by *Phytophthora nicotianae* var. *parasitica* (*Pnp*) were studied, using immuno-enzyme labeling technique on whole root segments. Lack of an appropriate stain to distinguish hyphae of *Gm* and *Pnp* inside tomato roots colonized by them necessitated the use of more precise techniques. Infection intensity by the pathogen was lower in mycorrhizal roots. Immunogold labeling of *Pnp* on cross-section of infected tomato roots showed that inter-or intra-cellular hyphae developed mainly in the cortex and their presence induced necrosis of host cells. The cell walls and the contents showed strong autofluorescence in reaction to the pathogen. The hyphae of *Gm* and *Pnp* were present in most cases in different root regions and sometimes in the same root tissues. The number of *Pnp* hyphae, growing in the root cortex, was greatly reduced in mycorrhizal root systems. In mycorrhizal tissues infected by the pathogen, arbuscule-containing cells surrounded by intercellular *Pnp* hyphae did not show any necrosis. These host cells exhibited only weak autofluorescence (Cordier et al. 1996).

2.3.2.4 Greenhouse Tests

The effects of AM fungi *Glomus fasciculatum* or *G. etunicatum* on the growth of three strawberry cultivars and development of root infection by *Phytophthora fragariae* were studied in the glasshouse. In two cultivars Cambridge Favourite and Elsanta, root necrosis was reduced by approximately 60 and 30 % respectively, compared with non-mycorrhizal plants. In the least susceptible cultivar Rhapsody, no significant reduction of root necrosis occurred on AMF-colonized plants (Norman et al. 1996). In another study, the influence of *G. mosseae* on the root infection by *Phytophthora nicotianae* var. *parasitica* in tomatoes was assessed using a sand culture system. The non-mycorrhizal plants showed appreciable reduction in plant weight and wide spread root necrosis, when inoculated with the pathogen. In contrast, AMF-colonized plants showed less adverse effects on root weight and root necrosis. The percentages of reduction of adventitious root necrosis and necrotic root apices ranged between 63 and 89 %. Although higher level of phosphorus supply increased plant development, disease spread was not decreased (Trotta et al. 1996).

The biocontrol potential of AM fungi has been assessed by setting up microcosms that permitted the root system to grow within a sand substrate in the gap between two glass plates and allowed to visualize the interactions between host

Table 2.6 Effect of *Glomus mosseae* on the infection foci induced by *Phytophthora parasitica* in tomato roots (Vigo et al. 2000)

Treatment	No. of infection loci per plant		Rate of necrosis spread (%) increase
	7 days	16 days	
AMF (–) + pathogen	122 a	510 a	38 a
AMF (+) + pathogen	74 b	360 b	34 a

Figures followed by the same letter in the same column are not statistically significant at $P < 0.05$

plant, AMF and the pathogen in situ. Tomato roots precolonized with *Glomus mosseae* were used as AMF inoculum and the root were examined for the presence of arbuscular vesicles and external mycelium. The AMF-colonized plants and plants not colonized by AMF were inoculated with zoospores of the pathogen *Phytophthora parasitica*. At harvest (81 days after sowing and 26 days after pathogen inoculation) necrosis of roots of noncolonized tomato plants was extensive, amounting to 61 % of the root system as against 31 % necrosis in AMF-colonized roots of tomato plants. In addition to disease suppression by AMF, the length of colonized root systems was increased by 50 %, indicating the additional benefit of obtaining better growth of tomato through AMF association (Table 2.6; Vigo et al. 2000). The biocontrol potential of three species of AM fungi *Gigaspora margarita* (GM), *Glomus fasciculatum* (GF) and *Glomus* sp. R10 (GR) against *Fusarium oxysporum* f. sp. *asparagi* (*Foa*) was evaluated under greenhouse conditions. Radicles of seedlings germinated on filter paper were inoculated using commercial inoculum. The AM-inoculated and noninoculated plants (NAM) were planted in bed soil with required nutrients. Infection of roots by AM fungi was ascertained by examining stained root segments under the microscope at 10 and 16 weeks of AMF inoculation. The conidial suspension of pathogen *Foa* grown in PDA was used for inoculation of AM and NAM plants. Disease index was scored using a disease rating scale of 1–5. Incidence and severity of Fusarium root rot were reduced by pre-infection with AM fungi, indicating the enhancement of tolerance of AM plants to infection by *Foa*. No significant difference in phosphorus concentration was observed in different treatments (Matsubra et al. 2001).

The biocontrol efficacy of the BCA may be inferred indirectly by determining the pathogen population remaining in the plants treated with the BCA. This approach was adopted to assess the capacity of the AMF *Glomus intraradices* to reduce the presence of *Fusarium solani* f.sp. *phaseoli* (*Fsp*) in bean plants and the surrounding mycorrhizosphere soil using a compartmentalized experimental system. The population of the pathogen and the AMF in plant tissues, the soil regions of the mycorrhizosphere (rhizosphere and mycosphere) and the bulk soil were quantified by employing specific PCR primers in real-time PCR assays, isolation-dependent methods and microscopic determination techniques. Bean plants precolonized by *G. intraradices,* followed by inoculation with *Fsp* remained healthy, while nonmycorrhizal plants exhibited distinct Fusarium root rot symptoms. The amount of *Fsp* genomic DNA was significantly reduced in mycorrhizal plants and mycorrhizosphere, compartment. The results suggested that reduction in root rot

symptoms and pathogen may be due to biotic and abiotic modifications of the mycorrhizosphere, following colonization with *G. intraradices* (Filion et al. 2003). The biocontrol potential of *Glomus mosseae* (BEG12) and *Pseudomonas fluorescens* A6R1 to suppress the development of root rot disease of tomato caused by *Rhizoctonia solani* was assessed. The epiphytic and parasitic growth of *R. solani* in the presence and absence of BCAs were determined by observations under the microscope. *G. mosseae* and *P. fluorescens* protected the plants in such a way that the treated plants overcame the growth depression caused by *R. solani,* as observed in the untreated control tomato plants. Significant suppression of epiphytic and parasitic growth of the pathogen along with increase in root length and number of root tips accelerated the growth of treated tomato plants challenged by the pathogen inoculation. The efficient disease suppression was attributed to the combined effects of both the mycorrhizal fungus and the rhizobacterial species (Berta et al. 2005). The AMF *G. mosseae*, *G. monosporum, G. deserticola, G. intraradices* and two unidentified species were evaluated for their ability to reduce the deleterious effects of tomato wilt pathogen *Fusarium oxysporum* f. sp. *radicis-lycopersici* (FORL) under greenhouse conditions. *G. mosseae* and *G. monosporum* improved the plant height, plant dry weight, fruit yield and fruit number of tomato plants inoculated with the AMFs. In mycorrhizal plants challenged with FORL, the root infection by the pathogen was significantly reduced, compared with non-mycorrhizal inoculated plants (Utkhade 2006).

The AM fungus *Glomus mosseae, Trichoderma harzianum* and *Pseudomonas fluorescens* were evaluated for their efficacy in suppressing the development of banana Panama wilt disease caused by *Fusarium oxysporum* f.sp. *cubense (Foc*) (race 1) was assessed, when inoculated in single, dual and tripartite combinations. The BCAs were allowed to colonize the plants up to 0, 45 and 90 days. Plants were then challenged with 50 g of *Foc* inoculum containing 1.5×10^6 CFU/g. Controls without treatment with BCA and inoculated with *Foc* were maintained. Enzyme-linked immunosorbent assay (ELISA) was employed to assess the populations of *Foc* every month. Growth parameters and yield of plants were recorded for each treatment. After 7 months, plants pre-inoculated with BCAs, *G. mosseae* + *T. harzianum* and challenged with *Foc* could sustain 61 and 70 % improvement in plant height and girth respectively and 75 % in bunch weight over plants not precolonized with BCAs but challenged with *Foc*. These control plants finally succumbed to the disease. ELISA assessment of *Foc* populations showed that the pathogen population was reduced to 0.58 OD in 7 months in *G. mosseae* and *T. harzianum* treatments compared to a level of 1.90 OD in control plants. The results suggested that protection to banana by the BCAs might be due to physical modifications in the cell wall, growth promotion and through induction of resistance to the Panama wilt disease (Mohandas et al. 2010).

The arbuscular mycorrhizal fungi (AMF) *Glomus mosseae, G. etunicatum, G. fasciculatum* and *Gigaspora margarita* were evaluated for their ability to suppress the development of Phytophthora blight of pepper seedlings caused by *Phytophthora capsici* and to promote the growth of pepper plants. Among the AM fungi tested, *G. mosseae* was the most effective in reducing the disease

severity due to *P. capsici* by 91.7, 43.0 and 57.2 % under pot, greenhouse and field conditions respectively. The phytoalexin capsidol concentration was increased in pepper plants pre-inoculated with *G. mosseae* and in the necrotic stems of pepper plants challenged with *P. capsici.* The results showed that the AM fungus could improve plant growth in addition to suppression of Phytophthora blight disease in pepper plants (Ozgonen and Erkilic 2007). Apple stem brown canker caused by *Botryosphaeria ribis* adversely affects growth, ultimately killing the shoots, limbs and even trunks of affected trees. Seven species of arbuscular mycorrhizal fungi (AMF) *Sclerocystis dussi*, *Glomus intraradices, G. fasciculatum, G. bagyaraji, G. leptotichum, G. monosporum, Gigaspora margarita* were evaluated for their efficacy in suppressing the fungal pathogen development. Two year-old potted apple plants were either pre-inoculated with AMF followed by stem inoculation with *B. ribis* or simultaneously inoculated with AMF and the pathogen. The intensity of canker was less severe in plants inoculated with AMF, relative to the non-inoculated control. Plants pre-inoculated with AMF were better protected compared with plants inoculated simultaneously with AMF and *B. ribis*. In addition, AMF inoculation improved survival and growth of plants, although the beneficial effects varied with different species of mycorrhizal fungi (Krishna et al. 2010).

2.3.3 Interaction Between Mycorrhizal Fungi and Fungal Biocontrol Agents and Bacterial Communities

In the plant rhizosphere, soil fungi including the ectomycorrhizal fungi compete with each other for nutrition, space and access to the suitable plant roots or substrates. It is possible that the ectomycorrhizal fungi and the saprophytic fungi *Trichoderma harzianum* and *T. virens* may interact affecting each other's development. The interactions between the ECM fungus *Laccaria laccata* and *Trichoderma* spp. were visualized using electron microscopy and gold cytochemistry techniques. In order to localize β-1,3-glucan, chitin and polysaccharides, anti- β-1,3-glucan antibody and WGA/ovomucoid-gold complex PATAg methods were applied. Concurrent cytoplasm disorganization and dissolution of β-1,3-glucan in the hyphal cell walls and conidia of *Trichoderma* spp. were observed. Digestion of polysaccharides and chitin of colonized fungal structures was seen subsequently. The results suggest that ECM fungi could parasitize fungal BCAs present in the soil indicating the possible negative effects of interaction between biocontrol agents (Zadworny et al. 2007). Several bacterial species are associated with AM fungi and they form essential living components of the soil microbiota. Ten isolates of bacteria out of a total 385 isolates from the spores of AM fungi exhibited the ability to inhibit the plant pathogens like *Erwinia carotovora, Phytophthora infestans* and *Verticillium dahlia*e. Colonization of roots of potato by AM fungi was enhanced by 7-folds in the presence of *Pseudomonas* FWC 70 isolate in greenhouse and by 6–9 folds in the presence of *Stenotrophomonas* FWC 94 and

Arthrobacter FWC 110 under field conditions. Many growth traits of potato were stimulated by these bacterial isolates, in addition to the inhibition of microbial plant pathogens. Protease(s), siderophores and indole acetic acid (IAA) were produced by all bacterial isolates. Chitinase production by *Stenotrophomonas* and phosphate-solubilizing activity by all *Pseudomonas* isolates, *Stenotrophomonas* FWC 14 isolate and *Arthrobacter* FWC 110 isolate were also recorded. The results of this study indicated that multifunctional capacity of some of the AMF-associated bacterial isolates was likely, due to the production of extracellular enzymes and bioactive compounds. The AMF-associated bacteria might contribute to the often described capacity of AM fungi to inhibit phytopathogens, acquire mineral nutrients and promote plant growth (Bharadwaj et al. 2008).

Arbuscular mycorrhizal fungi (AMF) have been reported to modify the bacterial community structure of the rhizosphere leading to the formation of mycorrhizosphere. Tomato plants were grown in a compartmentalized soil system and they were either exposed to direct mycorrhizal colonization or enrichment of the soil with exudates collected from mycorrhizal tomato plants. The plants were inoculated with *Phytophthora nicotianae*. Appropriate controls were maintained. At harvest, a PCR-denaturing gradient gel electrophoresis analysis of 16S rRNA gene fragments amplified from the total DNA extracted from each plant rhizosphere was performed. Root colonization with *Glomus intraradices* or *G. mosseae* caused significant changes in the bacterial community structure of tomato rhizosphere compared to non-mycorrhizal plants. On the other hand, enrichment with root exudates collected from mycorrhizal or non-mycorrhizal plants exhibited no significant changes. Further, infection of mycorrhizal or non-mycorrhizal plants with *P. nicotianae* did not alter the bacterial community structure significantly. It appeared that the rhizosphere bacterial community was less sensitive to pathogen invasion rather than to AM fungi (Lioussanne et al. 2010).

2.3.4 Interaction Between Mycorrhizal Fungi and Resistance-Inducing Chemicals

Sunflower is severely affected by the downy mildew disease caused by *Plasmopara helianthi*. The effects of the arbuscular mycorrhizal fungus (AMF) *Glomus mosseae* and two plant activators DL-β-amino-n-butyric acid (BABA) and acibenzolar-S-methyl (BTH) CGA 245704 on the development of downy mildew disease were assessed. The plant activators were applied as soil drenches and foliar sprays. Soil drenches of BABA and BTH (50 and 100 mg/kg soil) applied at 1 and 3 days prior to inoculation with the pathogen to mycorrhizal plants provided moderate protection against the pathogen (50–50 %). Morphological changes and decrease in mycorrhizal colonization in roots of BTH-treated plants were observed. Foliar spray treatment of BABA and BTH (400 and 200 μg/ml respectively) to mycorrhizal plants provided effective protection (about 80 %) against foliar infection by *P. helianthi.* In the in vitro tests,

germination of *G. mosseae* sporocarps increased with BABA treatment, while BTH treatment inhibited the sporocarp germination. The need to select the compatible combinations of biotic and abiotic inducers of disease resistance to maximize the effectiveness of protection to crop plants against the target pathogen (s) was suggested by Tosi and Zazzerini (2000).

Appendix 2.1: General and Selective Media for Isolation of Fungal Biocontrol Agents

A. **General media**

1. Czapek Dox agar

Solution A	
Sodium nitrate	40 g
Potassium chloride	10 g
Magnesium sulphate (hydrous)	10 g
Ferrous sulfate (hydrous)	0.2 g
Distilled water	1 l
Solution B	
Dipotassium hydrogen phosphate	20 g
Distilled water	1 l

(store the solutions A and B separately in a refrigerator)

Prepare the mixture of A and B	
Stock solution A	50 ml
Stock solution B	50 ml
Distilled water	900 ml
Sucrose (analar)	30 g
Oxiod agar No.3	20 g
Just before autoclaving and for 1 l	
Zinc sulfate (1.0 g/100 ml water)	1.0 ml
Cupric sulfate (0.5 g/100 ml water)	1.0 ml

2. Malt extract agar

White bread malt extract	20 g
Oxoid agar No.3	20 g
Tap water	1 l

3. Oat agar

Oat meal ground	30 g
Oxoid agar No.3	20 g
Tap water	1 l

4. Potato carrot agar

Grated potato	20 g
Grated carrot	20 g
Oxoid agar No.3	20 g
Tap water	1 l

5. Potato dextrose agar

Potato	200 g
Oxoid agar No.3	20 g
Dextrose	15 g
Tap water	1 l

   i. Potato dextrose agar (PDA) – for *Mucodor albus* and *M. roseus* (Stinson et al. 2003); PDA broth – *Trichoderma atroviride* (McLean et al. 2005).
   ii. Potato dextrose agar (PDA) (Anand and Reddy 2009) amended with colloidal chitin 0.2 % (or) amended with *Sclerotium rolfsii* cell wall chitin 0.2 %
   iii. PDA amended with chitosan (de Capedeville et al. 2002) chitosan concentrations 0.25–2.0 % (w/v) Adjust with acetic acid to pH 6.0
   iv. For fungi (Jensen et al. 2004)
   PDA amended with Triton X-100 2.2 g/l; chloramphenicol 0.5 g/l
   Potato carrot agar amended with chloramphenicol 0.5 g/l
   Low-nutrient agar (ASDA Micro)
   v. For yeasts
   Acidified yeast medium (YM) pH 3.7
   Osomophilic YM amended with glucose 40 % pH 4.5

6. V8 agar medium (Chen and Fernando 2006)

V8 juice	200 ml
$CaCO_3$	0.75 g
Agar	15.0 g
Distilled water	800 ml

7. Komada's selective medium for *Fusarium* spp. (Arie et al. 1995)

K_2HPO_4	1 g
KCl	0.5 g
$MgSO_4.\ 7H_2O$	0.5 g
Fe-EDTA	10 mg
L-asparagine monohydrate	10 mg
D (+) galactose	2.0 mg
Pentachloronitrobenzene (PCNB)	0.75 g (a.i)
Sodium chlorate	0.5 g
Sodium tetraborate Decahydrate	1 g
Chloramphenicol	0.25 g
Agar15 g	
Distilled water	1 l

8. Nutrient yeast dextrose agar (NYDA) for yeast BCAs (Droby et al. 2002)

Nutrient broth	8.0 g
Yeast extract	5.0 g
D-glucose	10.0 g
Agar	20.0 g
Distilled water	1 l

9. Solid medium for selecting BCAs (Khan et al. 2001)

Corn steep liquor	10.0 g
Yeast extract	1.0 g
KH_2PO_4	2.0 g
K_2HPO_4	2.0 g
NaCl	0.1 g
Glucose	15.0 g
Malt yeast extract	3.0 g
Malt extract	3.0 g
Peptone (type IV)	5.0 g
Tryptic soy broth	
agar (1/5 concentration)	pH 6.8
Difeo Lab (Detroit)	
Distilled water	1 l

10. Fungal endophytes: Oat meal agar (OMA) for *Phialocephala fortinii* (Narisawa et al. 2004)

Oat meal	10.0 g
Bacto agar	18.0 g
$MgSO_4.7H_2O$	1.0 g
KH_2PO_4	1.5 g
$NaNO_3$	1.0 g
Distilled water	1 l

11. Nutrient yeast dextrose agar (NYDA) for *Candida sake CPA-1* (Abadias et al. 2003)

Nutrient broth	8.0 g
Yeast extract	5.0 g
Dextrose	10.0 g
Agar	15.0 g

12. Rosebengal – Allisan – streptomycin – Previcur (RASP) selective medium (Metcalf et al. 2004)

$(NH_4)_2SO_4$	2 g
KH_2PO_4	4 g
Na_2HPO_4	6 g
$FeSO_4.7H_2O$	0.2 g
$CaCl_2$	1.0 mg
H_3BO_3	10 µg
$MnSO_4$	10 µg
$ZnSO_4$	70 µg
Agar	20 g
Cellulose powder	5 g
Distilled water	1,000 ml
Adjust to pH 4.0 before autoclaving	

13. Modified Nash and Snyder medium (MNSM) for *Penicillium oxalicum* (Larena and Melgarejo 2009a)

Peptone (Difco)	1.5 %
KH_2PO_4	2.0 %
$MgSO_4 . 7\ H_2O$	0.05 %
Agar	2.0 %
After autoclaving add streptomycin	300 ppm
Pentachloro nitrobenzene	1 : 1,000
Molal Na Cl	1

14. Trichoderma selective TSB medium (Xia et al. 2011)
Potato infusion, 200 g; glucose – 20 g; Chloramphenicol – 0.25 g; Rose Bengal – 0.15 g; Pentachloronitrobenzene – 0.15 g; Streptomycin 0.05 g; Baytan – 0.05 g; benomyl 0.0005 g; agar 20 g; distilled water – 1,000 ml

Appendix 2.2: Generation of Antibodies Against Fungi (Banks et al. 1992)

A. **Preparation of antigen**

(i) Prepare spore suspensions using 0.01 % Tween 80; wash thrice by centrifugation; inoculate 1 ml of spore suspension (10^6 spores/ml) into 100 ml of liquid medium supplemented with NaCl (100 g/l) and incubate at 25 °C for 7 days in the dark by placing the flask with contents on a rotary shaker

(ii) Transfer the mycelium by filtering into a sintered glass filter; wash with sterile water and then with sterile phosphate-buffered saline (PBS) containing 2.9 g $Na_2HPO_4 12H_2O$, 0.2 g KH_2PO_4, 8.0 g NaCl and 0.2 g KCl and 1,000 ml distilled water; freeze overnight at – 20 °C; thaw and transfer to centrifuge tubes and dry in vacuum dryer

(iii) Collect the mycelium and add 50 ml of liquid nitrogen; mince the mycelium in a blender for 1 min and grind in a mortar with a pestle to a fine powder

(iv) Suspend the mycelial powder in PBS (200 mg in 100 ml); centrifuge at 4,500 rpm (3,000 *g*) for 10 min at 4 °C and divide the supernatant containing soluble nitrogen into 0.5 ml aliquots and store at – 20 °C

(v) Estimate the total protein content of the antigen preparation

B. **Production of polyclonal antiserum**

(i) Mix soluble antigen preparation with equal volumes of Freund's complete adjuvant (Difco) to produce a final protein concentration of the mixture 1 mg/ml

(ii) Inject rabbits intramuscularly with 1 ml of the mixture at predetermined intervals

(iii) Bleed the animal at 4 weeks after the first injection and subsequently at 14, 16 and 18 weeks

(iv) Separate the serum after completion of clotting of blood cells followed by centrifugation

C. Production of monoclonal antiserum

(i) Mix soluble antigen preparation with an equal amount of Freund's complete adjuvant to yield a final protein concentration of 1 mg/ml
(ii) Inject a BALB/c mouse, after anaesthetization with 0.1 ml of the immunogen intraperitoneally and subsequently at 2, 4, 6 and 8 weeks after the first injection with PBS and remove the spleen after sacrificing the animal by cervical dislocation
(iii) Carry out fusion of splenocytes with selected myeloma cell line (PS-NS-1-Ag4) at a ratio of $1 \times 108 : 5 \times 107$ by gentle addition of 2 ml of 30 % polyethylene glycol (PEG) (w/v) over 60 s
(iv) Add 10 ml of warm serum-free RPMI 1640 medium (Gibco) over next 60 s with gentle stirring; add another 20 ml of RPMI and centrifuge for 3 min at 400 g at room temperature
(v) Suspend the pellet of cells in 50 ml of growth medium (RPMI 1640) with 20 % Myelone fetal calf serum (FCS) (v/v); dispense cell suspension into five 96-well microplates at 100 µl/well
(vi) Add 110 µl of hypoxanthine aminopterin-thymidine (HAT) medium diluted to 1:50 in growth medium to each well in the fusion plates
(vii) Add growth medium + HAT on 2, 4, 7 and 10 days by removing 100 µl of fresh medium
(viii) Screen the hybridoma cells for efficiency of antibody production by indirect ELISA procedure
(ix) Clone healthy growing hybridoma twice by limiting dilution in a non-selective medium; preserve by freezing slowly in 7.5 % dimethyl sulfoxide (DMSO) and store in liquid nitrogen

Appendix 2.3: Detection of Fungi by Enzyme-Linked Immunosorbent Assay (ELISA) Test (Bossi and Dewey 1992)

A. **Preparation of antigen**

(i) Prepare surface washings of the fungus grown on PDA for 17–20 days at 21 °C, using 5 ml/petridish of phosphate-buffered saline (PBS) containing 8.0 g NaCl, 0.2 g KCl, 1.15 g Na_2HPO_4. 0.25 g KH_2PO_4, and water 1,000 ml at pH 7.2 and remove the wash suspension by suction
(ii) Centrifuge the wash fluid for 3 min at 13,000 *g* to remove the fungal debris and dilute the supernatant with PBS to have 10-fold dilutions
(iii) Remove the high MW carbohydrates and glycoproteins by passing the cell-free wash fluid through a Centricon 30-kDa filter (Amicon No. 4208) to prevent induction of nonspecific antibodies; freeze-dry the filtrate and redissolve the contents in 1 ml of distilled water and use it as the antigen

B. **Enzyme-linked immunosorbent assay (ELISA)**

(i) Coat the wells (in triplicate) in the 96-well microtiter plates with PBS surface washing fluid (50 µl/well) overnight and wash the wells four times allowing two min for each washing followed by a brief washing with the distilled water
(ii) Air-dry the plates in a laminar flowhood and seal them in a polythene bag and store at 4 °C
(iii) Incubate the plates successfully with hybridoma supernatants for 1 h, then with a 1/200 dilution of a commercial goat antimouse polyvalent (IgG+IgM) peroxidase conjugate and finally with PBS with 0.05 % Tween-20 (PBST) for 1 h more
(iv) Add the substrate solution containing tetramethyl benzidine (100 µg/ml) for 30 min
(v) Maintain the controls in incubated tissue culture medium containing 5 % fetal bovine serum (FBS) in place of hybridoma supernatant
(vi) Stop the reaction by adding 3 M H_2SO_4 (50 µl/well); determine the intensity of color developed in each well using ELISA reader at 450 nm
(vii) Absorbance levels more than three times greater than those of controls indicate positive reaction and presence of antigen protein

Appendix 2.4: Characterization of the Antibody Specific for Fungal Biocontrol Agent by Western Blotting Technique (Thornton et al. 2002)

A. **Polyacrylamide gel electrophoresis (PAGE)**

(i) Use the system with 4–20 % gradient polyacrylamide gels (Bio-Rad) under denaturing conditions; denature the samples by heating at 95 °C for 10 min in the presence of β-mercapto-ethanol prior to gel loading
(ii) Separate the proteins for 1.5 h at room temperature
(iii) Use prestained broad-range markers (Bio-Rad) as standards for molecular mass determination
(iv) Stain the gels for total protein with Coomassive brilliant blue

B. **Western blotting**

(i) Transfer the separated proteins electrophoretically to a PVDF membrane (Immuno-Blot PVDF; Bio-Rad) and wash the membranes thrice with phosphate-buffered saline (PBS)
(ii) Block the non-specific binding areas in the membranes with PBS containing 1 % bovine serum albumin (BSA) for 16 h at 4 °C
(iii) Incubate membranes with MAb supernatant diluted (1:2) with PBS containing 0.5 % BSA (PBSA) for 2 h at 23 °C and wash the membranes thrice with PBS

(iv) Incubate the membranes with goat anti-mouse IgM (μ-chain specific) alkaline phosphatase conjugate (1 : 15,000) diluted in PBSA for 1 h and wash the membranes twice with PBS and once with PBST
(v) Stop the reaction by immersing the membranes in double distilled water and air dry between sheets of Whatman filter paper
(vi) Label the proteins immobilized on the membranes using a commercial glycoprotein kit as per the manufacturer's instructions (Bio-Rad)

Appendix 2.5: Detection of Fungal Biocontrol Agents by Enzyme-Linked Immunosorbent Assay (ELISA) (Thornton et al. 2002)

(i) Use microtiter plates (96 wells); incubate with selected antibody solution (200 μl) for 1 h and incubate with either a goat-anti-mouse polyvalent (IgG IgA and IgM) peroxidase conjugate (1:1,000) or a goat-anti-mouse IgM (μ – chain specific) peroxidase conjugate (1 : 5,000) for further 1 h
(ii) Transfer tetramethyl benzidine substrate solution to each well and incubate for 30 min
(iii) Stop the reaction by adding 3 M H_2SO_4
(iv) Record the absorbance at 450 nm using an ELISA reader
(v) Wash the wells four times of 5 min each with phosphate buffered saline (PBST) containing 0.02 M PO_4, 0.85 % NaCl and 0.05 % Tween 20 after each incubation mentioned above [steps (i) and (ii)]

Appendix 2.6: Identification of *Rhodotorula mucilaginosa* by Dot Blot Hybridization Technique (Utkhade and Cao 2005)

A. **PCR amplification**

(i) Extract the DNA from plant and fungal samples using the Fast DNA kit as per the manufacturer's instructions
(ii) Perform amplification using specific primers that are specific to septate fungi and yeasts and amplify the sequences of ITS1, 5.8 S ribosomal gene and ITS 2 portions of rDNA; 5′ AACTTGGTCATTTAG AGGAAGTAA (SF-UP 18570) and 5′GTTTCTTTTCCTCGC CTTAT TGATATGG (UN-LO28S-22)
(iii) Using a thermocycler (Robocyler, Stratagene, CA, USA) carry out PCR reactions in 20 μl volumes with final concentration of reagents as detailed below:
1 × reaction buffer, 100 μM of each of d-NTP, 500 nM of each primer and 1.25 U *Taq* polymerase and a drop of mineral oil overlaid on the reaction mixture.

(iv) Carry out 29-cycles at 94 °C for 45 s, at 58 °C for 45 s and at 72 °C for 45 s
(v) Maintain a positive control with the BCA (*R. mucilaginosa*) DNA and a negative control with all reagents + distilled water for each run
(vi) Analyze the PCR products with standard electrophoresis procedure on 1 % minigels, stained with ethidium bromide and visualize under UV light

B. **Hybridization reaction**

(i) Prepare nylon membrane blots (Roche Diagnostics, Canada) using a manifold device (Schleicher and Schuell, Manifold, USA)
(ii) Prepare aliquots (5 µl) of PCR product denatured in 0.5 M NaOH (500 µl) for 5 min at 37 °C; mix with 12 × SSC (500 µl; saline sodium citrate) and spot 200 µl aliquots onto nylon membrane through the wells of the manifold device
(iii) Dry the membranes immediately at 120 °C for 30 min to fix the DNA onto the membrane and cool the membrane
(iv) Prehybridize the membrane for 2.5 h in hybridization buffer containing 6 × SSC 1 % skim milk powder, 0.2 % sodium dodecyl sulfate (SDS) and 0.1 mg/ml of Poly (A) at 42 or 48 °C
(v) Hybridize the membrane overnight (about 16 h) in hybridization solution containing optimum concentration of probe (0.2 pmol)
(vi) Wash the membrane twice for 10 min each in 2 × SSC 0.2 % SDS at hybridization temperature and twice for 10 min each in 0.1 × SSC, 0.02 % SDS at the same temperature
(vii) Incubate the membrane at room temperature for 30 min in a 1:20,000 dilution of anti-digoxigenin AP (Roche Diagnostics, Canada) and wash as per the manufacturer's instructions; generate the signal using the CDP substrate (Roche Diagnostics) and capture the signal on scientific imaging film (X-OMAT Kodak, Rochester, NY)

Appendix 2.7: Assessment of Biocontrol Activity by In Vitro Tests

A. **Inhibition of growth of fungal pathogens (Weller et al. 1985)**

(i) Mark two lines perpendicular to each other passing through the center of the bottom of a sterile petriplate using a glass marking pencil; pour about 15 ml of potato dextrose agar (PDA) or King's medium B in each plate; tilt the plate gently so that the medium spreads uniformly in it; allow the medium to set
(ii) Cultivate the test antagonists in appropriate medium (PDA for fungi and King's medium B for bacteria or other specific media) for the required period
(iii) Using a sterile cork borer (6 mm dia), transfer one culture disk of each test antagonist to the plates containing medium already prepared; position the disks at equidistant points near the edges of the lines marked on the plates; incubate for 2 days at room temperature

(iv) Grow the pathogen in an appropriate medium; using the sterile cork borer (6 mm dia), punch out disks of pathogen culture; place one disk at the center of the plate in which antagonist cultures have already been placed; incubate the plate at room temperature

(v) Measure the zones of inhibition formed due to the activity of each test antagonist; select the antagonist causing maximum inhibition of the pathogen

(vi) To determine the spectrum of activity of the antagonist reverse the positions of antagonist and pathogen; place the culture disks of the antagonist at the center of the plate and the disks of pathogens at the periphery of the plate

B. **Leaf disk inoculation technique (Falk et al. 1996)**

a. Preparation of inoculum of pathogen (*Plasmopara viticola*)

(i) Inoculate the grapevine leaves from plants (cv. Riesling) grown in the growth chamber by spraying a suspension of sporangia obtained by washing from sporulating lesions; place the inoculated leaves inside the moist chamber in a growth chamber at 21 °C with a 12-h photoperiod

(ii) Wash the sporulating lesions formed on inoculated leaves with distilled water from a nonchlorinated fluorocarbon aerosol sprayer; adjust the concentration of sporangia to 1×10^5 per ml using a hemocytometer

(iii) Place the suspension at 22 °C for 20–30 min for the release of zoospores before inoculation onto test leaf disks/leaves

b. Preparation of the biocontrol agent (BCA) (*Fusarium proliferatum* isolate G6)

(i) Multiply the BCA in potato-dextrose-agar (PDA) medium for 2–3 weeks at 20–25 °C; scrape the mycelium from the surface of the medium and homogenize after adding enough sterile water by vortex mixing

(ii) Filter the suspension of conidia and hyphae through a double layer of cheese cloth; adjust the concentration of microconidia to 1×10^6 per ml using a hemocytometer

c. Application of BCA and pathogen

(i) Prepare leaf disks (2 cm dia) from grapevine leaves of plants grown in the growth chamber; arrange 5–6 inverted leaf disks (lower surface facing upward) on moistened filter paper (Whatman No. 3) placed in each sterile petri plate (9 cm diameter)

(ii) Spray the conidial suspension of the BCA until the leaf disks are wet; maintain suitable control leaf disks sprayed with sterile water alone

(iii) Allow the droplets to dry; seal the dishes with parafilm and then place them in the growth chamber with a 12-h photoperiod for 24 h at 21 °C

(iv) After incubation for 24 h, on each leaf disk place five 10 µl drops of the pathogen suspension containing released zoospores; thoroughly blot the moisture on the leaf disk with absorbent tissues at 20 h after inoculation

(v) Reseal the petri plates and place them in the growth chamber
(vi) To assess the effect of the BCA, collect the sporangia formed on each leaf disk using 2–3 ml of distilled water, after 7 days of incubation in the growth chamber; calculate the number of sporangia from each leaf disk using a hemocytometer
(vii) Assess the resporulation of the pathogen visually in each leaf disk and quantify after 24–48 h as done before; repeat the experiment three times
(viii) To assess the efficacy of the BCA as post-inoculation application, place five 10 µl drops of the suspension of sproangia and zoospores of the pathogen on the undersurface of each leaf disk; seal the plates and incubate
(ix) Blot out the moisture on leaf disks after 20-h incubation, reseal and place the petri plates in the growth chamber for 5 days
(x) Place one 10 µl drop of the conidial suspension of the BCA on the sporulating lesion in each leaf disk; blot out the moisture after 20 h; reseal the plates and incubate for 7 days
(xi) Quantify the sporangia produced in each leaf disk as done in step (vi) above; assess resporulation after 4 days as done in step (vii)

C. **Petal disk assay (Gould et al. 1996)**

a. Preparation of biocontrol agent (*Pseudomonas fluorescens*)

(i) Multiply the BCA on 30 ml of Luria-Bertani (Difco) broth for 48 h at room temperature
(ii) Centrifuge at 3,000 g for 5 min; reject the supernatant; resuspend the pellet in Tween 20 solution (0.03 %) in water
(iii) Adjust the optical density to 0.3 at 595 nm to have a concentration of 105 CFU of bacterial cells per ml

b. Preparation of pathogen

(i) Multiply the pathogen (Botrytis cinerea) in malt extract agar (MEA) for 5 days at 21 °C
(ii) Transfer conidia, using sterile forceps to 1 ml sterile water kept in a microcentrifuge tube, disperse the conidia by vortexing for 30 s to have a uniform suspension
(iii) Adjust the concentration of conidia to 10^3 per ml using a hemocytometer

c. Preparation of test flowers and petal disks

(i) Raise ultrawhite hybrid petunias in a glasshouse from seeds for 3–4 months; do not use any fungicide or pesticide
(ii) Collect 3rd, 4th or 5th fully opened flowers from the floral apex; surface sterilize by immersing them in 0.05 % sodium hypochlorite solution three times; rinse the flowers in 5 changes of sterile water; select flowers undamaged by disease or pest
(iii) Punch out petal disks (14 mm dia) using a sterile cork borer

d. Assay of biocontrol activity

(i) Dip the petal disks in the suspensions of BCAs at the rate of 4 disks per isolate or BCA to be tested; dip the control disks in sterile water with Tween 20 (0.03 %)
(ii) Place the BCA-treated and control disks individually in the wells of a sterile multiwell plate with 6 lanes of 4 wells each in the following sequence
Lane 1 – Well 1 – molten MEA medium; wells 2–4-check petal disks
Lane 2 – Well 1–4 – check petal disks
Lane 3–6 – One isolate or BCA for each lane
(iii) Cover the loaded plates with sterile lids; place them in a plastic box lined with wet paper towels
(iv) Incubate in a lighted, continuous-mist chamber with a relative humidity of about 90 % and a 12-h photoperiod at 15 ± 2 °C for 24 h
(v) Dispense aliquots of pathogen inoculum at the center of all petal disks in all treatments (lanes 2–6) and to the first well in lane 1 with MEA medium; do not inoculate the petal disks in the three wells in lane 1; incubate in the incubation chamber for 6 days
(vi) Assess the extent of disease suppression using a 0–4 scale based on the number of petal disks colonized by the pathogen in a given lane allotted for each BCA or isolate
(vii) Repeat the test, if the pathogen does not sporulate in the well with MEA (lane 1, well 1)

Appendix 2.8: Biopriming of Carrot Seeds with Biocontrol Agent *Clonostachys rosea* Effective Against *Alternaria* spp. (Jensen et al. 2004)

A. **Cultivation of antagonists**

Use nutrient-rich media (a) PDA amended with Triton X-100 (2.2 g/l) and chloramphenicol (0.5 g/l) and (b) potato carrot agar amended with chloramphenicol (0.5 g/l) for isolating the fungal biocontrol agents

B. **Seed Coating (Non-primed)**

(i) Scrap the fungal spores on the surface of PDA after adding sterile water (5 ml); filter through nylon mesh (38 μM) and adjust the conidial concentration 1 × 107 CFU/ml
(ii) Coat the carrot seeds by shaking the seeds (1 g/treatment) with adjusted conidial suspension (4 ml) on a shaker at 130 rpm for 10 min; keep the seeds on filter paper and air-dry in a laminar hood for 1 h
(iii) For commercial preparation (dry formulations), suspend in sterile water; shake on a vortexer for 1 min; adjust the spore concentration to 1 × 107 CFU/ml and air-dry as indicated above (step ii)

C. **Hydropriming of carrot seeds**

(i) Use aerated water (500 ml) to be imbibed by carrot seeds (50 g) for 16 h; surface-dry the seeds for 1 h; determine water content at regular intervals to bring the moisture contents (MC) to 38 and 40 %
(ii) Incubate the seeds in plastic containers with two 1-mm holes in the lid at 15 °C for 13 days

D. **Biopriming of carrot seeds**

(i) Prepare the clay inoculum of the BCA (*C. rosea*); (2.5 g clay inoculum/500 ml water); apply during imbibition of seeds or dust the inoculum onto seed after drying the seeds at 0.01 g inoculum/g seed at 40 % MC
(ii) Determine the MC during priming at 7 and 14 days by drying 1-g seed samples at 130 °C for 1 h
(iii) Air dry the bioprimed seeds in a laminar hood overnight at 22 ± 3 °C to an MC of 7.8 ± 0.2 % before use in bioassays and blotter tests
(iv) Store the hydroprimed and bioprimed seeds in air tight containers at 4 °C

E. **Bioassay of biocontrol potential**

(i) Plant seeds treated with different BCAs and untreated control seeds after washing in coarse sand moistened with tap water (3:1, v/v) kept in plastic boxes, after covering with transparent plastic foil
(ii) Place the planted boxes in a growth chamber at 20 ± 1 °C with 12-h photoperiod; water the seedlings using an atomizer after 8 and 14 days after planting
(iii) Record seedling emergence, dead and wilted plants (removed as and when noted) and confirm the post-emergence infection by incubating affected plants in three layers of filter paper under near UV light to induce sporulation of *Alternaria* spp.

Appendix 2.9: Identification of Ectomycorrhizal Fungi by Polymerase Chain Reaction (PCR) (Kulmann et al. 2003)

A. **DNA extraction**

(i) Use mycelium (0.05–0.2 g) or washed mycorrhizal roots (3.5 mm); transfer the samples to sterile Eppendorf cup (2 ml) filled with silanized sand and a ceramic sphere and extract the DNA with a QiaGen DNEasy DNA Extraction Kit as per the manufacturer's instructions
(ii) Purify the DNA using standard electrophoresis in 1 % agarose gel and stain with ethidium bromide along with known standards

B. **PCR analyses**

(i) Use ITS-specific nucleotides ITS1 primer (TCCGTAGGTGAACC TGCGG) and ITS 8 primer (ACAGGCATGCTCCTCGGAA)
(ii) Carryout the PCR amplification reactions in aliquots of 50 µl containing 20 mM Tris HCl (pH 8.4), 50 m KCl, 1.5 mM $MgCl_2$, 3 % DMSO, 100 pmol

of each primer ITS1, ITS8, 0.4 mM of each dNTP and 2.5 units of *Taq* polymerase and 0.1~100 ng DNA

(iii) Use the thermocycler for amplification with an initial denaturation step at 95 °C for 5 min followed by 35 cycles of 95 °C for 5 min followed by 35 cycles of 95 °C for 1 min, 68 °C for 1 min and 72 °C for 1 min and complete the reactions by a final 2 min extension step at 72 °C

(iv) Resolve the amplicons by electrophoresis in a 1.5 % agarose 7AE gel followed by staining with ethidium bromide

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Chapter 3
Mechanisms of Action of Fungal Biological Control Agents

Microorganisms present in or on the plants, soil and air are identified by applying various techniques and their biocontrol potential against target pathogen(s) is assessed simultaneously (Chap. 2). It is essential to understand how the biotic biocontrol agents work as well as their limitations and requirements, for exploiting their potential in the most effective manner for crop disease management. Various investigations have shown that the mechanisms biocontrol activities of fungal biocontrol agents (BCAs) are many and varied. Various mechanisms may operate in different species of BCAs within a genus, as in *Trichoderma* and even one species may suppress the phytopathogens through two or more mechanisms. Preferably the BCAs may be placed together on the primary mechanisms such as parasitism, antibiosis, competition for nutrients and/or space, prevention of colonization of specific tissues of the host by the pathogen and induction of local and or systemic resistance to the target pathogens. In addition, promotion of plant growth may also enhance the level of resistance to microbial pathogens as in the case of mycorrhizal symbiosis with plants (Narayanasamy 2002, 2011).

3.1 Types of Antagonism

The development of microbial plant pathogens may be adversely affected by fungal biocontrol agents through three types of antagonism: (i) direct antagonism, (ii) indirect antagonism and (iii) mixed-path antagonism (Pal and Gardener 2006). Direct antagonism reflects the ability of the fungal BCA to parasitize and kill the pathogen or its propagules like sclerotia. The fungal BCA can penetrate and destroy the resting spores of the pathogen. In the case of indirect antagonism, there is no physical contact between the BCA and the pathogen. The BCA may enhance the level of resistance by activating the host defense mechanisms. Several fungal BCAs have been demonstrated to induce resistance in plants against several microbial plant pathogens. Competition between the BCA and pathogen for space or nutrients also limits the pathogen development indirectly by starving the pathogen out or

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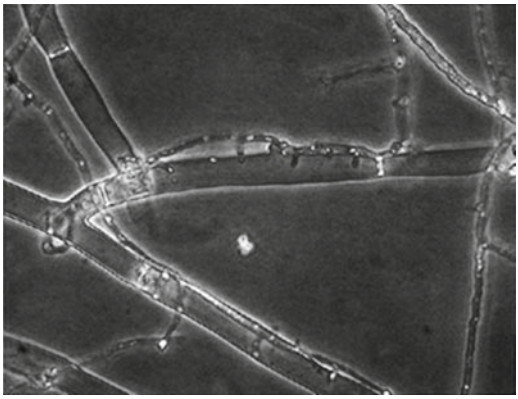

Fig. 3.1 Mycoparasitic activity of *Trichoderma virens* against *Rhizoctonia solani*, causing root rot diseases. Formation of haustoria of BCA within the large hyphae of the pathogen can be visualized using light microscope (Courtesy of Howell 2003 and with kind permission of The American Phytopathological Society, MN, USA)

preventing access to the plant tissues required for pathogen development. Mixed-path antagonism includes antagonistic activities based on the ability of the BCA to produce various kinds of enzymes, antibiotics or toxic metabolites inhibitory to pathogens. These different types of antagonism exhibited by fungal BCAs leading to suppression of development of microbial pathogens causing economically important crop diseases are discussed.

3.1.1 Mycoparasitism

The biocontrol agent is able to parasitize the pathogen and derive nutrition from the host pathogen. Several fungal parasites such as *Trichoderma virens* may function as an aggressive mycoparasite of fungal pathogens. It may parasitize not only the hyphae of many fungal species, but can also penetrate and destroy the resting bodies (sclerotia) that can help the pathogen overwinter and resist adverse environmental conditions. *T. virens* penetrates the hyphae and forms haustoria for absorption of nutrients from *Rhizoctonia solani* causing root rot diseases of many crops (Howell 2003; Fig. 3.1). In addition, destruction of these resting bodies will result in reduction in the inoculum potential in the soil (Tu 1980; Howell 1987). *Coniothyrium minitans* also attacks the hyphae and sclerotia of *Sclerotinia sclerotiorum*. On the other hand, the fungal BCA *Pythium oligandrum*, attacks living hyphae of the pathogen like *Pythium ultimum* and other *Pythium* spp. *P. oligandrum* was reported to be parasitic on the pathogens such as *Gaeumannomyces graminis* var. *tritici, Fusarium nivale* and *Phialophora graminicola* (Deacon 1976). *Pythium* spp. have been demonstrated to exhibit mycoparasitism on *Botrytis cinerea* causing gray mold diseases of several crops. *P. contiguanum* entered into the hyphal cells of *B. cinerea*, coagulated its protoplasm and finally emptied the cell contents (Paul 2000). *P. bifurcatum* coiled around the hyphae of *B. cinerea* and consumed the host protoplasm, finally leaving emptied host hyphal cells (Paul 2003). In a later study, *P. citrinum* was found to be an aggressive mycoparasite of *B. cinerea*. This BCA did

not coil around the mycelium of the host, but similar coagulation of protoplasm of hyphal cells was induced by this BCA, as in the case of other *Pythium* spp. studied earlier (Paul 2004).

The mycoparasitic activity of *Pythium oligandrum* on the sclerotia of *Botrytis cinerea* and *Sclerotinia minor* was assessed. The oomycete BCA should successfully enter *B. cinerea* sclerotia only through breaches at the junction of rind cells and corresponding to gaps in melanin deposits. As there were no breaches on the sclerotia of *S. minor,* the BCA ingress into the sclerotia stopped at the inner layer. On the other hand, *P. oligandrum* extensively colonized the cortical and medulla areas of *B. cinerea* sclerotia by intercellular growth. Colonization was associated with severe chitin degradation of all host cell walls which occurred at some distance from *P. oligandrum* hyphae. The hyphae of *P. oligandrum* showed the presence of wall thickenings, suggesting that these thickenings might represent defense-like reactions of the BCA, during the interaction with the pathogen sclerotial cells, constituting a harsh environment unsuitable for the survival of the BCA (Rey et al. 2005).

Coniothyrium minitans (Cm) is a mycoparasite on *Sclerotinia sclerotiorum* (Ss). The effect of oxalic acid (OA) degradation on the β-1, 3-glucanase activity of *Cm* which is involved in the mycoparasitism was assessed. OA was degraded by 86–92 % by *Cm* grown at 20 °C for 15 days in potato dextrose broth (PDB) medium and the pH of the cultures was increased from 3.4–4.8 to 8.3–8.6. In dual cultures of *Cm* and *Ss*, spread of *Cm* on to colonies of *Ss* was correlated with the elevation of the ambient pH from 2.9 to 6.6. Increase in the ambient pH was also evident on flower petals of oilseed rape inoculated with *Cm* and *Ss,* when they were incubated on water agar amended with 0.1 % (w/v) bromophenol blue for 6 days, compared with those inoculated with the pathogen alone. The leaf blight incidence of oilseed rape caused by flower petals inoculated with the BCA and the infection by the pathogen was lower significantly, compared to flower petals inoculated with *Ss* alone. OA degradation was correlated with the enhanced production of β-1,3-glucanase by *Cm* and the stimulated activity of this enzyme. The yield of β-1,3-glucanase produced by *Cm* was positively correlated ($R = 0.9439$, $P < 0.01$) with the ambient pH ranging from 3 to 8, implying that the increase in ambient pH caused by OA degradation may be responsible for enhanced production of β-1,3-glucanase by *Cm* in OA-containing medium. Inhibition by OA of the activity of β-1,3-glucanase produced by *Cm* was observed and the degree of inhibition was positively correlated to the concentration of OA ranging from 4 to 32 mM. The optimum ambient pH for the enzymatic reaction of β-1,3-glucanase of *Cm* ranged from 4.0 to 6.0. The results suggested that degradation of OA by *Cm* might nullify the effect of pH conditioned by OA and might improve mycoparasitism of *Cm* and *Ss* by stimulating production of β-1,3-glucanase by the BCA and/or the activity of this enzyme. Degradation of OA by *Cm* might also be a mechanism by which the BCA might protect plants from pathogen attack (Ren et al. 2007).

The role of oxalate degradation in the mycoparasitism of *Coniothyrium minitans* on *Sclerotinia sclerotiorum* was investigated. Three strains of *S. sclerotiorum* differed in their ability to produce oxalic acid (OA) on potato dextrose agar (PDA)

and Maxwell agar medium (MAM) and their mycelial susceptibility to infection by *C. minitans*. The strain PB produced negligible oxalate, while strain A5 produced greater amounts of oxalate than that produced by strain PK. Colonies of strains PB and PK formed on PDA were more susceptible to invasion by *C. minitans* than colonies of strain A5. Further, amendment of synthetic oxalate in PDA (0.25–2.00/g) suppressed the aggressiveness of parasitism by *C. minitans* on colonies of *S. sclerotiorum* strain PB. The results suggested that infection of hyphae of *S. sclerotiorum* was negatively affected by the presence of oxalate. The role of oxalate degradation by the fungal BCA in its mycoparasitism on *S. sclerotiorum* provides a key clue for improvement of the biocontrol potential of *C. minitans* (Huang et al. 2011). *Coniothyrium minitans* has been shown to be very effective against *Sclerotinia sclerotiorum* infecting several crops including winter lettuce and it is marketed as Contans. However, *C. minitans* was found to be ineffective against *S. minor* causing lettuce leaf drop disease. The efficacy of *C. minitans* against four major mycelial compatibility groups (MCGs) was evaluated in vitro at different stages sclerotial development of *S. minor.* The pathogen formed fewest sclerotia in plates that were inoculated with *C. minitans* at mycelial stage of the pathogen. The response of MCGs was inconsistent and variable. Treatment with Contans under field conditions reduced lettuce drop incidence and the number of sclerotia of *S. minor* in the soil (Chitrampalam et al. 2011).

Trichoderma asperellum isolates 697-7, PR10, PR11 and PR12 were mycoparsitic on *Phytophthora capsici*, *P. citrophthora* and *P. palmivora* causal agents of cocoa black pod disease. Culture filtrates (CFs) of the BCA isolates contained high laminarinase activity and lesser level of carboxymethyl cellulose activity which could be involved in the degradation of cell walls of the pathogen during mycoparasitism. Spraying cocoa trees with the suspensions of *T. asperellum* isolates significantly reduced cocoa pod infection by *Phytophthora* spp., compared with untreated controls in both short-term and long-term field screening experiments (Tondje et al. 2007). Green fluorescent protein (GFP) gene (*gfp*) from the jelley fish *Aequorea victoria* was used as a reporter gene to transform *Trichoderma virens* strain 110 to study its mycoparasitic activity on the sclerotia of *Sclerotium rolfsii*, *Sclerotinia sclerotiorum* and *S. minor*. Colonization of the sclerotia was tracked by fluorescent microscopy. Intracellular growth of *T. virens* in the cortex of *S. rolfsii* and intercellular growth in the medulla of *S. rolfsii* and *S. sclerotiorum* were observed. The uniform distribution of BCA mycelium just beneath the rind of the sclerotia of both *S. rolfsii* and *S. sclerotiorum* suggested that the BCA could parasitize the sclerotia through several randomly distributed entry points on the sclerotia (Sarrocco et al. 2006).

The biocontrol activity of *Trichoderma harzianum* against *Phytophthora capsici* alone or in combination with a compatible bacterial BCA *Streptomyces rochei* was evaluated. *T. harzianum* was able to not only arrest the spread of mycelial growth of *P. capsici* in the petriplate, but also invaded the whole surface of the pathogen colony and sporulated over it. The hyphae of the pathogen were surrounded by those of the fungal BCA, resulting in their subsequent disintegration and eventual suppression of the growth of *P. capsici,* as observed under the scanning electron microscope (SEM). On the other hand, *S. rochei* secreted an antifungal compound

(1-propanone-4-chlorophenyl) primarily responsible to its biocontrol activity (Ezziyyani et al. 2007). In a later investigation, mycoparasitism of *Sclerotinia sclerotiorum* by *Trichoderma harzianum* was studied by employing nucleic acid-based techniques to detect and quantify the genomic DNAs of both the BCA and pathogen. Sclerotia of *S. sclerotiorum* were incubated on *T. harzianum* cultures. Germination of sclerotia by producing mycelium was reduced by 50 % within 1 day and the decrease in germination continued with lapse of time after incubation. Quantification of *Sclerotinia* DNA in the older sclerotia by quantitative PCR assay revealed a decrease in the genomic DNA, indicating decrease in pathogen population. In contrast, the *Trichoderma* DNA registered an increase and the increase persisted in the older sclerotia, reflecting the higher population of *T. harzianum.* Fresh sclerotia did not seem to be affected by *T. harzianum* (Kim and Knudsen 2009).

Coprinellus curtus strain GM-21 was able to suppress the bottom-rot disease of Chinese cabbage caused by *Rhizoctonia solani*. The BCA inhibited the pathogen development by hyphal interference. The antifungal spectrum of GM-21 included *Fusarium* spp. in addition to *R. solani*. Hyphal interference between strain GM-21 and *Fusarium* spp. causing crown rot and root rot disease of tomato and also melon wilt disease was also observed under light microscope (Nakasaki et al. 2007). The mechanism of antagonism of *Trichoderma atroviride* against *Rhizoctonia solani* AG3 causing black scurf disease of potato was studied using confocal microscopy. The antagonist mycelium could be easily differentiated from the pathogen by hyphal morphology. Hyphae of *T. atroviride* established close contact with those of *R. solani* by coiling. The coils were very dense encircling the pathogen hyphae very tightly. At 7 days after establishing the contact, the BCA hyphae penetrated *R. solani* hyphae resulting in loss of turgor. On the other hand, the endophytes *Phomopsis* sp., *Epicoccum nigrum* and *Alternaria longipes* exhibiting antagonism against *R. solani* did not either form coils around or penetrate into the hyphae of the pathogen. However, they induced abnormal morphology and lysis of pathogen cells, probably by producing antifungal compounds resulting in recognizable inhibition zones (Lahlali and Hijri 2010). The mechanisms of biocontrol activity *Pythium oligandrum* against *Rhizoctonia solani* AG-3 causing black scurf disease of potato tubers were studied. Seed tubers infected with black scurf sclerotia were dipped for a few seconds in a suspension of *P. oligandrum* oospores and were then air-dried. Confocal laser scanning microscopic observation with an immuno-enzymatic staining technique revealed that the hyphae of *P. oligandrum* had colonized the sclerotia and established close contact by coiling around *R. solani* hyphae present on the surface of seed tubers, in a manner similar to that observed in the dual-culture test. Quantification of *R. solani* DNA by PCR showed that the population of *R. solani* was reduced on the seed tubers treated with *P. oligandrum* compared with untreated control tubers (Ikeda et al. 2012).

The nature of interactions between mycoparasite *Cladosporium tenuissimum* and the bean rust pathogen *Uromyces appendiculatus* was investigated using light and scanning electron microscopy (SEM). When the urediniospore came into contact with ungerminated conidia of *C. tenuissimum,* germination of pathogen spore decreased. In contrast, *C. tenuissimum* continued its growth towards the rust spores

and coiled around their germ tubes. Penetration of urediniospore occurred rather enzymatically and/or mechanically through appressorium or infection cushion structures from which a thin penetrating hypha was produced. The hyphae of the BCA grew within the host spore, emptied its content and emerged profusely forming conidiophores and conidia. By applying the culture filtrates of *C. tenuissimum,* bean rust disease was entirely suppressed. But conidial suspension did not show any suppressive effect. Cladosporal and related compounds were isolated from culture filtrates of *C. tenuissimum* and these compounds may have a role in the antagonistic potential of this mycoparasitic fungus (Assante et al. 2004).

Five yeast strains *Pichia anomala* Moh 93, *P. anomala* Moh 104, *P. guilliermondii* Moh 10, *Lipomyces tetrasporus* Y-115 and *Metschnikowia lunata* Y-1209 were evaluated for their efficacy of antagonism against *Botryodiplodia theobromae* causing guava Diplodia rot disease. Direct interaction between the BCA and the pathogen was studied using the scanning electron microscope (SEM). *P. anomala* Moh 93 tenaciously adhered to the pathogen hyphae followed by accumulation of extracellular matrices around the hyphae of the pathogen. The hyphae were penetrated extensively by the yeast leading to the complete destruction of the pathogen cells. Further production of cellulase and pectinase enzymes in guava fruit infected by *B. theobromae* was significantly inhibited by the BCA, possibly resulting in the reduction in the fruit decay during postharvest stage (Mohamed and Saad 2009). The interaction between the yeast antagonist *Pichia guilliermondii* and *Botrytis cinerea* was observed under the scanning electron microscope (SEM). The conidia were induced to germinate with glucose and phosphate and penetrate the strawberry leaves. In the presence of *P. guilliermondii*, conidia either did not germinate or it could form only short germ tubes. The yeast cells were found attached to conidia or at a distance from them and some of its cells were in the process of budding. The conidia were found intact. But when *P. guilliermondii* was applied as a mixture containing the bacterial antagonist *Bacillus mycoides,* no germination of conidia could be seen. Most of the conidia were shrunken, with distorted surfaces or with severe breaks and loose cell walls (Fig. 3.2; Guetsky et al. 2002). The ability of yeasts to attack to the hyphae or conidia of fungal plant pathogens was considered as the initial step for the biocontrol activity of the yeast species against the target pathogen (s). Majority of the yeast isolates (292) from phylloplane was able to attach to the conidia of *Botrytis cinera*. But ten yeast isolates including eight isolates of *Cryptococcus laurentii*, one isolate of *C. flavescens* and one unidentified *Cryptococcus* sp. failed to attach to *B. cinerea* conidia. Production of copious extracellular polysaccharide (EPS) by all non-attaching yeasts on PDA was observed. Culture medium had significant influence on attachment of yeast cells to *B. cinerea*. Attachment of *Rhodotorula glutinis* PM4 with remarkable biocontrol activity was significantly at higher level at a concentration of 1×10^7 cells/ml, indicating the effect of yeast cell concentration on the level of attachment to pathogen conidia (Allen et al. 2004).

Pichia membranifaciens strain FY-101 isolated from grape skins effectively suppressed the development of the gray mold pathogen *Botrytis cinerea.* In the cocultured plates, a small zone of inhibition was seen around the yeast BCA.

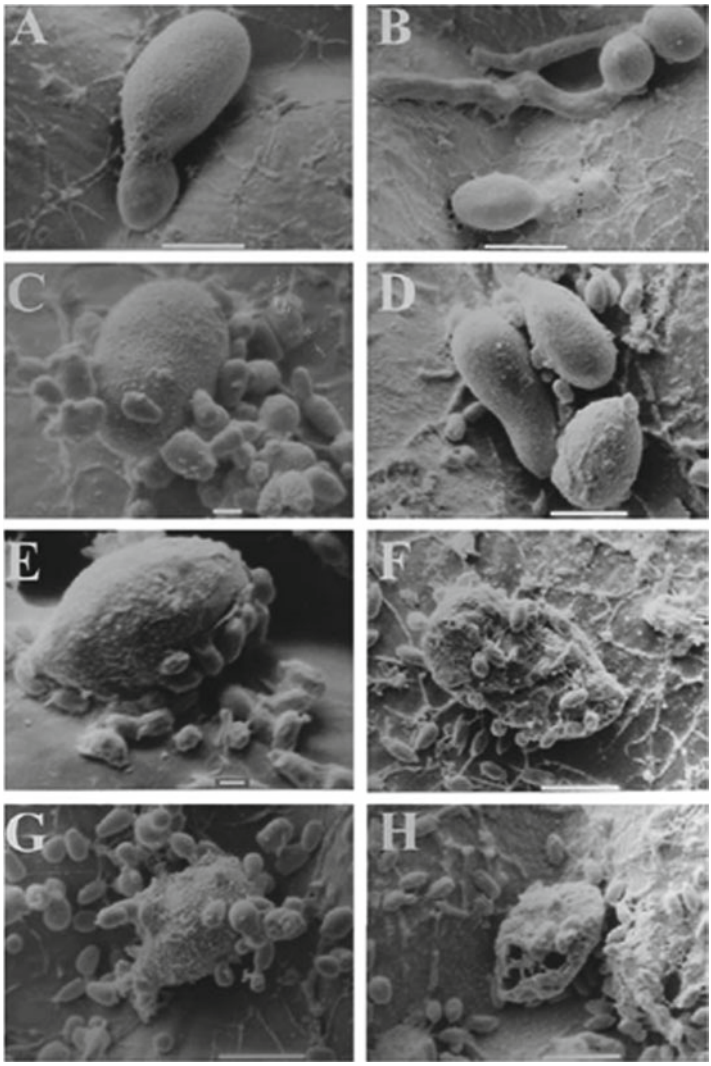

Fig. 3.2 Scanning electron microscopic (SEM) observations of interaction between *Botrytis cinerea* and *Pichia guilliermondii* (**a**) and (**b**): germinating conidia of *B. cinerea* at 6 (**a**) and 24 h (**b**) after application on strawberry leaves; (**c**) and (**d**): cells of *P. guilliermondii* attached to conidia of *B. cinerea* resulting in failure of germination or production of only short germ tubes (Courtesy of Guetsky et al. 2002 and with kind permission of The American Phytopathological society, MN, USA)

Hyphae developing in the vicinity of the zone of inhibition failed to sporulate. Microscopic observations showed that *B. cinerea* mycelium in contact with the BCA showed extensive coagulation of the protoplasm of *B. cinerea* and many empty hyphal cells could be visualized. The results indicated the mycoparasitic interaction of *P. membranifaciens* with the pathogen *B. cinerea* (Masih et al. 2001). In the case

of *Pythium lycopersicum* antagonistic to *Botrytis cinerea*, the gray mold pathogen, the hyphal interaction resulted in inhibition of growth and sporulation of *B. cinerea*. The robust mycelium of the pathogen became coagulated initially and tearing off the mycelium occurred later. These changes observed in vitro might explain the complete protection of the grapevine coinoculated with the BCA and the pathogen. The infected mycelium of *B. cinerea* lacked pathogenic potential to infect grapevine (Karaca et al. 2008).

The intercellular interaction between the antagonist *Verticillium lecanii* and the pathogen *Penicillium digitatum,* causing green mold was studied using transmission electron microscopy (TEM) and gold cytochemistry procedures. The growth of *P. digitatum* was inhibited by *V. lecanii* and this effect could be correlated with striking changes in the cells of *P. digitatum*, including retraction of the plasma membrane and cytoplasm disorganization. Deposition on the inner host cells surface of a chitin and cellulose-enriched material considered as a host structural defense reaction occurred afterwards. The accumulation of a new chitin correlated with a decrease in the amount of cell wall-bound chitin in the pathogen (Benhamou and Bordeur 2000; Benhamou 2004). *Acremonium strictum* has been shown to be a novel mycoparasite on *Helminthosporium solani,* causative agent of potato silver scurf disease. Both *A. strictum* and *H. solani,* are present invariably together. Repeated hyphal tip isolation technique was necessary to obtain axenic culture of *H. solani*. *A. strictum* was tightly linked to and partially dependent on *H. solani* in culture. It appeared that *A. strictum* was dependent on *H. solani* for its survival and for its growth in the culture. However, growth, sporulation and germination of *H. solani* were reduced in the presence of *A. strictum.* Observation under scanning microscope revealed shrivelled and shrunken conidia of *H. solani,* when present together with *A. strictum.* This effect may be apparently due to either direct parasitism or antifungal compounds secreted by the BCA. *A. strictum* could reduce sporulation of *H. solani* (Rivera-Varas et al. 2007). The nature of antagonism of the endophytic fungus *Piriformospora indica* against *Pseudocercosporella herpotrichoides* was studied using light microscopy. When *P. indica* was grown along with *P. herpotrichoides,* the hyphae of the pathogen appeared to be more irregular and curled. The hyphal tips exhibited more short branches (Serfling et al. 2007).

The sequence of events occurring during the interaction between the strain T472 of *Trichoderma harzianum* and *Gibberella zeae* was studied using scanning electron microscope (SEM). The autoclaved and mulched wheat straw was inoculated with *G. zeae* (control) and on straw treated with *T. harzianum* strain T472. An average of 167 perithecia were formed on untreated straw, whereas only an average of 15 perithecia were produced by *G. zeae* on straw treated with T472. The cells of the outer wall of the perithecia produced on treated straw were abnormal in appearance and unevenly distributed across the surface. Overgrowth of the perithecia by *T. harzianum* could seen clearly. At 15 days after inoculation (dai), mycelia and numerous spores of T472 could be visualized covering the surface of the young perithecia. Colonization of the perithecia by T472 could be observed at 21 dai. *T. harzianum* might secrete compounds that disrupt potassium and chloride ion transport into the perithecium. No direct penetration of perithecia by T472 could be seen. The BCA

colonized the substrate rapidly and out-competed the pathogen *G. zeae* causing Fusarium head blight (FHB) disease in wheat (Inch and Gilbert 2011). A multivariate weighted average (WA) regression approach showed that by using chemical signatures an effective method could be developed for predicting which *T. harzianum* isolates/compounds might be involved in reducing the number of perithecia produced by *G. zeae* (Inch et al. 2011).

Fusarium graminearum, one among the *Fusarium* spp. causing Fusarium head blight (FHB) disease of cereals produces the mycotoxins 3-acetyl- deoxynivalenol (3-ADON) and 15-acetyldeoxynivalenol (15-ADON). These mycotoxins induce serious ailments in human beings and animals, when the grains contaminated with mycotoxin are consumed. The mycoparasitic activity of *Sphaerodes mycoparasitica* on *F. graminearum* 3- and 15-ADON strains was determined in vitro using microscopic and PCR techniques. The germination of the ascospores of *S. mycoparasitica* was greatly enhanced in the presence of *F. graminearum* strains, indicating a compatible interaction between the BCA and pathogen strains. A quantitative real-time PCR was developed employing the *Fusarium*-specific (Fg16N) and trichothecene *Tri5* (TOX5-1/2)-specific primer sets. The amounts of DNA of *F. graminearum* 3-ADON and 15-ADON strains were reduced in the presence of *S. mycoparasitica,* indicating a significant reduction in pathogen population, when the BCA and the pathogen were coinoculated. The results showed that *S. mycoparasitica* was able to germinate in the presence of *F. graminearum* filtrates and also establish biotrophic mycoparasitic relations with two *F. graminearum* chemotypes suppressing their growth in vitro (Vujanovic and Goh 2011). *Talaromyces* sp. isolate KNB-422 was effective in suppressing the development of rice Bakanae disease caused by *Gibberella fujikuroi*. Green fluorescent protein (GFP)-labeled transformant was generated to visualize cell-to-cell interactions between the BCA and pathogen. The hyphal cell wall of *G. fujikuroi* collapsed and fluorescence of its cytoplasm disappeared at 3 days after contact with hyphae of *Talaromyces* sp. transformant. On inoculated plants, both the BCA and pathogen occupied the same regions of coleoptiles and roots, where the parasitic effect of *Talaromyces* sp. had to be exerted. The results suggested that the isolate KNB-422 acted on *G. fujikuroi* through mycoparasitism (Kato et al. 2012).

Ampelomyces quisqualis has been used as biocontrol agent against different powdery mildew pathogens infecting grapes, apple and roses. An isolate of this BCA has been commercialized and marketed as AQ 10 Biofungicide. *Ampelomyces* parasitizing clover mildew produced saprophytic phoma-like pycnidia in senescent clover leaf tissues at the end of the season and they survived until the next spring, suggesting that the BCA might overwinter in the field as saprophytic pycnidia in the leaf litter. Overwintering of *Ampelomyces* in the parasitized ascocarps of *Erysiphe necator* (syn. *Uncinula necator*) on the bark of grapevine stocks was reported by Falk et al. (1995). The mode of survival of *Ampelomyces* was studied by examining apple shoots and aerial parts of other plant species infected with powdery mildews during late winter and early spring of 1998–2003. The viability and subsequent mycoparasitic activity of the hyphae of *Ampelomyces* emerging from the overwintered fungal structures were assessed. The overwintered pycnidia, when placed

adjacent to the fresh powdery mildew colonies (*Podosphaera leucotricha*), initiated the life cycle. Likewise, thick-walled resting hyphae present in the dried powdery mildew mycelia also germinated giving rise to new intracellular pycnidia. On apple trees, the BCA overwintered as resting hyphae in the dried powdery mildew mycelia covering the shoots and in parasitized ascomata of *P. leucotricha* on the bark and scales of buds. About 31 % of the field samples of apple trees contained overwintered structures of *Ampelomyces.* The results indicated that the BCA could survive the winter in the field as pycnidia and resting hyphae in the dried mycelia of powdery mildew pathogens (Szentiványi and Kiss 2003).

The mycoparasites *Acremonium alternatum*, *Ampelomyces quisqualis* and *Lecanicillium lacanii* were evaluated for their efficacy in reducing the powdery mildew disease caused by *Sphaerothea fusca* on melon in greenhouses. Using microscopy, the effect of mycoparasitic fungi on the formation of infection structures such as haustoria, conidia and conidiophores was quantified. *L. lecanii* was found to be more efficient, when applied in the early stages of infection than the other mycoparasites (Romero et al. 2003). In a later study, the biocontrol potential of two mycoparasite products AQ10® containing *Ampelomyces quisqualis* and Mycotal® (*Lecanicillium lecanii*) as well as three strains of *Bacillus subtilis* was evaluated for the control of melon powdery mildew disease caused by *Podosphaera fusca* under greenhouse conditions. Observations under scanning electron microscope (SEM) revealed the mycoparasitic behavior of the fungal BCAs. The presence of the mycoparasites on melon leaves and extensive parasitism of *P. fusca* structures could be visualized. *L. lecanii* interacted with the pathogen ectoparasitically by penetrating the host hyphae. On the other hand, *A. quisqualis* induced typical swellings at the base of the conidiophores of *P. fusca* corresponding to the internal formation of pycnidia of the BCA. The conidia were also deformed in the presence of *A. quisqualis*. The fungal BCAs performed better under conditions of high relative humidity (90–95 %) (Romero et al. 2007).

Mycoparasitism of other obligate pathogens *Cronartium flaccidum* and *Peridermium pini* causing needle pine stem rust disease by a fungal BCA *Cladosporium tenuissimum* was studied, using light and scanning electron microscopy. The host-parasite interface was clearly visualized. The growth of *C. tenuissimum* was profuse and abundant in the vicinity of the rust aeciospores with the formation of bundles of hyphae that coiled around the pathogen spores. The BCA seemed to be strongly attracted to the host and attached to the rust spores either by producing appressoria of different shapes and sizes to establish an intimate relationship with the host. A felty, dark greenish-brown mycelium covered the spermatial and aecial fructifications on the bark of seedlings sprayed with the conidial suspension of the BCA. Typical sporulating structures (conidia and conidiophores) were formed in the decayed fructifications of the pathogen. As the BCA could destroy the fructification and the spores of the pathogen, the spread of the rust disease may be restricted to some extent (Moricca et al. 2001). The mycoparasitic activity of *Sphaerellopsis filum* (teleomorph: *Eudarluca caricis*) on *Melampsora larici-epitea* was assessed using willow leaf disc assay. Inoculum densities of *S. filum* were significantly correlated with the frequency of uredinia infected. Rust spore production was

negatively correlated with the frequency of uredinia infected, the number of *S. filum* pycnidia and the number of *S. filum* spores produced. This mycoparasite might be useful for the biocontrol of willow rust disease (Pei et al. 2003). Infestation of the coffee rust pathogen *Hemileia vastatrix* by the entomopathogenic fungus *Lecanicillium lecanii* was frequently observed under field conditions. The mycoparasitism of *L. lecanii* was demonstrated in vitro also. A search for spatial correlation between the attack of *L. lecanii* on the scale insect (*Coccus viridis*) and the incidence of rust in a commercial coffee crop was carried out. A weak but statistically significant effect of hyperparasitic control of coffee rust by *L. lecanii* through ant-coccoid mutualism, resulting in the spread of inoculum to the rust pathogen (Vandermeer et al. 2009). Direct predation of grapevine leaf rust pathogen *Phakopsora euvitis* by the coccinellid *Psyllobora rufosignata* was observed. The presence of the rust uredospores in the gut contents of *P. rufosignata* was detected after feeding on the infected leaves (Culik et al. 2011).

Studies on the molecular basis of mycoparasitism have provided an insight into the interaction between the BCAs and fungal pathogens. Mycoparasitism involves the activities of several cell wall degrading enzymes (CWDEs) including proteases, chitinases and glucanases (Inglis and Kawchuk 2002; Sanz et al. 2004). Purified host cell walls, substances secreted by hosts and also live host may stimulate the expression of the genes encoding these enzymes. Such enhanced gene expression can be expected to improve the biocontrol potential of the BCAs. Expression of novel genes in *Trichoderma hamatum* effective against *Sclerotinia sclerotiorum, S. minor, Rhizoctonia solani* and *Pythium* spp. causing diseases in a wide range of crops, was studied using subtractive hybridization (SSH) technique. The homologues of *chit 42* and *prb1,* two genes considered to be essential for mycoparasitism in other *Trichoderma* spp., were expressed at higher levels by *T. hamatum* during confrontation with *S. sclerotiorum*. However, the expression of *chit42* and *prb1* in *T. hamatum* in medium containing glycerol differed significantly from *T. atroviride,* suggesting that substantial differences might exist in mycoparasitism in these two BCA species. The sequence, Northern and Southern analysis of the subtraction products revealed 19 novel *T. hamatum* genes upregulated during mycoparasitism, representing a substantial increase in the number of *T. hamatum* genes. Four sequences had no significant similarity to any sequences in GenBank and they may be perhaps restricted to mycoparasites to facilitate mycoparasitism. The SSH technique was shown to be an effective method for identifying genes upregulated during mycoparasitism (Carpenter et al. 2005).

Fusarium solani causes root rot disease of common bean *(Phaseolus vulgaris*). *Trichoderma harzianum* is effective in suppressing the development of the disease through mycoparasitism and it has the potential to be used as an alternative to chemical control for the root rot disease. A transcriptome analysis was performed using expressed sequence tags (ESTs) and quantitative real time PCR (RT-qPCR) approaches for gaining insights into the biocontrol mechanism of *T. harzianum* for the suppression of the pathogen development. A cDNA library from *T. harzianum* mycelium (isolate ALL42) grown on cell walls of *F. solani* (CWFS) was constructed and analyzed. A total of 2,927 high quality sequences were selected from 3,845 and 37.7 % were

identified as unique genes. The gene ontology analysis indicated that majority of the annotated genes were involved in metabolic processes (80.9 %) followed by cellular processes (73.7 %). Twenty genes that encoded proteins with potential role in biological control were investigated. RT-qPCR analysis showed that none of these genes were expressed, when *T. harzianum* was challenged with itself. These genes showed different patterns of expression during in vitro interaction between *T. harzianum* and *F. solani* (Steindorff et al. 2012).

Mycoparasitism of *Sclerotinia sclerotiorum* by *Trichoderma harzianum* was studied by using green fluorescent protein (GFP)-transformed *T. harzianum* ThzID1-M3. A specific PCR primer/probe set for detecting the GFP-transformed isolate was developed. Quantitative real-time PCR was evaluated along with epifluorescence microscopy and image analysis to investigate dynamics of colonization of sclerotia in non-sterile soil. It was possible to quantify the amounts of ThzID1-M3 DNA and *S. sclerotiorum* DNA from individual sclerotia using the real-time PCR assay. Epifluorescence from the transformant was quantified using computer image analysis for estimating colonization on a per-sclerotium basis. Colonization of sclerotia by *T. harzianum* on agar plates was observed using confocal laser scanning microscopy to observe the GFP-fluorescing hyphae Thz ID1-M3. This procedure, although highly labor-intensive, provided high spatial resolution of colonization dynamics. Both methods quantified colonization of sclerotia by the BCA over a period of time. The real-time PCR provided a more precise assessment of the extent of sclerotial colonization and it could be more easily applied to sample entire sclerotia (Kim and Knudsen 2011).

Suppression of production and liberation of spores by employing biocontrol agents can be a successful approach for crop disease management. The isolates of yeast species *Candida sake, C. pulcherrima, Galactomyces geotrichum* and *Trichosporon pullulans* were evaluated for their ability to suppress liberation of conidia from *Botrytis cinerea*, the gray mold pathogen. The yeast cell suspension from each isolate was mixed with cellulose and dried. The product was milled into a fine powder. This yeast-cellulose formulation was applied as dry powder on sporulating colonies of *B. cinerea* on inoculated kiwifruit leaf disks. The yeasts attached to conidia and conidiophores of the pathogen colonies and significantly suppressed spore liberation. *C. pulcherrima* isolate 662 dib suppressed conidial liberation significantly (Table 3.1). The application of the yeasts to suppress conidial liberation could be an effective method to reduce airborne inoculum and to reduce consequent epidemic development. Selection of the yeast isolate and the cellulose component in the formulation were found to be important for suppression of spore liberation. The α-cellulose was highly effective in suppressing spore liberation irrespective of the yeast isolate (Cook 2002a, b).

The mechanism of biocontrol activity of two antagonistic yeast species *Pichia membranifaciens* and *Cryptococcus albidus* effective against three pathogens *Monilinia fructicola, Penicillium expansum* and *Rhizopus stolonifer* was studied in apple juice agar plates and apple wounds. Observations under light and scanning electron microscopes showed that *P. membranifaciens* exhibited stronger capacity of attaching to the pathogen hyphae than *C. albidus.* By applying sodium dodecylsulfate

Table 3.1 Effect of treatment with yeast isolate 662 dib on conidial liberation from *Botrytis cinerea* (Cook 2002a, b)

	Isolate 662 dib (mean spore density/mm)	
Inoculum dose	Untreated	Treated
2 Disks	5.62 a[a]	2.77 b
1 Disk	3.28 a	1.94 b
½ Disk	0.69 a	0.37 b
¼ Disk	0.38 a	0.17 b

[a]Treatments with the same letter are not significantly different at P>0.05 according to Fisher's least significant difference analysis

(SDS) and β-mercaptoethanol, it was possible to block the yeast attachment to the hyphae. Culture extract of *P. membranifaciens* showed higher β-1,3-glucanase and exo-chitinase activities than *C. albidus,* when the cell wall preparations of the fungal pathogens were used as sole carbon source. The results showed that firm attachment of yeast cells to hyphae and secretion of lytic enzymes might be the principal mode of action of the yeast species on the fungal pathogens causing post-harvest diseases of apples (Chan and Tian 2005). In another study, the biocontrol potential of *Pichia membranifaciens* against *Penicillium citrinum* and *Verticicladiella abietina* causing green mold decay in post harvest Chinese bayberries (*Myrica rubra*) was assessed. The washed cell suspensions of the yeast was more effective in protecting the Chinese bayberries than the yeast in culture broth at the same concentration. Higher the concentration of the yeast cells, lower was the disease incidence. The activities of the defence-related enzymes chitinase and β-1,3-glucanase were induced in Chinese bayberries treated with the yeast. The results showed that *P. membranifaciens* reduced the fruit decay possibly by directly inhibiting the pathogen growth and indirectly by inducing resistance to disease in treated fruits during postharvest storage (Wang et al. 2011).

3.1.2 Antibiosis

The fungal biocontrol agents may produce enzymes, antifungal and antibacterial compounds that can restrict the development of phytopathogens.

3.1.2.1 Production of Antifungal Enzymes

Degradation of fungal cell walls requires enzymes that can hydrolyze polymers of glucose with various glycosidic linkages. Chitinase and β-1,3-glucanase are considered to be important fungal cell wall-dissolving enzymes, because they attack the most common cell wall-forming polymers in fungi, β-1,3-glucan and chitin.

The BCAs secrete enzymes such as chitinases and/or glucanases that suppress the development of several fungal pathogens providing the basis for the concept of enzyme biosynthesis as a mechanism of biocontrol of microbial plant pathogens. These enzymes act on the pathogens by breaking down the polysaccharides, chitin and β-glucans that provide rigidity to the fungal cell walls, resulting in the loss of cell wall integrity and ultimate cell collapse. Inhibition of growth and sporulation of fungal pathogens by fungal BCAs may be partly due to the activity of the lytic enzymes including β-1,3-glucanases and chitinolytic enzymes. The antagonistic properties of *Trichoderma harzianum* against *Botrytis cinerea* were studied. *T. harzianum* antagonized the pathogen by antibiosis, leading to cell death followed by degradation of the cell wall by chitinolytic enzymes (Bélanger et al. 1995). In a later study, isolates of six unidentified *Trichoderma* spp. and *T. harzianum* were evaluated for their ability to produce chitinolytic enzymes and β-1,3-glucanases. All isolates of *Trichoderma* spp. and *T. harzianum* exhibited substantial enzymatic activities. The chitinase from *Trichoderma* spp. was purified and the hydrolytic action of the purified chitinase was assessed using scanning electron microscopy. The chitinase hydrolyzed the cell walls of *Sclerotium rolfsii,* but it had no effect on the cell wall of *Rhizoctonia solani* (Lima et al. 1997).

Trichoderma koningii (Tr5) did not invade healthy tissues of onion roots and it did not kill seedlings, but it colonized infected or damaged onion root tissue as secondary colonizer. *Trichoderma koningii* colonized onion roots infected by *Sclerotium cepivorum,* casual agent of Allium white rot disease, by producing hyphae that branched and spread throughout the root cortical tissues damaged by enzymes and toxins that diffused ahead of the pathogen hyphae and impeded the path of infection. Further, the pathogen hyphae became detached at septa; cell walls were hydrolyzed and many hyphal apices were burst. Lysis of the pathogen mycelial cells did not depend on the contact of the BCA with the pathogen hyphae, indicating that lysis was due to antifungal compounds secreted by the BCA. By applying PAGE technique, chitinolytic enzymes produced by *T. koningii* were detected. Four isozoymes (proteins) were detected in the chitinase medium on which *T. koningii* was grown. It produced two endochitinases (R_f 0.15 and 0.24) and two exo-acting chitinolytic enzymes (R_f 0.46 and 0.62) during degradation of crabshell chitin and *S. cepivorum* cell walls. Two proteins (R_f 0.24 and 0.46) detected in infected roots colonized by *T. koningii* might be involved in the process of antagonism by this BCA (Metcalf and Wilson 2001). Production of chitinolytic enzymes by *Trichoderma harzianum* strain T5 and their involvement in inhibiting the development of sugarcane red rot disease caused by *Colletotrichum falcatum* were investigated. *T. harzianum* strains exhibited greater chitinolytic activity in the presence of chitin. Strain T5 showed enhanced levels of *N*-acetylglucosaminidase and β-1,3-glucanase activities, when grown on minimal medium containing chitin or pathogen cell wall fragments. Inhibition of conidial germination and mycelial growth of *C. falcatum* was ascribed to the activity of chitinolytic enzymes of the BCA (Fig. 3.3; Viswanathan et al. 2003).

The development of cocoa witches' broom disease caused by *Crinipellis perniciosa* was impaired by the application of *Trichoderma* spp. isolates under field conditions.

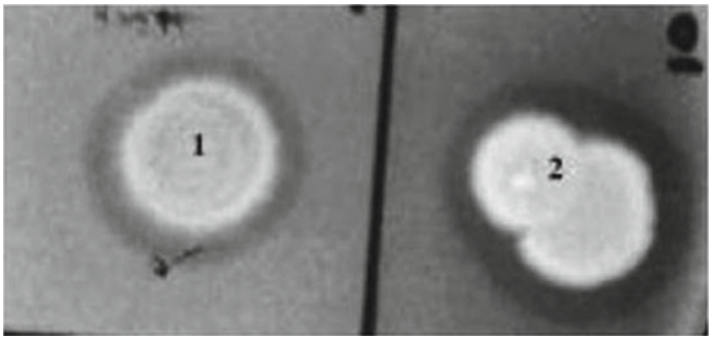

Fig. 3.3 Inhibition of mycelial growth of *Colletotrichum falcatum* by *Trichoderma harzianum* strain T5 in chitin-amended medium (Courtesy of Viswanathan et al. 2003 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

The most effective *T. harzianum* isolate 1051 was grown in *Trichoderma* liquid medium for investigating its ability to produce hydrolytic enzymes and to characterize them. Scanning electron microscopic (SEM) observations revealed the sites of hydrolysis on the hyphal cell walls of the pathogen. The SDS-PAGE analysis showed that the BCA produced several proteins in the medium. Two chitinases were detected by immunoblotting technique. Polyclonal antibody (PAb) specific to chitinase reacted with the 37.8-kDa protein. The partially purified chitinase from *T. harzianum* disrupted the cell wall of *C. perniciosa* as observed under SEM. The optimum pH and temperature for chitin hydrolysis by the partially purified chitinase were 4.0 and 37 °C respectively (De Marco et al. 2000). An amylase purified from the medium used for growing *T. harzianum* isolate 1051 was investigated for its hydrolytic activity on *C. perniciosa* using SEM. The amylase showed only a very discrete effect on the pathogen cell walls in contrast to the drastic hydrolysis of cell walls induced by chitinase produced by the same BCA isolate (de Azevedo et al. 2000). Multiple modes of action of BCAs on fungal pathogens have been reported, while assessing the effects of seed treatment with fungal BCAs. Seeds of maize inbred line Mo17 were treated with *Trichoderma harzianum* T22 which protected plants against the root pathogen *Pythium ultimum* and foliar pathogen *Colletotrichum graminicola*. The presence of T22 strain increased the protein levels as well as the activities of β-1, 3-glucanase, exochitinase and endochitinases in both roots and shoots. The BCA added to seed, soil or roots resulted in colonization, but little or no colonization of shoots. The role of the enzymes in the biocontrol potential was not clearly indicated, although the possible induction of resistance to these diseases was suggested (Harman et al. 2004a, b, c).

The complex process of parasitism involves different steps such as recognition of the host, attachment and subsequent penetration and killing of host cells. During this process, *Trichoderma* spp. secretes hydrolytic enzymes that hydrolyze the cell wall of the host fungus (Woo et al. 2006). The proteolytic activity of *T. harzianum* is a pre-requisite for the lysis of the protein matrix of the pathogen cell wall and for inactivation of the hydrolytic enzymes secreted by the pathogen, leading to decrease

in its pathogenicity. Isolates of *T. harzianum* with high potential for the secretion of hydrolytic enzymes may be obtained by screening the isolates from different natural sources such as compost or agricultural soils or through transformation of the fungus with multiple copies of the genes involved in the biosynthesis of these enzymes (Ruiz-Díez 2002; Rincón et al. 2008). *Trichoderma harzianum* was antagonistic to ten isolates of *Fusarium oxysporum* f.sp. *lycopersici* causing Fusarium wilt disease of tomato in different locations in three States in India. The fungal BCA inhibited the mycelial growth of the pathogen. The culture filtrate with volatile and non-volatile metabolites of *T. harzianum* showed inhibitory effect on all pathogen isolates (Mishra et al. 2010). The mechanism of biocontrol activity of strains of *Trichoderma* sp. on *Sclerotium rolfsii* and *Fusarium oxysporum* f.sp. *ciceri* was studied. The BCA strains were plated on media amended with colloidal chitin and cell wall extracts of *S. rolfsii*. Chitinolytic activity was detected in all isolates of *Trichoderma* spp. tested. Two strains had maximum endochitinase and exochitinase activity. In addition, these strains produced cellulase which may contribute to the biocontrol potential of *Trichoderma* sp. (Anand and Reddy 2009). *Trichothecium roseum* MML 003 was found to have strong suppressive effect on *Rhizoctonia solani,* causative agent of rice sheath blight disease. *T. roseum* showed neither mycoparasitic activity nor ability to produce siderophores and H_2O_2. The culture filtrate (CF) of *T. roseum* inhibited the mycelial growth and formation of sclerotia in *R. solani*. The sclerotial germination and viability were also significantly reduced by treatment with the CF. Suppression of sheath blight disease development was reduced under greenhouse condition. The results indicated that the antifungal compounds produced by the BCA was responsible for the biocontrol activity of *T. roseum* (Jayaprakashvel et al. 2010).

Trichoderma harzianum isolates have been employed against wide spectrum phytopathogenic fungi including *Fusarium oxysporum* f.sp. *melonis,* causative agent of Fusarium wilt disease of melon. *Trichoderma* spp. isolates (31) were analyzed by random amplified polymorphic DNA (RAPD)-PCR technique and five isolates of *T. harzianum* (T-30, T-31, T-32, T-57 and T-78) were selected. These isolates were characterized by their ability to secrete hydrolytic enzymes such as chitinases, glucanases and proteases. In the plate cultures, the greatest mycoparasitic activity was exhibited by the isolates T-30 and T-78, as reflected by the total and extracellular hydrolytic activities of *N*-acetyl glucosaminidase (NAGases), chitinase and β-1,3-glucanase which were greater than other isolates tested. The expression of genes encoding for NAGases (*exc1* and *exc2*), chitinases (*chit42* and *chit33),* proteases (*prb1)* or glucanases (bgn 13.1) activities and their respective enzymatic activities in vitro were measured. Different profiles of gene expression between various *T. harzianum* isolates were related to the activities values and dual plate confrontation test. The high NAGase activity observed for T-30 and T-78 corresponded with the levels of expression of the gene *exc1* for T-30, but not for T-78. The high NAGase activity of the isolate T-28 might be due to a higher expression of *exc1* over previous hours before sampling. The high chitinase activity of T-78, both total and extracellular, could be linked to the levels of expression of the genes *chit42* and *chit33*, since both were highest for this isolate. These two isolates exhibited the maximum

activity of β-1,3-glucanase. These values corresponded with the expression level of gene *bgn* 13.1 for T-30. The isolates T-30 and T-78 exhibited the greatest mycoparasitic potential against *F. oxysporum* f.sp. *melonis* (López-Mondéjar et al. 2011).

Ulocladium atrum strain 385, when applied on onion leaf tip and cyclamen, consistently reduced both sporulation of *Botrytis cinerea* causing gray mold diseases on several crops and development of disease symptoms. *U. atrum* 385 and two strains (18558 and 18559) were evaluated for their ability to produce enzyme that have antifungal activity. The enzymatic activities of the three strains along with *B. cinerea* during colonization phase of necrotic tissues were compared. *U. atrum* 385 exhibited the highest lipase, pectate lyase and cellobiase activities, while *B. cinerea* had maximum activity of endo-β-1,4-glucanase activity. Assessment of lytic activities that hydrolyzed fungal cell wall revealed higher β-1,3-glucanse activity of *U. atrum* 385 and this activity was induced by the presence of *B. cinerea* on necrotic strawberry leaflets. The results suggested that cell wall degrading enzymes (CWDEs) of plant and BCA might have a complementary role in the competitive colonization of dead strawberry leaves against pathogen development (Berto et al. 2001). In order to simulate lytic components existing in mulches suppressive to *Phytophthora cinnamomi,* two enzyme systems, cellulase (β-1,3-glucanse) and laminarinase (β-1,3-glucanse) were added to soil extracts. Cellulase inhibited significantly the development of zoosporangia and chlamydospores, when the mycelia were incubated in soil extract containing the enzyme extract at 10 units/ml and above. Zoospore production was also reduced by cellulase, while laminarinase had no effect. However, laminarinase was more effective in preventing encystment of zoospores, compared with cellulase. Low concentration of cellulase stimulated infection of excised roots by *P. cinnamomi*. In contrast, low concentration of laminarinase prevented pathogenic infection. The results suggested that each enzyme may have a role in the reduction of inoculum, although these enzymes may have different effects on the pathogen propagules (Downer et al. 2001).

Jungle soils containing high amounts of organic mulches have been reported to be suppressive to *Phytophthora cinnamomi* infecting avocado in Australia. In the Ashburner system, huge amounts of organic mulches that contain large quantities of cellulase are added to recreate suppressiveness to *P. cinnamomi*. Antifungal compounds including enzymes and antibiotics secreted exogenously may accumulate in soil, resulting in a suppressive environment harmful to zoosporangia, zoospores, oospores, chlamydospores and mycelium of the pathogen (Erwin and Ribeiro 1996). The cell walls of *P. cinnamomi* are composed of cellulose (β-1,4-glucans) and β-1,3 and β-1, b-linked glucans. Cyst cell walls of *Phytophthora* have primarily β-1,4-glucan linkages, while the hyphae have a lower content of cellulose (Bartnicki-Garua and Wang 1983).

Studies on the molecular biology of the confrontation between the biocontrol agents and fungal plant pathogens have provided evidence for the involvement of chitinases in suppression of pathogen development. The gene encoding for chitinase (*chit42*) in *Trichoderma virens* was disrupted or over-expressed. Decrease or increase in biocontrol activity of the transformants matched with the disruption or over-expression of *chi 42* gene in the cotton-*Rhizoctonia solani* pathosystem.

Since the differences in the biocontrol activity of the transformants and wild strain were less, other factors may also be involved in the biocontrol potential of the BCA (Baek et al. 1999). In another study, disruption of *ech42* gene of *T. harzianum* resulted in reduced biocontrol efficacy of the transformant against *Botrytis cinerea*. But the biocontrol efficacy of the transformant against *Pythium ultimum* remained unaltered. On the other hand, the biocontrol activity against *Rhizoctonia solani* was greater, when compared with wild strain of *T. harzianum.* These results suggested that factors other than chitinase activity might determine the efficiency of bioprotection offered by the BCAs (Woo et al. 1999). Transgenic apple plants incorporated with genes encoding both endo-and exo-chitinases of *T. atroviride* showed enhanced resistance to scab pathogen *Venturia inaequalis* (Bolar et al. 2000). Cotton plants transformed with endochitinase of *T. virens* exhibited greater resistance to seedling pathogens *R. solani* and *Thielaviopsis basicola* and leaf pathogen *Alternaria alternata* (Kenerley as quoted by Howell 2003) have also been generated.

Studies on the molecular genetics of the fungal biocontrol agents were performed to have an insight into the role of genes encoding the enzymes involved in the biocontrol activity. It has been difficult to clearly identify enzymes required for biocontrol activity due to the redundancy of the CWDE-encoding genes in the genome of *Trichoderma. T. harzianum* P1 strain effective against foliar and post- harvest pathogens such as *Botrytis cinerea* secretes several chitinolytic enzymes including *N*-acetyl-β-glucosaminidase (CHIT72), chitin 1,4-β chitbiosidase (CHIT 40) and a single 42-kDa endochitinase (CHIT42). The P1 strain was genetically modified by targeted disruption of the single copy *ech42* gene encoding for the secreted CHIT 42. The stable mutants lacked the *ech42* transcript, the protein and endochitinase activity in culture filtrates. Other chitinolytic and glucanolytic enzymes expressed during mycoparasitism were not affected by disruption of *ech42*. The mutant was as effective as P1 strain against *Pythium ultimum*, whereas its effectiveness against *B. cinerea* on bean leaves was reduced by 33 %. However, the endochitinase-deficient mutant was more effective against the soilborne pathogen *Rhizoctonia solani* than the wild-type strain P1. The results indicated that the biocontrol activity of *T. harzianum* might depend on the fungal pathogen involved in the interaction (Woo et al. 1999). Considerable efforts have been taken to detect and purify chitinolytic enzymes from *Trichoderma* spp. and to clone and characterize the genes encoding these enzymes involved in the biocontrol activity. *Trichoderma* chitinases seem to act synergistically, resulting in increased level of suppression of pathogens/disease in in vitro assays (Lorito et al. 1993) as well as in transgenic apple plants expressing endo- and exo-chitinase genes effective against scab pathogen *Venturia inaequalis* (Bolar et al. 2001). The mycoparasitic ability of *Trichoderma* spp. may be dependent on the joint power of a battery of different enzymes. *Trichoderma atroviride* strain P1 antagonistic to *Botrytis cinerea*, the gray mold pathogen, has a novel chitinase gene *ech30* encoding a 30-kDa protein. The Ech30 is a chitinase showing low sequence similarity to other *Trichoderma* chitinases. Polymerase chain reaction (PCR) screening indicated that one 306-bp DNA fragment had sequences similar to chitinases. The chitinase gene existed as a single copy gene in *T. atroviride.* Real-time quantitative RT-PCR assay revealed that expression of *ech30* gene was induced by

the presence of *B. cinerea* in plate confrontation assays, but hardly by chitin in liquid cultures. Cloning and expression studies using *Escherichia coli* showed that the gene *ech30* encoded an active chitinase which is included in family 18 chitinase (Klemsdal et al. 2006).

The antagonist *Trichoderma longibrachiatum* was shown to be effective against *Pythium ultimum* causing damping-off diseases. *T. longibrachiatum* was transformed with the gene *egl1* encoding β-1,3-glucanase enzyme. The transformants overexpressing the gene *egl1* were slightly more effective in reducing the disease incidence in cucumber than wild-type strain. As the antagonistic potential was not enhanced significantly, it was concluded that biosynthesis of several cell wall-degrading enzymes (CWDEs) might be necessary for efficient pathogen cell wall lysis (Migheli et al. 1998). *Trichoderma harzianum* is ubiquitous in the soil showing biocontrol activity against many fungal pathogens such as *Rhizoctonia solani, Sclerotinia sclerotiorum* and *Botrytis cinerea*. The principal mechanism of antagonism appears to be the release of lytic enzymes mainly chitinases, glucanases and proteases in the presence of sensitive host(s) (Chet and Chernin 2002). The biocontrol activity of several *Trichoderma* strains has been shown to be due to the action of fungal hydrolytic enzymes. The ability of these strains to produce extracellular proteases is known. Evidence is available indicating that the effectiveness of mycoparasitic activity of *Trichoderma* may also depend on their proteolytic abilities. The gene *prb1* of *T. harzianum* encoding a basic proteinase related to mycoparasitism was isolated and cloned. The biocontrol activity was improved in strains overexpressing the gene, showing the importance of proteases in the degradation of protein components of the host cell wall and in the lysis of whole host cells (Geremia et al. 1993; Flores et al. 1997).

Trichoderma harzianum T334, a potential biocontrol agent was able to produce low levels of protease constitutively. Mutants of T334 were generated by UV-irradiation. Some of the mutants were found to be more effective against *Fusarium culmorum, Pythium debaryanum* and *Rhizoctonia solani* in in vitro assays than the wild-type strain. They were better producers of extracellular trypsin- and chymotrypsin-like proteases with manifold levels of activities of the wild-type strain T334. The advantage of using mutants of BCA generated by UV-mutagenesis over the strain obtained through transformation, is the ease of getting registration for on field use (Sezekeres et al. 2004). Among the different enzymes released by *Trichoderma*, the aspartic proteases have a major role in their biocontrol potential. A gene (*SA76*) encoding an aspartic protease was cloned for 3′ rapid amplification of cDNA ends from *T. harzianum* T88. The coding regions of the gene was 1593-bp long, encoding a polypeptide of 530 amino acids with a predicted molecular mass of 55-kDa and pI of 4.5. The Northern blot analysis indicated that *SA76* was induced in response to different fungal cell walls. The aspartic protease gene was expressed functionally in *Saccharomyces cerevisiae.* The analysis of *SA76* expression confirmed that aspartic protease activity was induced in simulated parasitism by the presence of cell walls of *R. solani*, *Phytophthora sojae, Fusarium oxysporum* and *Sclerotinia sclerotiorum.* The increase in activity was due to induction at the transcription level, because the transcripts accumulated abundantly shortly after induction (Liu and Yang 2007).

Fusarium culmorum and *F. graminearum* causing Fusarium head blight (FHB) of wheat, maize and barley produce a mycotoxin trichothecene, deoxynivalenol (DON) harmful to humans and animals. *Trichoderma viride* P1 has a broad spectrum of biocontrol activity, as it produces an array of cell wall-degrading enzymes (CWDEs) that act synergistically to advance mycoparasitism. The most important of the CWDEs are the ECH42 endochitinase encoded by *ech42* gene and an *N*-acetyl-β-D-glucosaminidase encoded by *nag1* gene. Disruption of the *ech42* gene reduced the biocontrol activity of strain P1 against *Botrytis cinerea* (Woo et al. 1999). The *nag1* gene was induced by low-molecular weight chito-oligosaccharides and its own catabolic products, while *ech42* expression was indirectly induced by carbon starvation and other stress conditions (Mach et al. 1999). Expression of these genes contributing to biocontrol activity, was monitored in vitro and on crop residues of two maize cultivars by using *goxA* reporter gene fusions. The pathogen toxin DON repressed expression of *nag1* gene in *T. viride* P1. Expression of this gene was diminished to an extent of 50 % in maize residues, when the antagonist was placed in competition with DON-producing strains of *F. culmorum* and *F. graminearum*. By adding synthetic DON to assay mixtures with *Fusarium* strains that otherwise had no effect on the BCA, DON-induced repression could be reproduced, confirming the adverse effects of the pathogen on the expression of a key gene involved in the biocontrol activity of strain P1. On the other hand, expression of *Ech42* gene was neither positively nor negatively affected by DON or contact with *Fusarium* spp. The negative signaling could be an additional factor that may contribute to the inconsistent performance often observed with biocontrol agents (Lutz et al. 2003).

The effectiveness of *Clonostachys rosea* against *Fusarium culmorum* was shown to be correlated with secretion of CWDEs including chitinases and *N*-acetyl-β-d-glucosaminidase-encoding gene *cr-nag1* in *C. rosea*. Phylogenetically *cr-nag1* exhibited high sequence homogeneity to *N*-acetyl-β-d-glucosaminidase genes from other mycoparasitic fungi. Enzymatic assays and RT-PCR showed that the NAGase activity of *C. rosea* was specifically repressed in medium containing a high glucose content and is expressed in media containing chitin or *Fusarium culmorum* cell walls as sole carbon sources. *C. rosea* inhibited the mycelial growth of *F. culmorum* and *Pythium ultimum*. However, high expression of *cr-nag1* occurred only in the interaction between *C. rosea* and *F. culmorum,* but not with *P. ultimum.* The results indicated that although *C. rosea* could secrete, chitin-hydrolysing agents to target the cell wall of *F. culmorum,* it appeared to target *P. ultimum* by a different mode of action to suppress its development (Mamarabadi et al. 2009). Mycoparasitism exhibited by the fungal biocontrol agents involves the activity of several cell wall degrading enzymes (CWDEs) including proteases, chitinases and glucanases. The expression of the genes encoding these enzymes is enhanced in the presence of purified host cell walls, substances secreted by the host and also by the live host. The enhancement of expression of genes encoding CWDEs may improve the potential of BCAs. Two additional putative mycoparasitism-related genes were identified in *Trichoderma harzianum* by differential screening of a cDNA library for cDNAs expressed during growth in the presence of cell walls of *Rhizoctonia solani* (Vasseur et al. 1995; Rey et al. 2001). Expression of some of the genes involved in

mycoparasitism may depend on the contact of the BCA with the pathogen as in the case of one of the chitinases CHIT 73 of *T. hamatum* (Inbar and Chet 1995). Suppression subtractive hybridization (SSH) technique was applied to target novel mycoparasitic interaction of *T. hamatum* with *Sclerotinia sclerotiorum*. Nineteen novel genes of *T. hamatum* were identified and they showed enhanced level of expression during mycoparasitism compared to a *T. hamatum* control. Sequence analysis revealed some cDNA fragments had similarity to known fungal or bacterial genes. The proteins encoded by the novel genes included three monooxygenases, a metalloendopeptidase, a gluconate dehydrogenase, an endonuclease and a protein ATPase. The SSH was found to be an effective technique to identify the gene *prb1*, a gene known to be important in mycoparasitism (Carpenter et al. 2005).

The involvement of endochitinase of fungal biocontrol agents as a mechanism of biocontrol activity has been demonstrated. The effectiveness of a 42-kDa endochitinase coded by *ThEn42* gene from *Trichoderma harzianum* against Rhizoctonia root rot of barley caused by *R. solani* AG-8 and/or *R. oryzae* was assessed. Purified endochitinase strongly inhibited both *R. solani* AG-8 and *R. oryzae*. On the other hand, the endochitinase showed only moderate level of inhibition against *Gaeumannomyces graminis* var. *tritici* (wheat take-all disease) and it was ineffective against *Fusarium graminearum*, *F. pseudo-graminearum* and *F. culmorum* causative agents of wheat head blight disease (Wu et al. 2006). A two- dimensional gel electrophoresis (2-DE) technique was applied to obtain secreted protein patterns of *T. harzianum* ETS 323 grown in media containing glucose, a mixture of glucose and deactivated *Botrytis cinerea* mycelia, deactivated *B. cinerea* mycelia alone or deactivated *T. harzianum* mycelia alone. One L-amino acid oxidase (LAAO) and two endochitinase were specifically induced in the media containing deactivated *B. cinerea* mycelia. The results suggested that the cell wall of *B. cinerea* was the primary target of *T. harzianum* in its biocontrol activity (Yang et al. 2009). Proteomic, genomic and transcriptomic methods were applied for the isolation and characterization of a novel *Trichoderma* gene coding for a plant cell wall (PCW)-degrading enzyme (CWDE). A proteomic analysis, using a three-component (*Trichoderma* spp.-tomato plantlets-pathogen) system facilitated the identification of a differentially expressed *T. harzianum* endopolygalacturonase (endo-PG). A specific spot (0303) remarkably increased only in the presence of the soilborne pathogens *Rhizoctonia solani* and *Pythium ultimum* and corresponded to an expressed sequence tag (EST) from a *T. harzianum* T34 cDNA library that was constructed in the presence of PCW polymers and used to isolate the *Thpg1* gene. The *Thpg1*-silenced transformants had lower PG activity, less growth on pectin medium and reduced capacity to colonize tomato roots. The results were confirmed by real-time PCR assay which revealed that the presence of a pathogen in the system triggered the expression of *Thpg1* (Morán-Diez et al. 2009).

Resistance to exogenous and endogenous toxic compounds is one of the key characteristics for the ecological success of *Trichoderma* spp. Various special strains of *Trichoderma* are among the most resistant microorganisms to natural and synthetic chemicals and toxins and are able to degrade rapidly some of them including hydrocarbons, chlorophenolic compounds, polysaccharides and pesticides

(Rigot and Matsumara 2002; Harman et al. 2004a, b, c). Further, the ability of *Trichoderma* spp. to withstand different chemical stresses, including those associated with mycoparasitism has been demonstrated. An ATP-binding cassette transporter cell membrane pump was considered as an important component of resistance mechanisms that seemed to be supported by an extensive and powerful cell detoxification system. The encoding gene designated *Taabc2*, was cloned from a strain of *T. atroviride* and characterized. Expression of this gene was upregulated in the presence of pathogen-secreted metabolites, specific mycotoxins and some fungicides. Upregulation of this gene was also observed under conditions that stimulate the production in *Trichoderma* spp. of antagonism-related factors (toxins and enzymes). By generating deletion mutants, the key role of *Taabc2* gene in antagonism and biocontrol was demonstrated. The mutants showed enhanced sensitivity to inhibitory compounds either secreted by pathogenic fungi or produced by the BCA itself, under certain conditions. The mutant lost partially or entirely the ability to protect tomato plants against *Pythium ultimum* and *Rhizoctonia solani* causing damping-off and root rot diseases (Ruocco et al. 2009).

Pichia anomala strain K has been shown to be antagonistic to the gray mold pathogen *Botrytis cinerea*. The role of exo-β-1,3-glucanase produced by the BCA in its biocontrol potential was investigated. The synthetic medium amended with laminarin, a cell wall preparation (CWP) of *B. cinerea* or glucose was used to detect the exo-β-1,3-glucanase (EC3.2.1.58) activity of *P. anomala* strain K in the medium. The highest activity was induced in the culture media containing the CWP of *B. cinerea* as the sole carbon source. Exoglc 1, an exo-β-1, 3-glucanase was purified to homogeneity from culture filtrates of strain K containing a CWP. The Exoglc 1 caused stronger inhibitory effect on germ tube growth of *B. cinerea* than on conidial germination. In addition, morphological alterations including leakage of cytoplasm and cell swelling were also observed. Exo-β-3-glucanase activity could be detected in apples treated with the BCA strain. The results indicated that activity of the exo- β-1,3-glucanase could be an important mechanism of biocontrol activity of *P. anomala* strain K (Jijakli and Lepoivre 1998). The purified exo-β-1,3-glucanase from culture filtrate (CF) of strain (paexg2) strongly inhibited the germ tube growth of *B. cinerea,* in addition to inhibition of conidial germination and induction of morphological changes. The exo-β-1,3-glucanase detected on apples treated with strain K was similar to paexg2 in several properties. Two genes *PAEXG1* and *PAEXG2* coding for exo-β-1,3-glucanase were identified in the genome of strain K using polymerase chain reaction (PCR) with degenerate primers designed on the basis of conserved amino acid region and on the N-terminal sequence of paexg2 (Grevesse et al. 1998a, b). The segregation of *PAEXG1* and *PAEXG2* alleles in haploid segregants indicated that there was no relationship between exo-β-1,3-glucanase activity in vitro and their biocontrol potential against *B. cinerea* in apples (Grevesse et al. 1998b). The *PAEXG2* gene encoding for exo-β-1,3-glucanase was isolated from *P. anomala* strain K and the gene product was characterized. *PAEXG2* codes for an acidic protein consisting of 427 amino acids with MW of 45.7-kDa. Disruption of *PAEXG2* gene by insertion of the URA3 marker gene encoding orotidine monophosphate decarboxylase resulted in a reduction in biocontrol potential, as well

as in reduced colonization of wounds in apples. Disruption of *PAEXGs* led to loss of all detectable exo-β-1,3-glucanase in vitro and in situ. However, the biocontrol activity of strain K did not depend on the production of exo-β-1,3-glucanase (Grevesse et al. 2003).

The gene potentially involved in the biocontrol activity of *Pichia anomala* strain Kh5 were identified by applying cDNA-AFLP analysis. A total of more than 2,450 bands derived from the mRNA of strain Kh5 grown in the presence of cell walls (CW) of *Botrytis cinerea* were detected by employing 35 primer pairs in AFLP amplification. Eighty six bands corresponded to genes upregulated in the BCA grown in the presence of the cell wall fragments, compared with the BCA in the absence of cell wall fragments in the medium. Real-time RT-PCR assay confirmed the differential expression of the BCA in the presence of pathogen cell wall fragments. Following normalization of the results of RT-PCR using appropriate house-keeping gene *G2*, eleven genes showed marked increase in expression in the presence of cell wall fragments of *B. cinerea.* The overexpressed genes showed homologies to yeast genes with various functions, including β-glucosidase transmembrane transport, citrate synthase and external amino acid sensing and transport. Some of these functions had a bearing on biocontrol potential of *P. anomala* strain Kh5 (Massart and Jijakli 2006).

Trichoderma harzianum could adversely affect the pathogenic potential by reducing the activities of polygalacturonase (PG), pectin methylesterase (PME) and pectatelyase (PL) secreted by *Botrytis cinerea* resulting in reduced disease severity (Zimand et al. 1996). Another study showed that the extent of inhibition of production of enzymes involved in pathogenesis may determine the efficiency of biocontrol of two isolates of *T. harzianum.* Germination of conidia of *B. cinerea* on the surface of leaves of beans (*Phaseolus vulgaris*) and subsequent disease development were inhibited more effectively by the strain T-39 than NCIM 1185. Production of cutin esterase, exo-PG, endo-PG, PME and PL was inhibited to a greater extent by T-39 than by NCIM 1185 (Kapat et al. 1998). An interesting aspect of the concept related to enzyme biosynthesis in the BCAs as a mechanism in the biocontrol process was investigated. The enzymes like proteases produced by the BCAs may break down the hydrolytic enzymes secreted by the pathogens like *Botrytis cinerea*. The hydrolytic enzymes of the pathogen having an important role in pathogenesis may be broken down into peptide chains and/or the amino acids resulting in the loss of capacity to act on host plant cells. The protease solutions from *T. harzianum* on bean leaves partially deactivated hydrolytic enzymes. It reduced the disease severity by 56–100 %, when the solutions were applied on infected leaves (Elad and Kapat 1999).

3.1.2.2 Production of Antibiotics

Several fungal biocontrol agents have been demonstrated to produce antibiotics capable of inhibiting spore germination, mycelial growth and sporulation of fungal pathogens. *Gliocladium* (*Trichoderma*) *virens* produced powerful antibiotics

gliotoxin and viridin that could inhibit formation of sclerotia by *Sclerotinia sclerotiorum* and also parasitize the sclerotia produced already (Tu 1980). Howell and Stipanovic (1983) isolated and described a new antibiotic gliovirin produced by *G. virens,* strongly inhibitory to *Pythium ultimum* and *Phytophthora* sp. Howell et al. (1993) showed that the strains of *G. virens* could be divided into two groups (GV-P and GV-Q) based on the nature of the secondary metabolites produced. The P group strains produced gliovirin and heptelidic acid, whereas the Q group strains produced gliotoxin and dimethylgliotoxin. Both groups produced viridin and the phytotoxin viridol. The P group strains were effective against cotton seedling disease caused by *Pythium ultimum* which was strongly inhibited by gliovirin. But gliovirin was not inhibitory to *Rhizoctonia solani*, a component of cotton seedling disease. On the other hand, Q group strains producing gliotoxin effectively suppressed development of *R. solani*. The need to determine the antibiotic profiles of the BCAs is clearly brought out by the study to facilitate selection of effective BCA strain for application against target pathogen (s) (Howell et al. 1993). The biocontrol activity of *Cylindrocarpon olidum* var. *olidum* (*Coo*) on *Tilletia laevis*, causative agent of wheat common bunt disease was studied. Germination of the bunt teliospore was entirely inhibited in vitro on water agar medium supplemented with the culture filtrate of *Coo*, indicating the involvement of the compounds secreted by the BCA with antibiotic properties. Further, the biocontrol potential of *Coo* was demonstrated in field experiments by treating the seeds of wheat with the BCA. The bunt disease incidence was reduced from 82.9 to 40.4 % and from 81.4 to 42.0 % in 1995–1996 and 1997–1998 respectively, revealing the biocontrol potential of *Coo* under field conditions also (Yolageldi and Turhan 2005). The fungal strain *Clonostachys rosea* BAFC3874 isolated from soils suppressive to *Sclerotinia sclerotiorum* effectively inhibited the infection of the pathogen in pot-grown lettuce and soybean plants. Dual culture tests established that this strain produced antifungal compounds against *S. sclerotiorum* with secondary metabolism. *C. rosea* produced a microheterogenous mixture of peptides belonging to the peptaibols family. Further, mycoparasitic activity of *C. rosea* was also observed against *S. sclerotiorum* in the dual culture tests (Rodriguez et al. 2011).

The mechanism of biocontrol activity of *Trichoderma harzianum* (T-12) and *T. koningii* (T-8) against *Pythium* sp. infecting peas was investigated. Production of a toxic factor in the spermosphere was considered to be responsible for the suppression of the pathogen development (Lifshitz et al. 1986). A correlation between the biocontrol potential of *T. virens* (GL-21) and the production of the antibiotic gliotoxin was observed for the control of damping-off of zinnias caused by *Rhizoctonia solani* and *Pythium ultimum* (Lumsden et al. 1992). *T. koningii* was reported to produce many antifungal metabolites including antibiotics and CDWEs inhibiting the development of wheat take-all and root rot diseases caused by *Gaeumannomyces graminis* var. *tritici* and *R. solani* respectively (Benoni et al. 1990; Worasatit et al. 1994). *T. harzianum* and *T. koningii* isolated from roots of wheat produced cyclonerodiol and octaketodiol in common, while *T. harzianum* produced also three more metabolites, which were identified as octaketide-derived compounds using spectroscopic and chemical methods. *G. graminis* var. *tritici* was inhibited by all the

newly isolated compounds (Ghisalberti and Rowland 1993). *Trichoderma harzianum* produces trichodermin and a small peptide which could inhibit *Rhizoctonia solani*. The pathogen in retaliation secretes a coumarin derivative capable of inhibiting the mycelial growth of the BCA. However, inhibition of the BCA required a concentration than that was needed for the antimycotic compounds secreted by *T. harzianum* (Bertagnolli et al. 1998). In another investigation, the enhanced levels of endochitinase detected in the soybean rhizosphere were found to be due to the activities of *T. harzianum.* Effective suppression of *R. solani* was attributed to the endochitianse secreted by the BCA (dal Soglio et al. 1998).

The fungal biocontrol agents may produce different kinds of secondary metabolites, in addition to hydrolytic enzymes, that are inhibitory to the fungal pathogens at different stages of their life cycle. *Pichia guilliermondii*, the yeast biocontrol agent effective against *Botrytis cinerea* has multiple mechanisms of biocontrol activity. The supernatant separated from the BCA cell suspension suppressed the development of *B. cinerea,* implying the presence of an inhibitory compound in the cell suspension. At least some of the compounds were heat stable, since the inhibitory activity was retained even after heating. The secreted compound(s) had remote acropetal and to a lesser extent basipetal effects and it was not volatile (Guetsky et al. 2002). *Aspergillus giganteus* isolated from the field soil produced a basic low-molecular weight protein (with 51 amino acids) showing antifungal properties. This antifungal protein (AFP) was discovered accidently during anticancer screening. This AFP strongly inhibited the conidial germination and mycelial growth of isolates of *Botrytis cinerea* causing gray mold disease of geranium. The AFP induced swollen hyphal tips and reduced hyphal elongation. When applied on geranium plants, leaf infection by *B. cinerea* was significantly reduced indicating its fungicidal activity (Moreno et al. 2003).

Mutants of biocontrol agents may either lack the gene required for biosynthesis of the enzymes/antibiotics or possess the modified gene with low level of enzyme/antibiotic production. A mutant of *Trichoderma virens* was unable to synthesize gliovirin inhibitory to *Pythium ultimum* and consequently it could not reduce the infection of cotton seedlings by this pathogen causing damping-off disease. On the other hand, another mutant (GV-1) with enhanced gliovirin production was not more effective in controlling the damping-off disease, compared with wild-type strain of *T. virens* (Fig. 3.4) (Howell and Stipanovic 1983; Howell 2003). In another study, mutants of *T. virens* lacking the capacity of producing gliotoxin were shown to be ineffective for the control of Pythium damping-off disease (Wilhite et al. 1994). Ultraviolet irradiation is one of the methods of producing mutants of fungi artificially to study the functions of genes on the target fungus. Three mutants of *Trichoderma virens* produced by exposing the wild-type strain to UV-irradiation lost their ability to parasitize *Rhizoctonia solani*. However, the mutants had similar antibiotic biosynthetic capacity and biocontrol potential as the wild-type strain. The root rot disease of cotton due to *R. solani* was as effectively controlled as the parent strain of *T. virens,* indicating that the mycoparasitism-deficient mutants were equally efficient in protecting cotton plants against *R. solani*. Mycoparasitism of *T. virens* may have a less important role in its effectiveness of biocontrol of cotton root rot

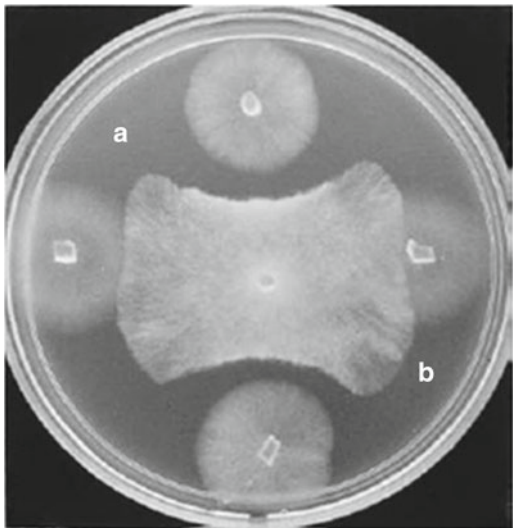

Fig. 3.4 Inhibition of mycelial growth of *Pythium ultimum* by *Trichoderma virens*-produced gliovirin (**a**): parent strain; (**b**): *Gliovirin-deficient* mutant (Courtesy of Howell 2003 and with kind permission of The American Phytopathological Society, MN, USA)

disease by *T. virens* (Howell 1987). The production of the antibiotic gliotoxin, as a mechanism in the biocontrol of *Trichoderma virens* against *Rhizoctonia solani,* has not been unequivocally established. The mutants of *T. virens* deficient in gliotoxin biosynthesis were equally effective in controlling cotton seedlings disease as the wild-type strain of *T. virens* (Howell and Stipanovic 1995). In the later studies, the biocontrol efficacy of parent strain of *T. virens* (G6-5) and the mutants deficient in both mycoparasitic and gliotoxin biosynthetic abilities was compared. The deficiencies of the mutants in the two parameters contributing to the biocontrol efficacy did not adversely effect their ability to protect the cotton plants against infection by *R. solani* and *Pythium ultimum*. These results indicated that both mycoparasitism and antibiosis may not be the primary mechanisms of *T. virens* (G6-5) for its bioprotection against *R. solani* and *P. ultimum* (Howell et al. 2000; Howell 2002).

A nonpathogenic *Fusarium oxysporum* was isolated from the soil suppressive to the fungal pathogen *Sclerotinia sclerotiorum.* The antagonistic activity of *F. oxysporum* strain S6 was demonstrated by dual culture technique. The toxic nonvolatile metabolites from *F. oxysporum* S6 were isolated by chromatographic techniques. They were purified and identified as cyclosporine A by spectroscopic methods. The antibiotic cyclosporine inhibited the growth and suppressed sclerotia formation. The antifungal activity against *S. sclerotiorum* was correlated with the presence of cyclosporine A by a dilution plate assay. The BCA also caused similar adverse effect on mycelial growth and sclerotial production by *S. sclerotiorum.* When the sclerotia were planted at the center of *F. oxysporum* colony, the percentages of germination of sclerotia were significantly reduced due to infection of sclerotia by the BCA. In the greenhouse test, the number of surviving soybean plants significantly increased, when the BCA and the pathogen were coinoculated. The results indicated that the antifungal activity of *Fusarium oxysporum* S6 against *S. sclerotiorum* was primarily due to the secretion of cyclosporine A by the BCA (Rodriguez et al.

2006). Inhibition of mycelial growth of *S. sclerotiorum* by *Trichoderma* spp. was attributed to production of volatile and non-volatile inhibitors under greenhouse and field conditions. *T. koningii, T. virens, T. ceramicum* and *T. viridescens* provided maximum protection to potatoes against the stem rot disease caused by *S. sclerotiorum* (Ojaghian 2011).

Fusarium moniliforme infecting cereals produces the secondary metabolite fusaric acid (FA). The interaction between *F. moniliforme* and two antagonistic isolates T16 and T23 of *Trichoderma harzianum* was investigated to assess the effects of the secondary metabolite of the pathogen on the BCA and the ability of the BCA to reduce the production of the toxic metabolites of the pathogen. The metabolites of *F. moniliforme* reduced the mycelial growth and conidial production by both isolates of *T. harzianum*. However, the isolates T23 and T16 degraded the metabolites of the pathogen by 51.4 and 88.4 % respectively in potato dextrose broth medium. The antifungal metabolite 6-pentyl-alpha-pyrone (6PAP) isolated from *T. harzianum* T23 decreased the FA content significantly. Dosages of 300 and 400 mg/l of PAP retarded FA accumulation by 62.5 and 77.2 % respectively. This study provides a direct evidence for the ability of the antifungal compound produced by the BCA to counteract the effects of the secondary metabolite produced by the fungal pathogen (El-Hasan et al. 2008). The antifungal secondary metabolites of *Trichoderma harzianum* have been shown to have important role in its biocontrol activity against *Fusarium moniliforme.* Production of viridiofungin A (VFA) by *T. harzianum* T23 in culture was recorded for the first time in this study. Bioautography assay showed that three fractions (F223, F323 and F423) were produced by isolate T23 and two fractions (F416 and F516) were isolated from isolate T16. These fractions exhibited pronounced fungitoxic activity against *F. moniliforme* and *Cladosporium* spp. The fractions F416 and VFA showed both volatile and non-volatile effects on test fungus, whereas F516 appeared to have only non-volatile activity. Reduced branching and thickened hyphae were attributed to the activity of these fractions. VFA seemed to have wider spectrum of antifungal activity against *Verticillium dahliae, Phytophthora infestans* and *Sclerotinia sclerotiorum.* VFA was found to be fungistatic rather than fungicidal in contrary to earlier reports. The metabolites of *T. harzianum* such as VFA, 6PAP, F416 and F516 did not show any antibacterial activity against both Gram-positive and Gram-negative bacteria (El-Hasan et al. 2009).

Acremonium zeae an endophyte was found to be antagonistic to kernel-rotting and mycotoxin-producing fungi *Aspergillus flavus* and *Fusarium verticillioides* in in vitro assays and it was able to interfere with infection of maize kernels (Wicklow et al. 2005). *A. zeae* produced pyrrocidines A and B, polyketide-amino acid-derived antibiotics. Pyrrocidine A inhibited the conidial germination of *A. flavus* and *F. verticillioides* more effectively than pyrrocidine B. In addition, pyrrocidine A exhibited potent antagonistic activity against major stalk and ear rot pathogens of maize, such as *Fusarium graminearum, Nigrospora oryzae*, *Stenocarpella* (*Diplodia*) *maydis* and *Rhizoctonia oryzae.* Maize seed-rotting fungi *Eupenicillium ochrosalmoneum, Alternaria alternata, Cladosporium cladosporioides* and *Curvularia lunata* were also inhibited by pyrrocidine A. The symptomless

Table 3.2 Suppression of development of diseases caused by fungal pathogens by chaetoviridins at different concentrations (Park et al. 2005)

		Control value (%)[a]		
Antifungal compound	Concentration (μg/ml)	Rice blast	Tomato late blight	Wheat leaf rust
Chaetoviridin A	250	99 ± 0.3[b]	87 ± 2.5	87 ± 2.5
	125	94 ± 1.1	50 ± 10	97 ± 0.3
	62.5	88 ± 5.2	0	83 ± 4.5
Chaetoviridin B	250	96 ± 2.1	0	91 ± 2.2
	125	96 ± 2.5	0	65 ± 5.7
	62.5	94 ± 1.2	0	0

[a]Control value (%) = 100 × (disease severity in control plants – disease severity in treated plants)/ disease severity in control plants
[b]Mean of six replicates ± SD

seedborne endophytes *F. proliferatum, F. subglutinans* and *F. oxysporum* showed little or no sensitivity to pyrrocidines. The results showed that pyrrocidine-producing endophyte *A. zeae* might effectively reduce the diversity and abundance of maize pathogen assemblages, while it was ineffective against protective endophytes, including mycoparasites (Wicklow and Poling 2009).

Chaetomium globosum is a common colonizer of soil and cellulose- containing substrates and it has been shown to be effective against fungal plant pathogens. Antibiosis, one of the different mechanisms, has been suggested as the mechanism of biocontrol activity of *C. globosum*. It produced chaetomin in liquid culture and its presence was found to be correlated with their activity against damping-off disease of sugar beet caused by *Pythium ultimum* (Di Pietro et al. 1992). A liquid culture of *C. globosum* F0142 exhibited high antifungal activity against *Magnaporthe grisea* causing rice blast disease and *Puccinia recondita* inciting wheat leaf rust disease. The culture filtrate suppressed the development of these diseases by more than 80 % even when diluted 90-fold. In addition, it showed moderate antifungal activity against *Phytophthora infestans* causing tomato late blight disease. Two antifungal compounds were purified from broth culture and identified as chaetoviridin A and B. Chaetoviridin A was more effective against these pathogens both in vitro and in vivo. Treatment of rice and wheat plants with chaetoviridin A (62.5 μg/ml) suppressed the development of rice blast and wheat leaf rust diseases by more than 80 % (Table 3.2; Park et al. 2005). *Pseudozyma focculosa* (syn. *Sporothrix flocculosa*) is a yeast-like fungus exhibiting strong antagonistic activity against powdery mildew fungi infecting rose and wheat (Hajlaoui and Bélanger 1993; Bélanger et al. 1994). Two antifungal fatty acids were produced by all *P. flocculosa* isolates except PH isolate and they were considered to mediate the biocontrol properties of the BCA. These effective isolates produced 9-heptadecenoic acid which might play a role in the selection of the most effective isolate of *P. flocculosa* for the biocontrol program (Avis et al. 2001). *Penicillium oxalicum* strain PY-1, isolated from the soil, produced antifungal substance capable of inhibiting the mycelial growth of *Sclerotinia sclerotiorum* causing stem rot disease of oilseed rape (*Brassica napus*)

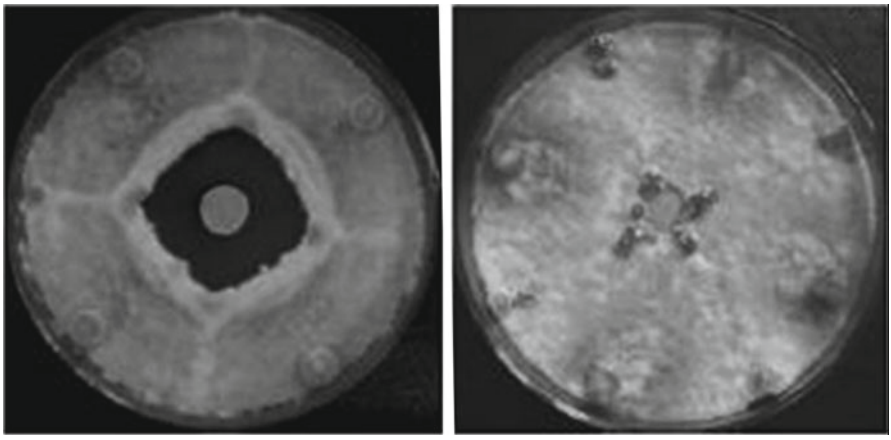

Fig. 3.5 Antifungal activity of the bioactive compound produced by *Penicillium oxalicum* strain PY-1 and isolated by high performance liquid chromatography (HPLC) Inhibition of mycelial growth of *Sclerotinia sclerotiorum* by the compound produced by strain PY-1 (Courtesy of Yang et al. 2008 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

in China. The antifungal compounds were extracted with ethyl acetate and further purified by high-performance liquid chromatography (HPLC). At least two active compounds that significantly inhibited mycelial growth were obtained (Fig. 3.5). Both spore suspension and culture filtrate reduced the size and number of lesions formed on oilseed rape leaves by *S. sclerotiorum.* No lesions/necrosis developed following application of undiluted culture filtrate, indicating the absence of detectable phytotoxicity of the antifungal compounds produced by *P. oxalicum* (Yang et al. 2008).

The *Gaeumannomyces-Phialophora* (G-P) complex consists of *G. graminis*, related anamorphic *Phialophora* spp. and other *Gaeumannomyces* spp. They are associated with cereal take-all diseases. The antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) is produced by *Pseudomonas* spp. in the rhizosphere of many crop plants. The sensitivity of the isolates of *G. graminis* var. *tritici (Ggt)* causing wheat take-all disease and the less virulent isolates of *Phialophora* was assessed using agar plate bioassay using plates containing PDA amended with 2,4-DAPG dissolved in methanol. The *Phialophora* isolates were substantially less sensitive to 2,4-DAPG than *Ggt* isolates with ED_{90} values of 11.9–48.2 and 3.1–11.1 μg/ml of 2,4-DAPG respectively. *Phialophora* spp. was shown to suppress take-all disease under field conditions. It is possible that *Phialophora* spp. might work in concert with 2,4-DAPG producers to suppress take-all disease. Roots of wheat or barley from take-all decline (TAD) field were able to support threshold population sizes of 2,4-DAPG producers required for take-all suppression and 95 % of the G-P complex isolates were *Phialophora* isolates with only 5 % *G. graminis* var. *tritici* isolates. Under such conditions, *Phialophora* isolates tolerant to 2,4-DAPG may play an important role in suppressing the incidence of wheat take-all disease (Kwak et al. 2010).

The process of utilizing the antimicrobial volatiles produced by fungal biocontrol agents is known as mycofumigation. The fungi *Muscodor albus* and *M. roseus* have been employed for mycofumigation to enhance the sugar beet stand and to decrease the disease severity due to *Rhizoctonia solani, Pythium ultimum* or *Aphanomyces cochlioides*. Five classes of compounds viz., alcohols, esters, ketones, acids and lipids were shown to be the key components of the mycofumigant gas volatiles. These compounds within each class were tested individually and combined into an artificial mixture in vitro against *P. ultimum, R. solani, Phytophthora cinnamomi, Verticillium dahliae, Fusarium oxysporum* f.sp. *betae* and *Sclerotinia sclerotiorum.* No single class of chemical was toxic individually to the test pathogens, as the natural volatiles from *M. albus.* The most effective single component was the esters (Strobel et al. 2001). In a later study, the efficiency of five different formulations containing *M. roseus* was assessed for the control of sugar beet Pythium damping-off and eggplant Verticillium wilt diseases. The Stabileze formulation was effective consistently in reducing the disease severity and population of *V. dahliae* in vivo. The results indicated that it is possible to maximize mycofumigation efficacy by selecting an appropriate formulation (Stinson et al. 2003).

Apple and pear are affected seriously by blue mold (*Penicillium expansum*) and gray mold (*Botrytis cinerea*), while peaches suffer heavily due to brown rot (*Monilinia fructicola*). The endophytic fungus *Muscodor albus* produces about 28 different volatile compounds which were shown to kill several fungal pathogens such as *Rhizoctonia solani, Sclerotinia sclerotiorum* and *Verticillium dahliae* (Strobel et al. 2001). The efficacy of the biofumigation with *M. albus* for the control of blue mold, gray mold and brown rot diseases in fresh fruits was evaluated. Biofumigation with *M. albus* provided excellent control of blue mold and gray mold of apples and also brown rot of peaches. Fumigation of apples for 7 days with the culture of *M. albus* grown on autoclaved grain gave complete control of both diseases, while all the fruits inoculated with the pathogens were infected. Likewise, peaches were entirely protected by fumigation with *M. albus* (Table 3.3). The important favorable attribute is that *M. albus* does not need to have direct contact with fruits to be treated. This differentiates it from other BCAs. It is remarkable that sub-micromolar concentrations of volatiles produced by *M. albus* were highly effective in controlling the postharvest diseases. It is unlikely that residues might accumulate to impermissible levels as do the fungicides being applied against these diseases (Mercier and Jiménez 2004). The biofumigant *Muscodor albus* has been reported to produce several volatiles that have antimicrobial properties. The efficacy of *M. albus* for the suppression of soilborne pathogen *Rhizoctonia solani* in greenhouse soilless growing mix was assessed. The treatment showed only local effect essentially indicating the inability of volatiles to move through the growing mix. The temperature range of 4–22 °C was found to be suitable for fumigation activity of *M. albus*. The ability of *M. albus* to control damping-off disease in broccoli seedlings declined rapidly after its incorporation in the growing mix. In treated mix, damping-off incidence remained at low level, regardless of planting time after treatment, suggesting that biofumigation could eliminate *R. solani* effectively. The experiments for root rot disease control in bell pepper caused by *Phytophthora*

Table 3.3 Efficacy of biofumigation with *Muscodor albus* in protecting apples against blue and gray mold diseases (Mercier and Jiménez 2004)

	Percent infection			
	Blue mold		Gray mold	
Treatments	7 days	14 days	7 days	14 days
Control	100	100	96	100
Treated 0 h post inoculation				
24 h	0	7 ± 6	0	0
48 h	0	0	0	0
72 h	0	4 ± 6	0	0
5 days	0	4 ± 6	0	0
Treated 24 h post inoculation				
24 h	7 ± 6	11 ± 16	0	0
48 h	7 ± 6	7 ± 6	0	0
72 h	4 ± 6	4 ± 6	0	0
5 days	0	0	0	0

capsici demonstrated the high level of protection to bell pepper by *M. albus*. Enhancement of plant growth following treatment with *M. albus* was believed to be due to control of other deleterious microorganisms that often contaminate commercial growing mixes (Mercier and Manker 2005). In the search for the endophytes producing volatile organic compounds (VOCs) with antibiotic activity against microorganisms, at least 12 isolates of *Muscodor albus* were found to produce biologically active volatile compounds (Strobel et al. 2007).

The effectiveness of *Muscodor albus* strain MFC2 as a biocontrol agent to protect kale (*Brassica oleracea*) against the root rot pathogen *Pythium ultimum* was assessed in the greenhouse conditions. The BCA was grown on potato dextrose agar (PDA) medium for 10 days and the BCA culture was able to kill the pathogen in vitro. The BCA culture was thoroughly mixed with commercial soil mix. The seeds of kale were sown in soil infested with *P. ultimum* and inoculated with BCA culture. Seedling emergence in pots inoculated with the BCA and pathogen was equal to a level close to that in the control without the pathogen. *M. albus* did not cause any adverse effect on seed germination and plant development. There appeared to be favorable effect on plant growth of kale in the control and pathogen-inoculated treatments up to 8 weeks after planting. The volatiles from *M. albus* might be responsible for the biocontrol activity against *P. ultimum* infecting kale plants (Worapong and Strobel 2009). The antimicrobial volatiles from *Muscodor albus* have been shown to effectively eliminate soilborne pathogens. The volatiles controlled damping-off of broccoli seedlings, when pots containing soil or soilless potting mix infested with *Rhizoctonia solani* were placed in the presence of active *M. albus* culture without physical contact in closed containers. Gas chromatographic analysis revealed that isobutyric acid and 2-methyl-1-butanol were released from the treated soil/ substrates. Production of isobutyric acid showed positive correlation with the extent of disease control. Amounts of isobutyric acid released from soil were several fold greater than that released from potting mix. In addition, higher amounts

of the BCA were required to achieve effective control of damping-off disease in soilless potting mix than in soil, suggesting that soil environment was better for the biological activity or viability of *M. albus* than the soilless potting mix (Mercier and Jiménez 2009).

The effectiveness of biofumigation with *Muscodor albus* for the control of post-harvest gray mold of table grapes caused by *Botrytis cinerea* was assessed individually as well as in combination with ozone or sulfurdioxide. The grapes were treated with ozone or sulfurdioxide during pre-cooling followed by exposure to continuous biofumigation by the volatiles produced by *M. albus*. Gray mold incidence was reduced in "Autumn seedless" grapes from 91.7 to 19.3 % by ozone and to 10 %, when combined with biofumigation. In organically grown "Thompson Seedless" grapes ozone fumigation and BCA biofumigation reduced the gray mold incidence to 9.7 and 4.4 % respectively, while the combined treatment reduced the disease incidence further to 3.4 %. Although the combination of ozone and *M. albus* reduced the decay significantly, it was less effective compared with standard sulfur- dioxide treatment. However, the combination of ozone and biofumigation may be preferable for organically grown grapes and also as an alternative to reduce the fungicide use for protecting the fruits against postharvest diseases (Gabler et al. 2010). High moisture content generally favors the development of storage fungi. Availability of water to the microorganisms is measured and expressed as water activity (a_w) which reflects the relationship between moisture in grains/foods and the ability of the fungi to grow on the stored materials. The effects of water activity on the production of volatile organic compounds (VOC) on *Muscodor albus* culture and their inhibitory effects on the growth of three potato pathogens *Fusarium sambucinum* (causing dry rot) *Helminthosporium solani* (causing silver scurf) and *Pectobacterium atrosepticum* (causing bacterial soft rot) were assessed. *M. albus* produced isobutyric acid, bulnesene, a sesquiterpene, an unidentified terpene, 2- and 3-methyl-1-butanol and ethanol. The level of these VOCs varied with a_w of the culture. The VOC was inhibitory to *F. sambucinum, H. solani* and *P. atrosepticum.* Biofumigation with *M. albus* significantly reduced dry rot and soft rot development and silver scurf was entirely controlled in inoculated potato tubers incubated at both 8 and 22 °C. The results indicated that a_w of culture significantly affected the production of VOC which in turn influenced pathogen development (Corcuff et al. 2011).

Another endophyte *Oidium* sp. isolated from *Terminalia catappa* (tropical chestnut) produced primarily esters of propanoic acid, 2-methyl- butanoic acid, and 3-methylbutanoic acid. Addition of exogenous volatile compounds such as isobutyric acid and naphthalene, 1,1-oxybis caused a dramatic synergistic increase in the antibiotic activity of the VOCs of *Oidium* sp. against *Pythium ultimum*. The development of the pathogen was entirely inhibited and consequent death of the pathogen. The results of experiments with different producers of VOCs suggested that the VOCs of different endophytic fungi might act both additively and synergistically to inhibit the development of other symbiotic and/or pathogens colonizing the same plant species (Strobel et al. 2008). The transition from vegetative growth to conidiation is marked in many fungi by enhanced production of secondary metabolites which include volatile organic compounds (VOCs). Their spectrum is characteristic for

each species (Calvo et al. 2002) and they may be produced during antagonistic interactions with other fungi (Hynes et al. 2007). Light and starvation (non-availability of nutrition) appear to be two important environmental stimuli inducing conidiation in *Trichoderma* spp. *Trichoderma atroviride* (formerly *T. viride), T. harzianum* and *T. longibrachiatum* were evaluated for their efficacy in producing VOCs that could induce conidiation. The biological activity of VOCs produced by fungi is their ability to influence the development of their own producer as well as the development of other fungi. VOCs being volatile may diffuse to a distance from the producing colony and act as pheromones mediating intercolony communication. Volatiles produced by conidiating colonies of *Trichoderma* spp. elicited conidiation in colonies that had not been induced previously by exposure to light. The inducing effect of volatiles was both intra-and interspecific. The eight-carbon VOCs could act as signaling molecules capable of regulating development and mediating intercolony communication in *Trichoderma* spp. (Nemčovič et al. 2008).

The synergism between enzymes and antifungal compounds resulting in enhanced biocontrol activity has been reported in certain cases. The synergistic effects of endochitinase and gliotoxin produced by *Trichoderma virens* on the germination of conidia of *Botrytis cinerea* were observed. Treatment of the conidia of *B. cinerea* with a combination of compounds secreted by *T. virens* was much more inhibitory than the treatment with either compound separately (Di Pietro et al. 1993). Likewise, a greater inhibitory effect on conidial germination and hyphal elongation of *B. cinerea* was recorded, when the pathogen was treated with the combination of hydrolytic enzymes and peptaibols produced by *T. harzianum*. But treatment with either the enzyme or antibiotic alone was less effective (Schirmbock et al. 1994). The level of synergism between the enzymes and antibiotics seems to be influenced by the sequence of their application. Synergism was found to be at lower level, when the treatment with antifungal compound preceded the enzyme application. It may possibly be due to the requirement of cell wall degradation by the enzyme for the commencement of activity of the antifungal compounds (Lorito et al. 1996).

The antagonistic yeasts such as *Pichia guilliermondii* and *Candida oleophila* have been developed as commercial produce for application against postharvest diseases of fruits. These BCAs provide insufficient and inconsistent levels of protection against target pathogens. Further, they are used against wound pathogens, but not against pathogens that invade directly through the cuticle and cause quiescent infections. Hence, the possibility of improving the biocontrol efficacy was explored by expressing a DNA sequence in yeast to allow for the production of an antifungal peptide that can act on the target pathogen. An approach to control the postharvest decay of tomato due to *Colletotrichum coccodes* was adopted by expressing a lytic peptide in *Saccharomyces cerevisiae.* The antimicrobial properties of cecropin A and B peptides have been demonstrated mostly against phytopathogenic bacteria. The cecropin A-based peptide inhibited the conidial germination of *Colletotrichum coccodes.* The DNA sequence encoding the peptide was cloned in pRS413, using the *Saccharomyces cerevisiae* invertase leader sequence for secretion of the peptide and expressed in yeast. By incubating the pathogen in the

presence of *S. cerevisiae* transformants Y-20 and Y-47, the fungal growth was entirely inhibited. The decay induced by germinated spores in tomato fruit was arrested. The mechanism of biocontrol activity of the peptide enabled a direct interaction between the antifungal peptide and the target pathogen membrane, resulting in localization and inhibition of further development of germinated spores of the pathogen in the wounded tissues. The lack of activity toward nontarget organisms by the peptide and use of the yeast as a delivery system deserve consideration for wider exploitation of this approach (Jones and Prusky 2002).

Application of mycoparasitic *Trichoderma* strains may be limited, since many strains of soil bacteria have been shown to suppress the activity of *Trichoderma* (Simon and Sivasithamparam 1989). Hence, it would be advantageous to identify a strain of *Trichdoerma* that can antagonize and degrade bacteria present in the compost or rhizosphere of plants. Eighteen *Trichoderma* strains were screened for their ability to degrade *Bacillus subtilis, B. megaterium, Escherichia coli*, *B. cereus* var. *mycoides, Micrococcus luteus, Pseudomonas aeruginosa* and *Serratia marcescens. T. harzianum* T-19 was found to have maximum degrading capacity among the four bacterial species tested. This strain produced at least three trypsin-like proteases, six chymotrypsin-like proteases and four NAGases as well as muramidase-like activity. Proteases, NAGases and muramidases appear to be very important for the degradation of bacterial cells. The ability to degrade bacteria present in the soil might enhance the effectiveness of *Trichoderma* spp. as a biocontrol agent applied for the control of soilborne pathogens (Manczinger et al. 2002).

Powdery .mildew disease caused by *Blumeria graminis* f.sp. *hordei* in barley may be able to inflict serious damages to susceptible barley cultivars, if effective management measures are not applied at right time. Pre-treatment with mycelial extracts or culture filtrates of taxonomically different fungi *Bipolaris oryzae, Pythium ultimum* and *Trichoderma harzianum* was evaluated for their efficacy in suppressing the development of powdery mildew disease in barley. The number of colonies formed on treated barley leaves was reduced by 70–98 % in the fourth leaf, compared with untreated control and on the second leaf, the percent reduction varied from 82 to 87 %. The colonies were also much smaller in size. The mycelial extracts of *B. oryzae* was the most effective in suppressing the powdery mildew development in barley leaves. Protection was limited to the leaf area treated with mycelial extract and no systemic effect of treatment was discernible. The results suggested that components of the mycelial extract interacted directly with the pathogen and antifungal effects of the compounds present in the extract were responsible for the protection of barley against the powdery mildew disease development (Haugaard et al. 2001). The effects of antifungal substances (AFS) produced by the fungal BCA *Coniothyrium minitans* (*Cm*) on *Sclerotinia sclerotiorum* (Ss) causing leaf blight disease of oilseed rape plants were assessed using modified Czapex-Dox (MCD) broth and potato dextrose broth (PDB). The mycelial growth of the pathogen was reduced by 41.6–84.5 % by the culture filtrates in broth medium. Retardation of mycelial development, morphological abnormality like hyphal swellings and cytoplasm granulation were also observed in colonies grown on PDA amended with culture filtrates from MCD. Sclerotia soaked in the filtrates

remained viable, but their myceliogenic germination was delayed. Although ascospore germination was not affected by the culture filtrates, the germ tubes were deformed and shortened with hyphal swelling, following treatment with filtrates from MCD. Incidence of leaf blight on leaves of oilseed rape was reduced following application of culture filtrates. The antifungal substances produced by the BCA could delay or inhibit the development of the pathogens at different stages of its life cycle, resulting in the suppression of the leaf blight disease of oilseed rape (Yang et al. 2007).

Suppression of Fusarium wilt of cucumber is brought out by different mechanism by *Trichoderma harzianum* strain SQR-T037 in cucumber continuous cropping (CCC) system. The allelochemicals exuded from cucumber cause stress and these chemicals have to be biodegraded for better growth of cucumber plants. The allelochemicals isolated from cucumber rhizosphere included 4-hydroxy- benzoic acid, vanillic acid, ferulic acid, benzoic acid, 3-phenylpropionic acid and cinnamic acid. The allelochemicals were completely degraded by SQR-TO37 after 170 h of incubation. Inoculation of SQR-TO37 in the CCC soil also resulted in degradation of allelochemicals exuded from cucumber roots. The degradation of allelochemicals was accompanied by significant decrease ($P \geq 0.05$) in the disease index and increase in dry weights of cucumber plants in pot experiments following application of *T. harzianum.* The results indicated that alleviation of allelopathic stress could be attributed to SQR-TO37 strain. It may be possible to resolve problems associated with monocropping by applying appropriate BCA capable of biodegrading allelochemicals (Chen et al. 2011). *Trichoderma harzianum* SQR-TO37, an effective biocontrol agent of *Fusarium oxysporum* f.sp. *cucumerinum* (Foc) infecting cucumber, produced several antifungal compounds. One such compound was purified and it was identified as 6-pentyl-α-pyrone (6PAP) using both mass spectrometry and nuclear magnetic resonance spectroscopy. The antifungal activity of 6PAP at different concentrations (50, 150, 350 and 450 mg/l) was assayed using growth inhibition tests in plates. Antifungal activity increased with increase in the concentration of 6PAP in general. At 350 mg/l, 6 PAP inhibited the mycelial growth and spore germination by 73.7 and 79.6 % respectively compared with control. Further, at a concentration of 150 mg/l, 6 PAP decreased sporulation and fusaric acid production (g/g dry mycelia) by *Foc* by 88 and 52.68 % respectively. Application of 6 PAP to cucumber continuously cropped soil reduced the pathogen population by 41.2 % and the incidence of cucumber Fusarium wilt disease by 78–89.6 %, in addition to promotion of cucumber plant growth (Chen et al. 2012).

Penicillium expansum, an important postharvest pathogen of apples and pears, also produces a mycotoxin patulin in the infected fruits making the infected fruits unfit and harmful for human consumption. Patulin contamination in derived fruit juices and baby foods has also been reported (Beretta et al. 2000). The efficacy of the biocontrol yeast *Rhodotorula glutinis* strain LS11, *Cryptococcus laurentii* LS28 and *Aureobasidium pullulans* LS30 was assessed for reducing the accumulation of pathogen-secreted patulin in apples. *R. glutinis* strain LS11 was the most tolerant to patulin. *R. glutinis* strain LS11 effectively protected the apples against infection by *P. expansum.* The presence of this BCA significantly lowered the mycotoxin levels

by 44.9 % at 4 days and 39.3 %, at 6 days after inoculation with the pathogen. Further, the yeast cells applied in the apple wounds were able to survive and multiply in the decaying apple tissue infected by *P. expansum.* The results indicated that *R. glutinis* had the potential not only to protect the apples from infection by *P. expansum,* but also could effectively prevent the accumulation of the mycotoxin patulin in infected apples (Castoria et al. 2005). The performance of 12 native isolates of *Debaromyces hansenii* existing in the marine environment and the pericarp of Mexican lime (*Citrus aurantifolia*) was evaluated. Isolates from Mexican lime pericarp were more effective both in in vitro and in simulated industrial packing-house conditions for the control of the postharvest blue mold disease on Mexican lime. The effectiveness of the BCA was partially linked to a rapid utilization of available sugars in the medium. Three isolates DbhBCS06, LL1 and LL2 of *D. hansenii* were more effective and they reduced the disease incidence up to 80 % after 2 weeks of storage (Hernández-Montiel et al. 2010).

3.1.3 Competition for Nutrients and Space

Rhizosphere competence is an important attribute for the biocontrol agent to be successful particularly against soilborne pathogens. If the BCA is unable to grow in the rhizosphere, phyllosphere, spermosphere or surface of other organs of plants, it cannot compete with other microorganisms or pathogens for nutrients and space for further prolification and establishment in different habitats. Different species of *Trichoderma* identified as biocontrol agents are added to the soil or used to treat the seeds. They are able to propagate readily along with the developing root system of the treated plants. The microorganism may compete for nutrients that may be in sufficient quantities or in an unavailable form. The microorganism with better uptake mechanism or capable of producing more efficient extracellular enzymes may be in an advantageous condition than others. Competition may occur for both carbon and nitrogen sources. The microorganisms may compete for utilizing the exudates from roots and other plant surfaces. The exudates may stimulate differentially the germination of spores of the pathogen and the biocontrol agents. The three-way interactions among plants, pathogens and biocontrol agents are complex and variable, depending on the environment existing in the soil and the microclimate around plants.

Nonpathogenic strain of *Fusarium oxysporum* have a vital role in soil microbial ecology and especially in the natural phenomenon of soil suppressiveness to diseases induced by pathogenic soilborne pathogens. Soils suppressive to Fusarium wilts supported a large population of nonpathogenic *Fusarium* spp. *Fusarium oxysporum* strain Fo47 is possibly the most intensively investigated isolate to determine the mechanisms of their biocontrol activity. Among the available methods to generate fungal mutants, insertional mutagenesis has been extensively employed to tag genes involved in the biocontrol activity of bacterial BCAs (Mirleau et al. 2000). This approach was applied to generate Fo47 mutants in their biocontrol activity

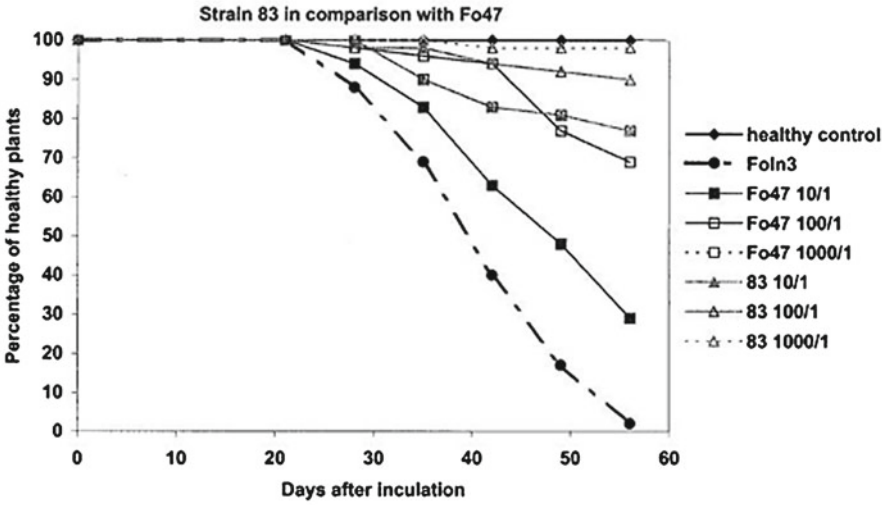

Fig. 3.6 Comparative efficacy of the mutant 83 and wild-type Fo47 (nonpathogenic) strain of *Fusarium oxysporum* in protecting flax plants against Fusarium wilt pathogen *F. oxysporum* f.sp. *lini* at different density ratios (Courtesy of Trouvelot et al. 2002 and with kind permission of The American Phytopathological Society, MN, USA)

against *F. oxysporum* f.sp. *lini* causing linseed wilt disease. The mutants were selected, based on their ability to grow and compete in soil, because the biocontrol activity of Fo47 was mainly based on mechanisms of competition involving a great saprophytic ability. The mutants were characterized by their saprophytic traits. Mutants 83 and 94, the most significantly affected in their biocontrol activity, had the same ability to grow and elongate on MMA-nitrate as the wild-type strain Fo47. The mutants 83 and 94 showed marked differences with respect to their antagonistic activity, whatever was the density ratio (10, 100 or 1,000). Mutant 83 inoculated in the ratio 10:1 was as effective as the parent strain Fo47 inoculated in the ratio 1,000:1, whereas mutant 94 inoculated in ratio 1,000:1 was no more effective than strain Fo47 inoculated in ratio 10:1 (Fig. 3.6). The results indicated that mutants were not impaired in their saprophytic phase. As the mutants were either less or more antagonistic than the wild-type strain, the biocontrol activity was not dependent entirely on the saprophytic capacity of the nonpathogenic Fo47 strain (Trouvelot et al. 2002).

A nonpathogenic strain *Fusarium oxysporum* F2 effectively reduced development of symptom in eggplant (brinjal) infected by *Verticillium dahliae* under greenhouse and field conditions. The dual plate confrontation test showed that the strain F2 did not act on the pathogen through parasitism or antibiosis. In order to determine the mechanism of biocontrol activity of the strain F2, the BCA and pathogen strains were transformed respectively with the EGFP and DsRed2 reporter genes to facilitate visualization of their presence on the root surface of eggplants. In addition, the real-time QPCR analysis was performed to monitor the ramification of both

fungi into the plant vascular system. The strain F2 colonized the root surface along the intercellular junctions excluding *V. dahliae* from the same ecological niche. The QPCR analysis also showed that application of F2 reduced the levels of *V. dahliae* vascular colonization along with the disease severity. The results of the split root experiment revealed that the strain F2 did not trigger the defence mechanisms of eggplant against *V. dahliae.* This investigation appeared to provide evidence that the mechanism of biocontrol activity of the strain F2 against *V. dahliae* was through the competition for space or nutrients on the root surface of eggplant (Pantelides et al. 2009). In the further study, the nonpathogenic *Fusarium oxysporum* F2 strain was applied by stem injection of a conidial suspension of this strain, instead of root drenching with the BCA suspension, since root drenching might adversely affect the native beneficial microbial community. Stem injection of the strain F2 at 7 days before transplanting the seedlings to soil infested by *V. dahliae* microsclerotia resulted in reduced disease severity, compared to untreated control plants. Ramification of F2 into the plant vascular system of egg plant stems was visualized by injecting an EGFP transformed F2 strain. The strain F2 colonized the plant vascular tissues effectively over a long period of time as determined by the levels of DNA. The QPCR analysis showed that the application of F2 reduced significantly the amount of *V. dahliae* DNA in the stem tissues compared to the untreated control plants (Gizi et al. 2011).

Competition for nutrients on plant surfaces is one of the mechanism of biological control against microbial plant pathogens that depend on external nutrition. It is difficult to demonstrate competition through rhizosphere competence as a major mechanism of biocontrol of crop diseases. The replacement of endogenous fungi including pathogens by *Trichoderma* species may be difficult to demonstrate. The BCA is able to suppress the growth of endogenous fungi on an agar medium masking their presence. For example, *T. virens* grew rapidly from the root segments of plants heavily infested with propagules of *Macrophomina phaseolina* at room temperature. However, incubation at 40 °C favored the development of *M. phaseolina*, but not *T. virens* (Howell 2003). Wheat head blight or maize ear rot diseases are caused by *Fusarium culmorum*, *F. graminearum, F. proliferatum* and *F. verticillioides*. Antagonists such as *Trichoderma* spp., non-pathogenic *Fusarium* spp. and isolates of *Clonostachys rosea* consistently suppressed sporulation of *F. culmorum, F. graminearum, F. proliferatum* and *F. verticillioides* on maize stalks. The biocontrol agents significantly reduced the colonization of stalk pieces by pathogenic *Fusarium* spp. significantly at several sampling dates. The pathogenic *Fusarium* spp. might be suppressed by the competition with the BCAs (Luongo et al. 2005). Colonization pattern of nonpathogenic *Fusarium oxysporum* endophytes was studied by using *F. oxysporum* isolates transformed with the green fluorescent protein (GFP) and red fluorescent prod (Ds Red) genes. It was possible to distinguish the transformants from other saprophytic strains. Root and rhizome tissue colonization by the transformants were similar to that of wild-type isolates. Ds Red transformants were difficult to visualize in tissues colonized by them. Use of GFP-transformed isolates provides the possibility of monitoring the BCA colonization in the presence of other saprophytic fungi (Paparu et al. 2009).

Pichia guilliermondii utilized nutrients more effectively, when cocultured with the pathogen *Penicillium digitatum* infecting grapefruit. Rapid multiplication and colonization of wound sites by the BCA was observed (Droby et al. 1989). The effect of nitrogenous compounds on the colonization of *Candida sake* (CPA-1) on apples and pears was determined, since the pome fruits are poor in nitrogenous compounds. Application of ammonium molybdate, calcium chloride and 2-deoxy-D-glucose enhanced biocontrol activity of *C. sake* against *Penicillium expansum.* In cold storage, the combination of ammonium molybdate and *C. sake* offered complete protection against blue mold caused by *P. expansum* on pears and reduced its severity by more than 80 % on apples (Nunes et al. 2001). Two yeast species *Candida guilliermondii* and *Saccharomyces cerevisiae* were evaluated for their biocontrol activity against *Penicillium expansum* causing blue mold disease of apples. The yeasts applied alone or in the presence of various additives reduced apple decay up to 100 %, compared with untreated control fruits. Dead yeast cells and the culture filtrates showed no inhibitory effect. Addition of a nutrient analogue 2-deoxy-D-glucose which could not be metabolized by *P. expansum*, inhibited the blue mold disease by giving advantage to the antagonist. Nitrogen is likely to be a limiting factor in the carbon-rich environment of apple wound. Nutrient competition for nitrates may play a major role in the biocontrol efficacy of the strains 3C-1b and F1 of *C. guilliermondii*. The reduction of decay due to *P. expansum* in the presence of both *C. guilliermondii* and *S. cerevisiae* may be partly due to the inhibitory effect $FeNO_3$ on the pathogen per se (Scherm et al. 2003).

The utilization of ^{14}C-glucose by the cells of the antagonist pink yeast *Sporobolomyces roseus* was higher to such an extent as to prevent the germination of conidia of *Botrytis cinerea* by nutrient deprivation (Filonow et al. 1996). The role of competition for sugars by *Cryptococcus laurentii* BSR-Y22 or *Sporobolomyces roseus* FS43-238 that effectively reduced gray mold caused by *B. cinerea* in apples at 22 °C was studied. The increase in populations of *C. laurentii* and *S. roseus* in wounds of apples was six to nine times from 1 to 7 days following inoculation, compared with *Saccharomyces cerevisiae* which had less antagonistic activity against *B. cinerea.* The BCA utilized greater amounts of ^{14}C-labeled fructose, glucose or sucrose than the conidia of *B. cinerea* during 48 h. The results suggested that these BCAs might act on the pathogens primarily by competing for nutrients (Filonow 1998). Among the yeasts tested, *P. guilliermondii* was the most effective in inhibiting the growth of *Ceratocystis paradoxa,* causing black rot of pineapple fruit. The mechanisms of biocontrol activity of *P. guilliermondii* appeared to be competition for space and nutrients (Reyes et al. 2004).

Attachment of the antagonist to the pathogen hyphae seems to be required for the strategy of competition for nutrients, as in the case of *Pichia guilliermondii* which attached itself to *Penicillium italicum* (Arras et al. 1998). But no such physical interaction appears to be necessary for the biocontrol activity of *Aureobasidium pullulans* against *B. cinerea*, *Penicillium expansum, Rhizopus stolonifer* and *Aspergillus niger* infecting table grapes and *B. cinerea* and *P. expansum* infecting apples fruits (cv. Royal Gala) (Castoria et al. 2001). The protocol developed by Janisiewicz et al. (2000) may be useful for assessing the need for direct contact

between the BCA and the pathogen. Rhodotorulic acid produced by *Rhodotorula glutinis* enhanced the biocontrol potential of its strains. Rhodotorulic acid reduced the growth of *P. expansum* in the absence of iron, but not in the presence of iron, indicating that antagonism of this BCA was due to siderophore and it was related to competition for iron (Calvente et al. 1999). *Candida oleophila,* primary component of the commercial product Aspire, was shown to efficiently compete with *P. digitatum,* causing green mold disease in citrus for nutrients released by injuries to the fruits (Brown et al. 2000). Postharvest fungal pathogens are able to invade tissues of fruits and vegetables primarily through wounds. Hence, wound competence is essential for biocontrol yeasts to successfully compete for space and nutrients against pathogenic fungi (Droby and Chalutz 1994). Competition for nutrients and space has been reported to be a major mechanism in the antagonism of biocontrol yeasts against postharvest fungal pathogens. Timely colonization of wounds by BCA and the number of live antagonist cells present in wound sites may be crucial for providing effective protection to fruits and vegetables under storage. Antagonistic yeasts have been selected mainly for their ability to rapidly colonize and grow on surface wounds and subsequently to out- compete the pathogen for nutrients and space. Besides competing for nutrients and space, the yeasts may act by parasitizing fungal postharvest pathogens through strong attachment to pathogen hyphae (Droby and Chalutz 1994). The isolates of the yeast species *Rhodotorula glutinis* and *Cryptococcus albidus* have to compete for nutrients with germinating conidia of *Botrytis cinerea* causing gray mold diseases of several crops (Elad 1996). Biological control depending on competition for nutrients may be made ineffective by enhancing the supply of relevant nutrients (Elad et al. 1994). The effectiveness of bioprotection achieved by competition for nutrients is determined by several biotic and abiotic factors and hence, its efficacy cannot be predicted accurately.

The yeast *Torulaspora globosa* effectively suppressed the development of *Colletotrichum sublineolum* causing anthracnose disease of sorghum. The BCA produced a killer toxin capable of causing hyphal deformities in the pathogen. The killer toxin could attack the cell membranes, decreasing the intracellular pH and cause an over flow of potassium ions and ATP. *Pichia guilliermondii*, the yeast antagonist was evaluated for its ability to compete for nutrients with *Botrytis cinerea* by adding increasing concentrations of adenine, histidine, folic acid and riboflavin. *P. guilliermondii* competed with *B. cinerea* for all nutrients except riboflavin. In contrast, the bacterial antagonist *Bacillus mycoides* did not compete for any of the nutrients tested. However, suppression of conidial germination and disease severity achieved by a mixture of the yeast and bacterial antagonists was significantly higher than that could be obtained by the antagonists individually. The combined effects of the two BCAs was, in most cases, additive (Guetsky et al. 2002). A strain of *Metschnikowia pulcherrima* (MACH1) was assessed for its potential as biocontrol agent against *Botrytis cinerea, Penicillium expansum* and *Alternaria alternata* infecting apples during storage at 1 °C for 8 months. Competition for iron appeared to be important for the suppression of pathogen development by the BCA in media amended with different concentrations of iron. The yeast strain MACH1 produced a wider pigmented inhibition zone against *A. alternata* and

B. cinerea in low iron amendments, whereas high concentrations of iron affected the biocontrol activity of the yeast. Failure of germination of conidia and mycelial degeneration were observed at the colored inhibition zone. Furthermore, reduced level of infection by both *A. alternata* and *B. cienrea* was observed following treatment of apples with MACH1 supplemented with low iron amendments compared to higher iron concentrations. No significant effect on infection by *P. expansum* was noted under increased iron amendments and without iron (Saravanakumar et al. 2008).

Colonization of wounds on fruits by antagonistic yeasts in time is crucial for successful competition for nutrients against pathogens. Wounding of plant tissues is associated with increased lypolitic acylhydrolase activity, phospholipase and lipoxygenase activation, formation free radicals and possibly reactive oxygen production. The microorganisms including the pathogens and the BCA that have to colonize fresh wounds may be required to cope with the oxidative stress caused, following wounding. Generation of the reactive oxygen species (ROS), superoxide anion and hydrogen peroxide (H_2O_2) in apple wounds immediately after wounding was observed. Two yeast species viz., *Crytococcus laurentii* LS-28 with higher antagonistic activity and *Rhodotorula glutinis* LS-11 with lower antagonistic activity against the postharvest pathogens *Botrytis cinerea* and *Penicillium expansum* were evaluated for their resistance to ROS. LS-28 showed faster and greater colonization of wounds than LS-11. In in vitro tests, LS-28 exhibited greater resistance to ROS-generated oxidative stress. Combined application of BCAs and ROS-deactivating enzymes in apple wounds resulted in higher levels of colonization of wounds and antagonistic activity of both antagonistic yeast species against *B. cinerea* and *P. expansum.* The results suggested that resistance to oxidative stress might be a pivotal mechanism of biocontrol yeasts antagonism against postharvest wound pathogens (Castoria et al. 2003). *Rhodotorula glutinis* was evaluated for its potential to suppress the development of *Botrytis cinerea.* Washed cell suspensions of *R. glutinis* protected peach fruits more effectively than the yeast in culture broth. Treatment of wounds with autoclaved cell cultures or cell-free cuture filtrate (CF) did not prevent decay. Rapid colonization of the yeast in wounds was observed during the first day at 20 °C and then the populations of the yeast stabilized for the remaining storage period. The living cells of the yeast inhibited spore germination and germ tube elongation of *B. cinerea. R. glutinis* in combination with salicylic acid reduced the average natural infection of peach fruit to 16.67 % as against 46.67 % in untreated control fruits (Zhang et al. 2008).

Cryptococcus laurentii was effective in reducing the infection of pear fruits by *Penicillium expansum* causing blue mold disease, but its efficacy declined rapidly with increase in incubation period. Application of the cytokinin N^6-benzyladenine (6-BA) alone or with *C. laurentii* increased catalase activity and inhibited the activities of peroxidase and lipoxygenase as well as ethylene production. 6-BA did not influence the population growth of *C. laurentii* in pear fruit wounds. Combination of 6-BA and the BCA could integrate the dual biological activities of the resistance inducer and *C. laurentii* inhibition resulting in improvement in the biocontrol of the blue mold disease (Zheng et al. 2007). The competitive ability of *Aureobasidium*

pullulans Ach1-1 for nutrients was assessed to determine the mechanism of biocontrol activity against the apple blue mold pathogen *Penicillium expansum.* The effect of the BCA strain Ach 1-1 on conidial germination was determined after a 24-h incubation period in the presence of increasing apple juice concentrations (0–5 %). Irrespective of the juice concentration, conidial germination was strongly promoted by apple juice. But in the presence of Ach 1-1, conidial germination was significantly reduced. In situ assays revealed high protective ability of Ach 1-1 against *P. expansum* on postharvest wounded apples. Application of high concentration of exogenous sugars, vitamins and aminoacids reduced the protective ability of *A. pullulans* strain. The results indicated that competition for apple nutrients, particularly amino acids might be the principal mechanism of biocontrol activity of strain Ach 1-1 against *P. expansum* on harvested apple fruit (Bencheqroun et al. 2007).

The antagonistic effects of yeast species *Aureobasidium pulluans, Metschnikowia pulcherrima* and *Pichia guilliermondii* were compared with that of commercially available *Candida oleophila*. In general, the yeast species tested, had higher level of inhibitory activity than *C. oleophila* against *Botrytis cinerea*. The composition of the media had significant impact on the biocontrol activity of the yeast species. Since competition for nutrients is one of the mechanisms of biocontrol activity of the yeasts, the exogenous supply of substances such as amino acids or carbohydrates enhances biocontrol capacity of antagonists against fungal pathogens. *Saccharomyces cerevisiae* showed higher antagonistic activity against *B. cinerea,* when tested on media with increased concentrations of glucose. As the in vitro testing did not provide results that can be correlated with effectiveness of biocontrol activity in vivo, the tests were performed using selected yeast strain on wounded and unwounded grape berries of cultivars Rebula and Chardonnay for the biocontrol activity against *B. cinerea.* The results showed that *S. cerevisiae* might be an effective biocontrol agent against the gray mold pathogen infecting grapes (Raspor et al. 2010).

3.1.4 Prevention of Colonization of Host Tissues by Pathogens

The biocontrol agents may prevent colonization of specific host tissues by the pathogen resulting in disease suppression. Treatment of cotton seeds with *Trichoderma* (*Gliocladium*) *virens* reduced colonization of cotton roots by *Fusarium oxysporum* f.sp. *vasinfectum* and reduced the incidence and severity of wilt disease also (Howell and Stipanovic 1995). The competitive colonization of plant necrotic tissue by the fungi may vary. If the BCA can colonize the senescent or necrotic tissue, it may effectively prevent colonization of plant tissues by the fungal pathogens. Ability of *Botrytis cinerea* causing gray mold diseases and the saprophytic antagonist *Ulocladium atrum* was compared using immunohistological approach. Colonization and sporulation were used as indicators for comparative resource capture and effectiveness of biological control of *B. cinerea* by *U. atrum*. Analysis of the extent to which sporulation of either fungus in cyclamen tissue could be reduced by co-inoculation with the other fungus at different times showed that *B. cinerea* could be entirely excluded by early pre-inoculation with *U. atrum*, but not vice versa. This

indicated that *U. atrum* could exploit resources in the leaf and made them inaccessible to the pathogen. The results of this study using a specific model, demonstrated that competition for resources could provide a sufficient biological explanation for the dynamic interactions between the BCA and the pathogen (Kessel et al. 2002).

Effectiveness of biocontrol of soilborne pathogens causing root diseases depends essentially on maintaining an adequate population level of biocontrol agents at target sites and the timing of application. The nonpathogenic *Fusarium oxysporum* strain Fo47 protects tomato roots against infection by *F. oxysporum* f.sp. *radicis-lycopersici* (FORL), causing tomato foot and root rot (TFRR) disease. When tomato seedlings were planted in sand infested with spores, Fo47 hyphae attached to the root earlier than FORL. When the inoculum concentration of Fo47 was increased, root colonization by the pathogen was arrested at the stage of initial attachment to host plant root. The percentage of spores of Fo47 germinating in the tomato root exudate in vitro was higher than that of FORL. By using different autofluorescent proteins as markers and observing under confocal laser scanning microscope, the pathogen and the BCA could be visualized simultaneously on tomato roots and colonization of tomato root surface by them was quantified. The preferential germination of Fo47 spores by root exudates components was believed to reduce pathogen growth toward tomato roots and consequently to reduce the number of FORL hyphae that compete for attachment sites on roots (Bolwerk et al. 2005).

The binucleate *Rhizoctonia* (BNR) has been shown to be effective as a biocontrol agent of *Rhizoctonia solani* causing stem and root rot disease of poinsettia (*Euphorbia pulcherrima*). The rhizosphere competence and ability to maintain adequate population levels are important requirements of BCA for successful control of soilborne pathogens. In addition, the timing of BCA application has been shown to be an important factor. For example, during propagation of poinsettia, one application of *Burkholderia cepacia,* the bacterial BCA suppressed stem rot, while BNR isolate was not effective. In contrast, one application of BNR isolate after transplanting rooted poinsettias was more effective than the bacterial BCA. Highest root colonization by BNR isolates occurred, when the bacterial BCA was applied at propagation, followed by BNR application after transplanting. Both BNR isolates and *B. cepacia* were found to be good colonizers of poinsettia roots and maintain the initial high population levels up to 5 weeks after application (Hwang and Benson 2002). Binucleate *Rhizoctonia* (BNR) was found to be a potential antagonist protecting crops against soilborne pathogens. The BNR isolates were consistently isolated from hypocotyls and roots of cotton, indicating that colonization of root tissues was associated with control of *R. solani* infecting soybean plants (Khan et al. 2005).

The yeast species *Candida valida*, *Rhodotorula glutinis* and *Trichosporon asahii* were evaluated for their ability to colonize the roots of sugar beet and to assess their biocontrol potential against *Rhizoctonia solani* causing post-emergence damping-off of seedlings. The three yeast species were effective as BCAs against *R. solani*. This study appears to be the first to report the usefulness of yeasts for controlling soilborne diseases. The root colonization plate assay showed that *C. valida* and *T. asahii* colonized 95 % of the roots after 5 days, while *R. glutinis* colonized 90 % of the roots after 8 days. Population density assessment indicated that the three yeast species were present at all depths of the rhizosphere soil adhering to the tap

roots up to 10 cm. The yeast species promoted plant growth, when applied individually or in combination. Further, there was antagonism between the yeast species tested and the biocontrol efficacy was enhanced due to combination of some yeast species. The yeast species exhibited high levels of rhizosphere competence as reflected by the extent of their colonization of roots (El-Tarbily 2004).

Fungal endophytes have been shown to colonize the plant tissues in which they develop inter- and intracellular structures. Colonization of plant tissues by endophytes occurs through several steps such as host recognition, spore germination, penetration of epidermis and tissue colonization. Fungal endophytes are generally believed to protect plants against fungal pathogens by rapid colonization, resulting in exhaustion of limited available substrates. This situation denies the pathogen required niche for colonization and further development. Naturally occurring root endophytic fungi such as *Heteroconium chaetospira* and *Phialocephala fortinii* have been reported to effectively suppress Verticillium wilt of eggplant (Narisawa et al. 2002). Colonization patterns of the endophytes *Phialocephala fortinii* and a dark septate endophytic (DSE) fungus were studied, along with *Verticillium longipsorum,* causative agent of Verticillium yellows disease of Chinese cabbage. Hyphae of *P. fortinii* and DSE taxon extensively colonized the roots of Chinese cabbage seedlings without causing any observable external symptoms. Hyphae of *P. fortinii* grow along the surface of the root and formed microsclerotia on or in the epidermal layer, whereas the hyphae of the DSE taxon heavily colonized some root cortical cells. *P. fortinii* suppressed the effects of postinoculated virulent strain of *Verticillium* in vitro. The DSE taxon was able to colonize Chinese cabbage roots and suppressed the development of Verticillium yellows. The protective values of the DSE taxon against the disease were significantly higher compared to other fungal endophytes as reflected by higher marketable quality of the produce obtained from DSE taxon-treated plots (Narisawa et al. 2004).

The biocontrol potential of fungal root endophytes *Acremonium blochii*, *A. furcatum, Aspergillus fumigatus, Cylindrocarpon destructans, Fusarium equiseti, Phoma herbarum* and *P. leveillei* was tested by the dual culture technique. All isolates could colonize the rhizosphere and frequently the root cortex without inducing any observable symptom. The plant growth was not adversely affected. Some isolates significantly reduced the intensity of symptoms of take-all disease in barley and also reduced the presence of the pathogen *Gaeumannomyces graminis* var. *tritici* in the roots (Maciá-Vicente et al. 2008). In a later study, the endophytic fungi following colonization of plant roots have been shown to confer benefits to the host plant species like protection against abiotic or biotic stresses or plant growth promotion. *Fusarium equisetti*, a naturally occurring endophyte in vegetation under stress and *Pochonia chlamydospora*, parasitic on nematode eggs have the ability to colonize roots of non-host plants endophytically and to protect them against fungal plant pathogens under laboratory conditions. The effects of these two fungi on plant growth and incidence of take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (*Ggt*) were investigated. Both fungi colonized barley roots endophytically and competed with other fungal root colonizers present in the rhizosphere. *F. equisetti* isolates reduced the mean root lesion length caused by *Ggt*. However, no clear cut suppressive effect of the endophyte could be seen (Maciá-Vicente et al. 2009).

Pollen grains disseminated from alfalfa anthers may be found on petals in large quantities, could stimulate germination of conidia and germ tube growth of the gray mold pathogen, *Botrytis cinerea* as well as the fungal BCAs *Coniothyrium minitans* and *Clonostachys rosea* (Li et al. 2002, 2003). The ability of the fungal BCAs to compete with *B. cinerea* for the nutrients and colonize the florets to prevent the infection of pods and seeds of alfalfa was assessed. The fungi *C. rosea*, *Gliocladium catenulatum, Trichothecium roseum* and *Trichoderma atroviride* could effectively inhibit the sporulation of *B. cinerea*. On detached alfalfa florets *C. rosea* and *G. catenulatum* effectively colonized young petals of alfalfa at the anthesis stage, resulting in better pod formation and protection of young pods. They also effectively colonized senescent petals of alfalfa at pod development stage and prevented infection of pods and seeds. *T. atroviride,* although effective in in vitro tests, was less effective, compared to *C. rosea* and *G. catenulatum* in in vivo experiments. *C. rosea* and *G. catenulatum* could colonize senescent petals of alfalfa which provided nutrients to the pathogen, as evidenced by formation of conidia and conidiophores profusely, indicating their greater saprophytic colonization ability, compared with *T. atroviride.* In addition, *C. rosea* and *G. catenulatum* might be less sensitive to lack of available moisture than *T. atroviride,* during colonization of alfalfa petals, implying their suitability as BCA against *B. cinerea* (Li et al. 2004). The biocontrol efficacy of a marine antagonist *Rhodosporidium paludigenum* in suppressing the postharvest decay of Chinese winter jujube caused by *Alternaria alternata* was assessed. The BCA was able to colonize the wounds on the jujube fruits rapidly during the first 48 h at 25 °C. The concentration of the BCA had significant adverse effect on the development of *A. alternata*. As the yeast population increased, the disease incidence and intensity decreased. The BCA did not affect the fruit quality parameters during 21 days of storage at 25 °C. The results indicated that *R. paludigenum* was effective in suppressing the fruit decay by rapid colonization of all sites that are required for pathogen development (Wang et al. 2009).

Aflatoxin contamination of maize grains due to *Aspergillus flavus* is of great concern, because of the ailments caused in humans and animals. The efficacy of two non-aflatoxigenic isolates of *Aspergillus flavus* CT3 and K49 in reducing aflatoxin levels in maize grains was assessed. The non-toxigenic isolates CT3 and K49 reduced aflatoxin levels by 61 and 76 % respectively. The sclerotia-producing strain K49 showed more rapid growth and grater ability to colonize maize grains than the non-sclerotia producing CT3 strain, when they were inoculated on maize, indicating its greater ecological (spermosphere) competence. The indigenous non-aflatoxigenic strain K49 has the potential for use as a biocontrol agent to reduce aflatoxin contamination (Abbas et al. 2006).

3.1.5 Induction of Resistance in Plants to Diseases

The concept of inducing resistance to crop diseases by inducing natural disease resistance (NDR) mechanisms operating in existing cultivars has attracted the attention of the researchers all over the world, since development of disease resistant cultivars

through breeding and/or biotechnological approaches is difficult or time-consuming or not feasible. Biotic and abiotic agents have been used as inducers of disease resistance in a wide of range of agricultural and horticultural crops. Different fungal species identified as biocontrol agents (BCAs) have been demonstrated to induce resistance to crop diseases, in addition to other mechanisms of biocontrol activity against microbial plant pathogens.

The possibility of inducing resistance in cucumber, muskmelon or watermelon by employing the pathogen *Colletotrichum lagenarium* causing anthracnose disease was first demonstrated by Ku (1987, 1990). Primary inoculation of cotyledons with this pathogen induced systemic acquired resistance (SAR) to several diseases caused by fungi, bacteria and viruses, in addition to the anthracnose disease. The binucleate *Rhizoctonia* (BNR) species was able to induce resistance to *Rhizoctonia solani* causing root rot disease and *Colletotrichum lindemuthianum* causing anthracnose disease in bean (*Phaseolus vulgaris*), when the bean hypocotyls were inoculated with the BCA prior to challenge inoculation with the fungal pathogens. Thus, biotic inducer of resistance elicited significant systemic increase in the activities of defense enzymes peroxidases, β-1,3-glucanases and chitinases. The increases in activity of peroxidase and glucanase (2–8 folds) were positively correlated with levels of induced resistance (Xue et al. 1998). BNR isolates obtained from soybean cultivars with different levels of resistance to *R. solani* were evaluated for their biocontrol efficacy. In addition to disease control, the BNR isolates significantly increased the height of soybean plants, indicating their ability of growth promotion in treated plants. It was considered that the mechanism of biocontrol of *R. solani* by BNR might be a novel form of induced resistance (Khan et al. 2005).

Resistance of tomato to wilt disease caused by *Fusarium oxyporum* f.sp. *lycopersici* could be induced by inoculating the nonpathogen *Penicillium oxalicum* resulting in reduction in disease severity, area under disease progress curve (AUDPC) and extent of stunting of plants. Histological observations revealed that BCA-inoculated plants did not lose the cambium, had lower number of bundles and less vascular colonization by the pathogen. Renewal or prolonged cambial activity in treated plants leading to the formation of additional secondary xylem may be a reason for reduction disease severity. As there was no detectable adverse effect on tomato cultivars susceptible and resistant to wilt disease, *P. oxalicum* can be applied to protect the susceptible tomato cultivars (de Cal et al. 1997, 2000). *P. oxalicum* appears to be primarily functional through induction of resistance to diseases caused by fungal pathogens. Pectinases from *P. oxalicum* could induce resistance in cucumber against *Cladosporium cucumerinum* (Peng et al. 2004). Further, this BCA possessed the ability to improve soil nutrition by producing acidic compounds which solubilize barley soluble phosphates and consequently the treated plants have greater biomass (Shin et al. 2005). However, presence of potent antifungal compounds in the culture filtrates of *P. oxalicum* has also been reported (Yang et al. 2008).

The nature of determinants of induction of resistance to plant diseases may vary depending on *Trichoderma* spp. interacting with the fungal or bacterial pathogens. Many classes of compounds capable of inducing disease resistance are released by *Trichoderma* into the zone of interaction with the pathogen. The ability of

Trichoderma harzianum T39 to induce resistance to grape downy mildew disease caused by *Plasmopara viticola* was assessed under greenhouse conditions. The strain T39 reduced disease severity on grapevine without a direct inhibitory effect on sporangial germination of *P. viticola*. Plant-mediated resistance was activated after a preventive T39 treatment, in a manner similar to that observed for benzothiadiazole (BTH) elicitation. Optimal disease suppression (63 %) could be achieved by applying *T. harzianum* more than once at 48–72 h before inoculation with the pathogen. Systemic activation of grapevine defense systems was observed, when *T. harzianum* was applied on leaves. The untreated leaves on the opposite side were resistant to downy mildew disease. In addition, treatments of basal leaves induced acropetal resistance in untreated leaves (more than 40 % disease reduction). However, root treatments did not induce resistance to a significant level in the leaves. The systemic resistance was homogenously activated, independently of leaf position on the shoots. The results suggested that induced resistance by *T. harzianum* followed different pathways other than of salicylic acid (SA)-dependent BTH elicitation of disease resistance (Perazolli et al. 2008).

In a later study, the molecular mechanisms activated by *Trichoderma harzianum* T39 and the energy costs of the induced resistance in terms of plant growth were investigated. The strain T39 reduced downy mildew disease severity on susceptible grapevines under controlled greenhouse conditions by a direct modulation of defense-related genes and the activation of priming for enhanced expression of these genes after pathogen inoculation. The stronger local than systemic modulation of defense-related genes corresponded to a higher local than systemic disease control in T39-treated plants. The absence of any negative effect of T39 treatment on grapevine growth, shoot and root weight, leaf dimension and chlorophyll content indicated the activation of a priming state. This was in contrast to the effect of benzothiadiazole (BTH) treatment. Priming of defense gene expression by T-39 treatment recorded a level higher than that of BTH treatment. The modulation of marker genes suggested the movement of jasmonic acid and ethylene signals in the defense processes induced by T39, in contrast to the SA pathway activated by BTH. The results indicated that the strain T39 could be used for suppressing the grapevine downy mildew disease development without apparent costs for grapevine plant growth (Perazolli et al. 2008).

Trichoderma harzianum spores (10^5/ml) were used to inoculate roots of 7-day old cucumber seedlings in an aseptic hydroponic system. Defense responses were initiated in both roots and leaves of treated cucumber plants. The hyphae of *T. harzianum* penetrated the epidermis and upper cortex of the cucumber root. Marked increases in peroxidase activity (associated with production of fungitoxic compounds) and chitinase activity and deposition of callose-enriched wall apposition on the inner surface of cell walls were observed in treated plants (Yedidia et al. 1999). In a later study, production of an array of pathogenesis-related (PR) proteins, including a number of hydrolytic enzymes was observed, following inoculation of cucumber roots with *T. harzianum* (T-203). There was similarity in responses of cucumber plants treated with *T. harzianum* or the chemical inducer 2,6-dichloroisonicotinic acid, indicating that the process of induction of resistance in plants follows similar

Table 3.4 Effect of colonization of maize roots by *Trichoderma harzianum* T22 on mean lesion size on maize leaves at 7 days after inoculation with *Colletotrichum graminicola* (Harman et al. 2004c)

Seed treatment	Leaf treatment	No.of lesions per leaf*	Mean lesion length (mm)*
None	None	0.4 a	7.5 a
T 22	None	0.0 a	–
None	Wounded	1.4 a	33 c
T22	Wounded	1.6 b	22 b

*Figures followed by the same letter are not significantly different at P = 0.05 by Fischer's protected least significant different test

host responses, irrespective of the nature of inducer of disease resistance (Yedidia et al. 2000). Treatment of seeds of maize inbred line Mo17 with *Trichoderma harzianum* T22 resulted in colonization of plant roots, but little or no colonization of shoots or leaves. Seedlings grown in the presence of T22, either in treated or untreated soil were larger in size than that in the absence of *T. harzianum*. The presence of T22 increased protein levels and activities of β-1,3-glucanase, exochitinase and endochitinase in both roots and shoots. Plants grown from T-22-treated seeds showed less intensity of symptoms of anthracnose and greater enzyme activity following inoculation with *Colletotrichum graminicola*. As the BCA was separated from the pathogen, root colonization by T22 offered protection to anthracnose by inducing systemic resistance in maize against *C. graminicola* (Table 3.4; Harman et al. 2004a, b, c). A differentially expressed *T. harzianum* endopolygalacturonase (endo-PG) gene was identified by proteome analysis and the production of endo-PG remarkably increased in the presence of *Rhizoctonia solani* and *Pythium ultimum.* The endo-PG encoding gene was necessary for active root colonization and induction of plant defense responses by *T. harzianum* T34. Assays to determine disease intensity in vivo showed that *Botrytis cinerea*-induced leaf necrotic lesions on tomato were slightly smaller in plants colonized by the *T. harzianum* transformant (Morán-Diez et al. 2009).

The influence of genetic variability among wild and cultivated tomato lines on the outcome of the interaction with strains of *Trichoderma harzianum* and *T. atroviride* was investigated. The beneficial effect of the BCA strains on the plant growth and development of systemic resistance to the gray mold disease caused by *Botrytis cinerea* was clearly observed for some tomato lines, but not all lines were tested. In the case of line M82, treatment with biocontrol agents had no beneficial effect or was even detrimental. Studies on the expression of defense-related genes suggested that the BCA strains were able to trigger, in the responsive lines, a long-lasting up-regulation of the SA pathway in the absence of a pathogen, possibly activating a priming mechanism in the plant. Consequently, challenge inoculation with *B. cinerea* on plants pretreated with *Trichoderma* was followed by enhanced activation of jasmonate-responsive genes, eventually boosting systemic resistance to the pathogen in a plant genotype-dependent manner. The results indicated that at least in tomato, the *Trichoderma*-induced systemic resistance mechanism appeared to be much more complex than considered so far and the ability of the plant to benefit from the interaction with the BCA may be genetically improved (Tucci et al. 2011).

A suspension of *Crinipellis perniciosa*-chitosan filtrate (MCp) significantly delayed the development of vascular wilt disease of tomato caused by *Verticillium dahliae.* Activation of synthesis of PR proteins with tissue lignification in tomato leaves was observed, although the in vitro growth of *Xanthomonas vesicatoria* was not affected (Cavalcanti et al. 2007). The efficacy of a heterogeneous chitosan suspension (MCp) and a commercial plant activator acibenzolar-*S*-methyl (ASM; Bion® 50) for inducing resistance in cocoa against *V. dahliae* was assessed. The MCp and ASM enhanced the level of protection to susceptible cocoa cv. SIAL 70 against Verticillium wilt. Treatment with MCp reduced Verticillium wilt severity to a level that was equivalent of 80 % of ASM protection level. Local induced resistance was associated basically with peroxidase (POX) and polyphenoloxidase (PPO) activities in leaves and with lignin deposition at 13 days after application. Local induction of resistance was confirmed by the increase in the activities of chitinase and β-1-3-glucanase in the leaves at 4–18 days after treatment with MCp and ASM. Treated with MCp and ASM and plants challenged with the pathogen showed in the leaves and hypocotyls, increased levels of lignin deposition which was associated with cocoa defense strategy against Verticillium wilt pathogen (Cavalcanti et al. 2008).

The dynamics of expression of defense response genes in the root tissues of potato plantlets were investigated following treatment with *Trichoderma harzianum* and challenged by *Rhizoctonia solani*. Analysis of gene expression showed induction of *PR1 at* 168 h post-inoculation (hpi) and phenylalanine-ammonia lyase (PAL) at 96 hpi. In plants inoculated with *T. harzianum* strain Rifai MUCL 2907, induction of *PR1'*, *PR2* and *PAL* at 48 hpi in plants inoculated with *R. solani* and induction of *LOX* at 24 hpi and *PR1, PR2, PAL* and *GST1 at* 72 hpi in plants inoculated with both BCA and pathogen were recorded. The results suggested that in the presence of the BCA isolate, the expression of *LOX* and *GST1* genes might be primed in potato plantlets with *R. solani* at an early stage of infection (Gallou et al. 2009). Using a multi-analysis technique to hormone quantification of endogenous levels of salicylic acid (SA), jasmonic acid (JA), abscisic acid (ABA) and 1-aminocyclopropane-1-ca rboxylic acid (ACC), the ET precursor were analyzed in melon plants inoculated with *Glomus intraradices* and *T. harzianum* in the presence of *F. oxysporum* f.sp. *melonis* (*Fom*). Infection by *Fom* activated a defensive response in plant, mediated by plant hormones SA, JA, ET and ABA, similar to the one produced by *T. harzianum.* Both *T. harzianum* and *G. intraradices* attenuated the plant response mediated by ABA and ET elicited by pathogen infection. In addition, *T. harzianum* attenuated the SA-mediated plant response. A synergistic effect of *T. harzianum* and *G. intraradices* in reducing the disease incidence was observed. But no such effect was noted in the hormonal disruption induced by the pathogen. The results suggested that the mechanisms of biocontrol activity of *T. harzianum* might be induction of plant basal resistance and the attenuation of the hormonal disruption induced by *F. oxysporum* f.sp. *melonis* causing Fusarium wilt disease of melons, whereas the mechanisms involving *G. intraradices* appeared to be independent of SA and JA signaling (Martínez-Medina et al. 2010).

The role of oxidant-antioxidant metabolites of the *Trichoderma harzianum* isolates in the development of resistance in sunflower against *Rhizoctonia solani*

was investigated. Changes in the apoplast of sunflower challenged by *R. solani* in the presence/absence of *T. harzianum* NBRI-1055 were determined. Analysis of oxidative stress response revealed a reduction in hydroxyl radical concentration. The protection by the BCA strain against the pathogen was associated with the accumulation of the reactive oxygen species (ROS) gene network involving catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and ascorbate peroxidase (APx). In NBRI-1055-treated plants challenged with *R. solani,* these enzymes registered maximum activity after different periods (7–8 days). The enhanced enzymatic activities was accompanied by inhibition of lipid and protein oxidation in *Trichoderma*-treated plants. In addition, synthesis of secondary metabolites of phenolic nature was stimulated by the BCA strain reaching a 5-fold concentration. Strong antioxidant activity at 8 days post-inoculation resulted in the systemic accumulation of phytoalexins. The results suggested that the mechanism of biocontrol activity of *T. harzianum* against *R. solani* might be related to neutralizing *R. solani-induced* oxidative stress (Singh et al. 2011).

Trichoderma virens, an effective biocontrol agent against cotton root rot disease caused by *Rhizoctonia solani,* has been demonstrated to induce defense related compounds in the roots of cotton. The effect of seed treatment with BCA on elicitation of defense responses of cotton plants was assessed. The role of terpenoid compounds in the control of cotton root rot disease was studied by analyzing the extracts of cotton roots and hypocotyls grown from *T. virens*-treated seeds. Terpenoid synthesis and peroxidase activity were enhanced in the roots of treated plants, but not in the untreated controls. The terpenoid pathway intermediates deoxyhemigossypol (dHG) and hemigossypol (HG) strongly inhibited the development of *R. solani,* indicating that terpenoid production is the major contributor for the control of the root rot disease. Furthermore, a strong correlation between the biocontrol and induction of terpenoid was revealed, when the strains of *T. virens* and *T. koningii* were compared. The results indicated that induction of resistance by *T. virens* occurred through the activities of terpenoids acting as elicitors of defense responses in cotton (Howell et al. 2000). In the further study, it was observed that heat stable proteinaceous compounds were elicited following treatment of roots with effective strains of *T. virens*. One compound had a MW between 3 and 5 K and it was sensitive to proteinase K. Several bands could be recognized in the gel after subjecting the active material to SDS-PAGE. One band exhibited cross-reaction with an antibody to ethylene-inducing xylanase from *T. viride*. Another band (18 K) induced production of terpenoids, in addition to increasing the peroxidase activity, in cotton radicals and this protein showed highest similarity to a serine proteinase from *Fusarium sporotrichoides* (Fig. 3.7; Hanson and Howell 2004).

Trichoderma hamatum strain 382 induced resistance in cucumber against root rot, crown rot, leaf and stem blight caused by *Phytophthora capsici*. The effectiveness of protection provided was equal to that offered by the chemical inducer benzothiadiazole (BTH). The biotic inducer remained spatially separated from *P. capsici* in plants in split root and leaf blight bioassays. The results suggested that resistance induced by *T. hamatum* was systemic in nature (Khan et al. 2004). Treatment of pepper seeds with spores of *T. harzianum* significantly reduced stem necrosis caused

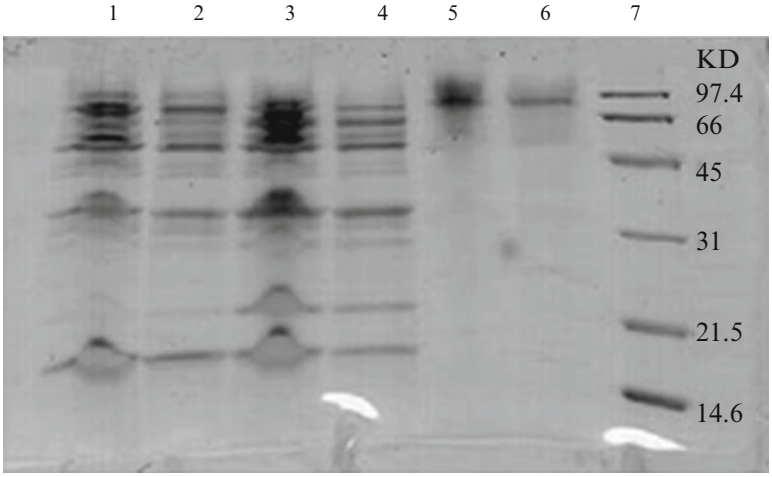

Fig. 3.7 SDS-PAGE analysis of culture filtrates (CFs) of effective and ineffective biocontrol strains of *Trichoderma virens*. Several bands are detectable only in effective strains of *T. virens*. *Lanes 1* and *2*: CF from *T. virens* strain G6 (biocontrol-effective); *Lanes 3* and *4*: CF from *T. virens* strain G6-5 (biocontrol-effective); *Lanes 5* and *6*: CF from *T. virens* strain G6-4 (biocontrol-ineffective) and *Lane 6*: Size marker (Courtesy of Hanson and Howell 2004 and with kind permission of The American Phytopathological Society, MN, USA)

by *P. capsici*. Similar reduction in disease was also seen following drenching the roots of pepper plants with spore suspension of *T. harzianum*. Isolation of the pathogen from necrotic zones and not the BCA suggested that there was no direct contact between the pathogen and the BCA in the zones of isolation. The percentage of *P. capsici* isolated at 9 days after inoculation was higher in nontreated inoculated plants than in treated inoculated plants. *T. harzianum* introduced into the subterranean part of the plants could induce a systemic defense response against *P. capsici* in the aerial parts of the plants. Concentration of capsidol in stems of treated inoculated plants was >7-folds greater than in non-treated inoculated plants at 6 days after inoculation. The capsidol concentration was reduced later. Accumulation of capsidol in the earlier stages of BCA-pathogen interaction with pepper plants might contribute to enhancement of resistance to the disease (Ahmed et al. 2000).

The nonpathogenic Fo47 strain of *Fusarium oxysporum* used to coat the seeds or roots of tomato seedlings reduced wilt disease incidence from 100 to 75 %. The hyphae could be observed only just below the crown region and it is likely that reduction in disease incidence might be due to induction of resistance to *F. oxysporum* f.sp. *radicis-lycopersici* (FORL). Inoculation of tomato with Fo47 acted via a systemic acquired resistance (SAR)-like mechanism (Duijff et al. 1998; Bolwek et al. 2005). Nonpathogenic binucleate *Rhizoctonia* spp. (np-BNR) has been reported to protect plants against damping-off and crown and root rot diseases caused by *Pythium* spp. and *Rhizoctonia solani.* In the greenhouse or field evaluation, the np-BNR strain 232-CG was shown to elicit induced systemic resistance (ISR) in the stem and cotyledons of bean to challenges with *R. solani* (AG-4) or *Colletotrichum*

lindemuthianum causing root rot and anthracnose diseases respectively (Xue et al. 1998). The mechanism of biocontrol activity of np-BNR was studied in comparison with chemical inducer benzothiadiazole (BTH) against *Rhizoctonia solani* and *Alternaria macrospora* causing pre-and post-emergence damping-off and leaf spot diseases of cotton. Pretreatment of cotton seedlings with np-BNR isolates protected the plants effectively against a virulent strain of *R. solani* (AG-4). Several isolates significantly reduced disease severity. The combination of BTH and np-BNR provided significant protection against seedling rot and leaf spot in cotton. However, the degree of disease reduction obtained with np-BNR treatment alone was comparable to the effectiveness of combined treatment. The results indicated that np-BNR isolates could protect cotton from infections by both root and leaf pathogens and they were more effective than the chemical inducer BTH (Jabaji-Hare and Neate 2005).

Trichoderma hamatum 382 (T382), binucleate *Rhizoctonia* (BNR) isolates BNR 621 and P9023 were evaluated for their potential to induce systemic resistance in geranium against Botrytis blight disease caused by *Botrytis cinerea*. The strain T382 and isolate P9023 induced resistance in geranium plants raised in potting mix amended with the BCA 2 weeks prior to inoculation with the pathogen, when grown under environments either highly or less conducive to disease development. The BCAs did not exhibit any direct inhibitory effect on conidial germination or lesion enlargement, when extracts of BCA-treated leaves were tested. Lesion area development depended on the interval between application of inducing agents and detachment of leaves for inoculation. In geranium leaves detached and inoculated at 7 days after spray application of formulations of BNR 621 and P9023, AUDPC calculated from lesion area was smaller than in T382 and inoculated (pathogen alone) control ($P < 0.0001$). On the other hand, leaves detached and inoculated at 14 days after treatment with T382 formulation had a smaller AUDPC from lesion area than plants treated with BNR 621 ($P < 0.0001$). Restriction of lesion development might play a role in the suppression of Botrytis blight in geranium (Olson and Benson 2007).

Trichoderma asperellum (= *T. harzianum*) could penetrate the roots of cucumber seedlings and colonize the epidermis and outer root cortex (Yedidia et al. 1999), resulting in induction of host plant resistance to pathogens infecting upper plant parts. Inoculation of roots with *Trichoderma* was shown to be effective against fungal pathogens (Harman et al. 2004a, b, c). The local and systemic expression of defense-related genes was analyzed in cucumber seedlings inoculated with *T. asperellum* strain T203 and challenged with *Pseudomonas syringae* pv. *lachrymans* (*Pst*) causing bacterial leaf spot of cucumber. Analysis of signal molecules involved in defense mechanisms and application of specific inhibitors indicated the involvement of jasmonic acid and ethylene in protective effect conferred by *Trichoderma* spp. against *Psl.* Further, examination of local and systemic gene expression by real-time RT-PCR analysis showed that the strain T203 modulated the expression of genes involved in the jasmonate/ethylene signaling pathways of ISR (*LOX1, Pal1, ETR1* and *CTR1*) in cucumber plants. The results implicate that the main signal transduction pathway, through which *Trichoderma*-mediated ISR was activated, used JA and

ethylene as signal molecules, as indicated by the involvement of several JA/ethylene pathway-related genes. Furthermore, the results demonstrated that the *Trichoderma*-induced state sensitized the plant to respond more efficiently to subsequent pathogen attack. This sensitization was apparent from both reduction in disease symptoms and the systemic potentiation of the PR genes *chit1,* β-1,3-glucanase and peroxidase. The ISA mediated by *Trichoderma* spp. appeared to depend on the wide spectrum of the potentiated gene expression (Shoresh et al. 2005).

The nature of determinants of induction of resistance to plant diseases may vary depending on the fungal biocontrol agent interacting with fungal or bacterial plant pathogens. Many classes of compounds capable of inducing resistance are released by *Trichoderma* spp. into the zone of interaction. Proteins form the major group of compounds present in fungal pathogens and BCAs and they have enzymatic or other activity that adversely affect the pathogen development. Fungal proteins such as xylanase, cellulase and swollenin are secreted by *Trichoderma* species. These proteins appeared to induce only localized plant reactions and necrosis (Fuchs et al. 1989; Brotman et al. 2008). Other proteins and peptides were also found to be active in inducing terpenoid phytoalexin biosynthesis and peroxidase activity. *T. virens* produced a small protein SMI with hydrophobin-like properties (Djonovic et al. 2006). *T. harzianum* T22 secreted a hydrophobin-like protein capable of inducing resistance as well as enhancing root development (Ruocco et al. 2007). A group of metabolites known as peptaibols are linear short-chain length (<20 residues) peptides of fungal origin produced by the nonribosomal peptidesynthase. Their ability to elicit plant defense responses has been demonstrated by Viterbo et al. (2007). Oligosaccharides and low-molecular weight compounds are released from fungal or plant cell walls by the activity of enzymes secreted by *Trichoderma* spp. (Harman et al. 2004a, b, c). These secondary metabolites are able to induce the expression of PR proteins and reduce disease symptoms systemically (Vinale et al. 2008).

Induction of resistance to angular leaf spot disease of cucumber (*Pseudomonas syringae* pv. *lachrymans*) by application of *Trichoderma asperellum* to the root system was reported by Yedidia et al. (2003). *T. asperellum* activated metabolic pathways in cucumber involved in plant signaling and biosynthesis, eventually leading to systemic accumulation of phytoalexins, as in the case of beneficial plant growth-promoting rhizobacteria (PGPR). Penetration of the epidemics and subsequent ingress into the outer cortex of cucumber seedlings by *Trichoderma* requires secretion of cell wall lytic enzymes. Two differentially secreted arabinofuranosidases were detected by SDS-PAGE procedure, when *T. asperellum* was cultivated in the presence of cucumber roots. In addition, an aspartyl protease was also detected. Differential mRNA display performed on *Trichoderma* mycelia interacting and non-interacting with plant roots showed that another aspartyl protease was present along the differentially regulated clones. RT-PCR assays revealed that the proteases were induced in response to plant roots attachment and were expressed in planta. The gene *pap*C (similar to *pap*A from *T. harzianum*) was induced in plate confrontation assays with *Rhizoctonia solani*. The expression studies indicated that *T. asperellum papA* was upregulated during the first 48 h of interaction by cell wall proximity.

The gene *papB* did not seem to be regulated by the presence of the pathogen. The results suggested that the protease identified, might play a role in *Trichoderma* both as a mycoparasite and as a plant opportunistic symbiont (Viterbo et al. 2004).

The potential of 28 *Trichoderma* isolates to induce systemic resistance in tomato against *Xanthomonas euvesicatoria* (*Xe*) and *Alternaria solani* (*As*) causing bacterial spot and early blight diseases respectively was assessed. All isolates were able to colonize the root system of tomato plants. Treatment of the soil with *Trichoderma* isolates provided protection to tomato plants to varying degrees from 24 to 96 %, against *Xe* and 31 to 95 % against *As*. The most efficient isolates in reducing the severity of bacterial spot and early blight diseases were IB 28/07, IB 30/07, IB 37/01 and IB 28/07, IB 30/07 and IB 42/03 respectively. Two isolates IB 28/07 and IB 30/07 were effective against both diseases. The isolate IB 28/07 conferred resistance against both diseases at all time intervals confirming its ability to reduce the severity of both diseases up to 21 days after treatment of tomato plants. The isolate IB 28/07 was not antagonistic to both pathogens. The results indicated that the isolate IB 28/07 of *Trichoderma* spp. was able to promote the growth of tomato plants and to effectively reduce the severity of both bacterial spot and early blight diseases, possibly by inducing defense responses in treated plants (Fontenella et al. 2011).

Trichoderma viride with multiple mechanism of biocontrol activity against fungal pathogens was evaluated for its effectiveness against *Fusarium oxysporum* f.sp. *adzuki* and *Pythium arrhenomanes* infecting soybean. The BCA exhibited mycoparasitic behavior under in vitro conditions. The pot assays showed that *T. viride* suppressed the development of diseases due to *F. oxysporum* f.sp. *adzuki* and *P. arrhenomanes*. In addition, *T. viride* enhanced growth of root and shoot systems as well as pod yield of treated plants by 5 and 1.6 times, compared with plants inoculated with *Pythium* and *Fusarium* alone respectively. *T. viride* appeared to be an avirulent opportunistic symbiont in the rhizosphere of soybean plant. Further, *T. viride* enhanced resistance against the secondary infection of soybean by the fungal pathogens (John et al. 2010).

Trichoderma harzianum isolate T39 has been shown to be versatile in its biocontrol activity against several phytopathogens. The mechanisms of biocontrol activity of T39 against foliar pathogens *Botrytis cinerea*, *Peronospora cubensis*, *Sclerotinia sclerotiorum* and *Sphaerotheca* (syn. *Podosphaera fusca)* infecting cucumber was investigated. Activation of defense responses locally as well as systemically in cucumber plants treated with T39, was observed. Cells of T39 applied to the roots and dead cells applied to leaves of cucumber induced resistance to powdery mildew disease (Elad 2000). Application of T39 suppressed the enzymes of *B. cinerea* such as pectinases, cutinase, glucanase and chitinase required during different stages of disease development (pathogenesis), through the secretion of protease by the BCA on the plant surfaces. *T. harzianum* T39 did not act on the pathogen either by mycoparasitism or by producing antibiotics. The results indicated that T39 was able to act on the fungal pathogens through a combination of several modes of action to provide effective protection to the crop plants simultaneously against several diseases (Elad 2000). Treatment of onion seeds with *T. harzianum* strains TR1C7 and TR1C8 induced acceleration of production of antifungal compounds suppressing development of black mold disease caused by *Aspergillus niger* (Özer 2011).

Biocontrol agents have been applied as seed treatment to suppress pathogenic infection of plants. *Idriella bolleyi* was used for treating barley seeds for protecting the plants against root and leaf infection by *Bipolaris sorokiniana*. The treatment improved the systemic resistance on both leaves and roots of young plants to subsequent infection with the necrotrophic pathogen *B. sorokiniana. I. bolleyi* induced biochemical defense responses in plants as reflected by the slight accumulation of pathogenesis-related (PR)-proteins. However, the accumulation was not as great as when the roots were inoculated with *B. sorokiniana.* The BCA colonized the seeds and roots under field conditions. Two months after sowing, frequencies of *I. bolleyi* were higher on plants treated with the BCA than on control plants where colonization occurred naturally from field soil (Liljeroth and Bryngelsson 2002). *Trichoderma* spp. was evaluated for its ability to stimulate systemic induced response in wheat plants against *Septoria tritici* causing wheat leaf blotch disease. The BCA was applied as foliar spary or seed coating and the extent of leaf necrosis and pycnidial coverage were recorded at 21 days after inoculation with *S. tritici. T. harzianum* Th5 was the most efficient in restricting the progress of leaf blotch. Seed coating was more effective than foliar application of the BCA. The antifungal activity increased in plants growing from seeds coated with Th5 strain, as indicated by the assessment of leaf apoplast antifungal proteolytic activity. The increase was considered to confer resistance to the susceptible wheat cultivar against leaf blotch pathogen. The endogenous germin-like protease inhibitor coordinated the proteolytic action. The results suggested that the BCA application might stimulate a biochemical induced response against leaf blotch (Cordo et al. 2007).

Among the three *Colletotrichum* spp. considered to cause anthracnose disease of strawberry, *C. acutatum* (M11) was pathogenic, while *C. fragariae* (F7) was non-pathogenic inducing no visible symptoms. The avirulent strain F7 prevented the growth of *C. acutatum*, when inoculated prior to the pathogenic strain M11. The effectiveness of protection depended on the interval between the inoculations with F7 and M11. The development of F7 on plant without inducing symptoms and absence of antagonistic effect on M11 in vitro suggested that avirulent strain might trigger plant defensive responses against M11. Further, the detection of an early oxidative burst occurring within 4 h after first inoculation, in addition to anatomical alterations associated with induction of defense response indicated that F7 elicited one or more diffusible compounds effective against the pathogen (Salazar et al. 2007). The mechanism of suppression by *Chaetomium globosom* isolate NC-1 of wheat tan spot caused by *Pyrenophora tritici-repentis* was studied. Application of *C. globosom* or its culture filtrate resulted in the accumulation of extracellular proteins in host tissues, indicating activation of host defense systems. The intracellular washing fluid from leaves treated with *C. globosom* did not contain any inhibitory substances. The antagonistic activity of the endophytic BCA might be due to activation of host defense systems rather than direct antagonism (Istifadah and McGee 2006).

The mechanism of biocontrol activity of the avirulent isolate of *Colletotrichum fragariae* (M23) against the virulent isolate of *C. acutatum* (M11) causing anthracnose disease of strawberry was investigated. Treatment of strawberry cv. Pájaro with the culture filtrate (CF) of M23 at 3 days prior to pathogen inoculation significantly reduced disease severity and the development of symptoms was entirely inhibited,

when the plants were pretreated at 7 days before the challenge inoculation with M11. Similar enhancement of disease suppression was achieved, when a single leaf was sprayed with CF, suggesting the development of systemic resistance in strawberry against anthracnose pathogen, since no direct inhibitory effect of the CF on the pathogen growth was observed. In addition, accumulation of reactive oxygen species (ROS) and deposition of lignin and callose considered to be associated to plant defense were also recorded, following treatment of strawberry plants with CF of M23. Induction of resistance in other strawberry cultivars by the CF suggested that the response to CF treatment was not cultivar-specific. The results suggested that treatment with CF of the avirulent strain was able to induce resistance, because of the production of defense-eliciting molecules by this BCA strain (Chalfoun et al. 2011).

Many *Phytophthora* spp. secrete elicitins which may enhance defense reactions against microbial plant pathogens. The effects on the elicitins cryptogein and capsicein on cork oak root infection by *Phytophthora cinnamomi* were investigated by determining cytological and physiological changes in treated plants in comparison to untreated controls. The development of the pathogen in root tissue and its effects on total fatty acid (TFA) composition of roots and leaves were analyzed in seedlings. In elicitin treated roots, 2 days after inoculation, *P. cinnamomi* showed loss of viability and membrane degradation that were restricted to the inter cellular spaces of the cortical parenchyma and did not reach the vascular cylinder. Electron dense materials accumulated in the intercellular spaces of the cortex adjacent to the disorganized hyphae, suggested to be related with defense reactions. Cryptogein induced higher levels of lipid synthesis in leaves which might facilitate preservation of membrane stability of host plant cells. The results indicated a resistance response of cork oak against *P. cinnamomi,* following treatment with elicitins produced by *Phytophthora* spp. (Medeira et al. 2012).

Production of mutants through UV mutagenesis has been demonstrated in microorganisms and plants. Generation of nonpathogenic mutants from the wild-type plant pathogens has been attempted to identify nonpathogenic mutants with biocontrol activity against the pathogen. The nonpathogenic mutant strain (path-1) of *Colletotrichum magna* retained the wild-type phenotype, colonized cucurbit and other hosts and protected plants against the wild-type *C. magna* and *Fusarium oxysporum* f.sp. *niveum* in watermelon by priming the host defense response (Prusky et al. 1994). Two nonpathogenic mutants (4/4 and 15/15) were obtained from the cucurbit wilt pathogen *Fusarium oxysporum* f.sp. *melonis* (FOM) (race 1, 2) by a continuous dip-inoculation technique following UV mutagenesis. The strain 4/4 did not induce any visible symptoms or deleterious effect on muskmelon, while strain 15/15 caused mortality of susceptible cultivars to a lesser extent, compared to the wild-type strain. The strain 4/4 could colonize 100 % of the roots and 30–70 % of the lower stem tissues in 7 days after inoculation of seedlings. Significant reduction in seedlings mortality was observed in seedlings treated with 4/4 strain followed by challenge inoculation with FOM. The nonpathogenic strain lacking only pathogenicity may be expected to compete more efficiently with the pathogen, when

compared with other BCAs that may require different set of conditions for their survival and development in the soil environments (Freeman et al. 2002).

The fungal entomopathogens, *Beauveria bassiana* and *Lecanicillium* spp. are able to suppress soilborne fungal pathogens such as *Pythium* spp., *Rhizoctonia solani* and *Fusarium* spp., as well as airborne powdery mildew pathogens. *B. bassiana* can endophytically colonize a wide array of monocot and dicot plant species. It produces an array of bioactive metabolites that have been shown to arrest the growth of fungal pathogens in vitro. This BCA induced systemic resistance, when endophytically colonized cotton seedlings were challenged with the bacterial blight pathogen *Xanthomonas campestris* pv. *malvacearum. Lecanicillium* spp. could colonize plant roots also and induce systemic resistance against powdery mildew pathogens. Comparison of these entomogenous fungi with *Trichoderma harzianum* showed that some fungal traits which are of importance for insect pathogenicity are also involved in the biocontrol of microbial plant pathogens (Ownley et al. 2010).

Nonpathogenic isolates of *Fusarium oxysporum* (npFo) were evaluated for their ability to induce systemic resistance (ISR) and defense responses against *F. oxysporum* f.sp. *asparagi* (*Foa*) infecting asparagus. In the split-root experiments, roots inoculated with npFo exhibited a hypersensitive response and those subsequently inoculated with *Foa* exhibited resistance. Development of ISR in npFo-treated plants resulted in significant reduction in the number of necrotic lesions and reduced wilt disease severity, compared with untreated control plants. In hyphal-sandwich root inoculation experiments, activities of POX and PAL and lignin content were higher in npFo-treated plants and increased more rapidly than in npFo-untreated plants after *Foa* inoculation. Presence of antifungal compounds in the exudates of roots inoculated with *Foa* was observed for npFo-treated plants, but not for npFo-untreated plants. The results indicated that the isolates of npFo may function as inducers of systemic acquired resistance (SAR) and defense responses against *Foa* invasion in asparagus (He et al. 2002). The soilborne nonpathogenic *Fusarium oxysporum* strain Fo47 has been shown to be an effective biocontrol agent. A nonpathogenic mutant generated from *F. oxysporum* f. sp. *melonis* (*Fom*) (rev 157) did not protect muskmelon plant against infection by the pathogenic strain *Fom24* and it was also unable to protect the nonhost flax (linseed) plant against the flax wilt pathogen *F. oxysporum* f.sp. *lini* (*Fol*). In contrast, the parental strain *Fom24* of the mutant rev157 could protect flax plants against *Fol.* The results suggested that mutation in rev157 did not alter the capacity of the mutant in its mycelial growth and penetration into the roots and possibly affected the traits responsible for interaction within the plants. Studies on the comparative molecular genetics of the pair of strains *Fom24*/rev157 may be useful to identify genes involved in the biocontrol potential of *F. oxysporum* (L'Haridon et al. 2007).

Nonpathogenic isolate *Fusarium oxysporum* (Fo47) has been demonstrated to reduce the severity of symptoms induced by *Verticillium dahliae* (Verticillium wilt) and *Phytophthora capsici* (Phytophthora blight) in pepper plants. The isolate Fo47 did not protect pepper plants against infection by *Botrytis cinerea* on leaves treated with the BCA. *V. dahliae* colonies were inhibited by Fo47, whereas the growth of *P. capsici* was not affected in the presence of Fo47. It was considered that at least

part of the protective effect observed against *V. dahliae* was due to antagonism or competition for nutrition. In order to determine the role of induction of resistance as a mechanism of biocontrol activity of Fo47, three defense genes previously related to pepper resistance were monitored over time. These genes encoded a basic pathogenesis-related (PR)-1 protein (*CABPR1*), a class II chitinase (CACHI2) and a sesquiterpene cyclase (CASC1) involved in the synthesis of capsidol, a phytoalexin. These three genes were transiently up-regulated in the roots by Fo47 in the absence of inoculation with the pathogen, but in the stem only *CABPR1* was up-regulated. In the plants inoculated with *V. dahliae* prior to the treatment with Fo47, three genes had a higher relative expression level than the control in both roots and stem of pepper plants, indicating the involvement of induction of resistance as another mechanism of the biocontrol activity of Fo47 in treated pepper plants (Veloso and Díaz 2012).

The yeast BCA *Pichia guilliermondii* acts through multiple mechanisms on fungal pathogens like *Botrytis cinerea*. Application of *P. guilliermondii* to the root zone resulted in some adverse effect on *B. cinerea* on the foliage, implying the activation of defense mechanisms of plants. Disease suppression achieved by this mechanism was moderate (3.9). *P. guilliermondii* could also suppress pathogen development by reducing conidial germination and penetrating ability of *B. cinerea* (Guetsky et al. 2002). The endophyte *Piriformospora indica* was able to colonize roots of wide range of plant species and increase their biomass. The efficacy of *P. indica* in colonizing roots of winter wheat and protecting the cultivars against pathogens infecting roots, stem base and leaves was assessed. In greenhouse experiments symptom severity due to powdery mildew (*Blumeria graminis* f.sp. *tritici*) stem base disease (*Pseudocercosporella herpotrichoides*) and root infection (*Fusarium culmorum*) was significantly reduced. As the powdery mildew pathogen infecting wheat leaves was spatially well separated, root colonization by the endophyte might have induced systemic resistance, resulting in reduction in disease severity. Increase in concentration of hydrogen peroxide, after infection by *B. graminis* was also detected in *P. indica* colonized plants, providing evidence for the possibility of induction of systemic resistance as one of the mechanisms of biocontrol activity of this BCA (Serfling et al. 2007).

The ability of inducing resistance response in tomato against the tomato bacterial spot pathogen *Xanthomonas vesicatoria* by a heterogenous chitosan suspension (MCp) from *Crinipellis perniciosa* mycelium was compared with the commercial resistance inducer acibenzolar-*S*-methyl (ASM) (Bion® 50WG). Four days after treatment with MCp and ASM, tomato plants were inoculated with a virulent strain of *X. vesicatoria*. MCp-treated plants exhibited significant responses reaching 87 % of ASM protection performance. Changes in pathogenesis-related enzymes, lignin deposition and synthesis of soluble phenolic compounds were determined. Operation of the phenomenon of induced resistance (IR) was revealed by the enhancement of peroxidase (POX), polyphenol oxidase (PPO) and chitianse activities at 1–72 h after spraying. Increase in lignin deposition in treated and inoculated plants was also observed. The results suggested that enhancement of POX and PPO activities, improvement of lignification and to a lesser extent increased activity of CHI might reflect induction of defense responses by the MCp treatment (Cavalcanti et al. 2007).

The potential of *Penicillium citrinum* BTF08 was assessed for its biocontrol efficacy in suppressing the development of Fusarium wilt disease of banana caused by *F. oxysporum* f.sp. *cubense* race 4 (FocR4). The possibility of inducing resistance in banana plants by *P. citrinum* against FocR4 was examined using the biochemical markers as the basis. Changes in peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) were determined at different stages of host plant-pathogen interaction. Colonization of *P. citrinum* resulted in enhancement of significant levels of PO and PPO in plants penetrated with *P. citrinum,* compared with plantlets inoculated with pathogen only. The treatment with *P. citrinum* reduced the percentages of disease incidence and severity and delayed symptom progression. However, at the end of 28 days, all plants succumbed to Fusarium wilt with 80 % disease incidence and 42 % disease severity. The results showed that *P. citrinum* was not efficient in protecting the banana plants against Foc R4 causing Fusarium wilt disease (Ting et al. 2012).

Oligandrin, an elicitin-like protein isolated from the fungal mycoparasite *Pythium oligandrum* was evaluated along with crude glucans obtained from the cell walls of *P. oligandrum* and crab shell chitosan for their ability to induce resistance to tomato wilt pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) in tomato root tissues. These compounds were applied to decapitated tomato plants and induction of defense mechanisms in root tissues was monitored. A significant decrease in disease incidence was observed in oligandrin- and chitosan-treated plants, while glucans failed to induce resistance response. In oligandrin-treated tomato plants, restriction of fungal growth to the outer root tissues, decrease in pathogen viability and formation of aggregated deposits accumulating at the surface of invading pathogen hyphae were the striking features of the defense responses. The results established that oligandrin had the ability to induce systemic resistance in tomato and that exogenous foliar application of the fungal protein could sensitize susceptible tomato plants to react rapidly and efficiently to infection by FORL. Reduction in disease incidence might be primarily through enormous accumulation of fungitoxic compounds at sites of attempted pathogen penetration (Benhamou et al. 2001).

Pythium oligandrum induces resistance in host plants against fungal pathogens. Four elicitin like proteins (POD-1, POD-2, POS-1 and oligandrin) produced by the BCA were indentified as elicitor proteins. Two groups of *P. oligandrum* isolates were differentiated based on the nature of cell wall proteins (CWPs) as D-type containing POD-1 and POD-2 and the S-type isolate containing POS-1. The distribution of genes encoding these elicitin-like proteins among ten *P. oligandrum* isolates was analyzed using a genomic fosmid library of the D-type isolate MMR2. By employing Southern blot analyses, the isolates were divided into the same two groups, as those based on the CWPs. The D-type isolates contained pod-1, pod-2 and two oligandrin genes designated *oli-d1* and *oli-d2*, while S-type isolates had *pos-1* and one oligandrin gene *oli-s1*. These genes were single copies present only in *P. oligandrum*, but not in nine other *Pythium* spp. All the genes were expressed during colonization of tomato roots by *P. oligandrum,* as revealed by RT-PCR assays. The results lend support to the suggestion that these genes encode potential

elicitor proteins, resulting in the enhancement of resistance in plants against pathogens. The investigation on the genetic relationships between the D-type and S-type isolates of *P. oligandrum* suggested that the D-type isolates might be derived from S-type isolates by gene duplication and deletion events (Masunaka et al. 2010).

In an another investigation, the cell wall protein (CWP) fraction of *Pythium oligandrum* (Po) was sprayed on sugar beet leaves and the treated leaves were screened for induced expression of defense related genes and for resistance against *Cercospora* leaf spot. In a western blot analysis, the CWP was primarily retained on the surface of leaves without degradation for at least 48 h after application of CWP. In northern blot analyses, four defense-related genes (β-1,3-glucanase, acidic class III chitinase, 5-enol-pyruvylshikimate-phosphate synthase and oxalate oxidase-like germin) were expressed more rapidly in CWP-treated leaves, compared to control leaves. When CWP was applied to a suspension of cultured cells of sugar beet, an oxidative burst was observed, but not in control treatment. In growth chamber trials, the severity of disease was significantly reduced in the CWP-treated leaves, following inoculation with the leaf spot pathogen. CWP had no direct inhibitory activity against *C. beticola* in in vitro assays. The results suggested that CWP retained on the sugar beet leaves might induce expression of disease resistance genes, resulting in the suppression of disease development (Takenaka and Tamagake 2009). The ability of *Pythium oligandrum* to induce resistance against black scurf disease of potato caused by *Rhizoctonia solani* was determined using potato tuber disk assay. Treatment of tuber disks with the cell wall protein fraction of *P. oligandrum* enhanced the expression of defense-related genes such as 3-deoxy-d-arabino-heptulo-sonate-7-phosphate synthase, lipoxygenase and basic PR-6 genes and reduced severity upon challenge with *R. solani,* compared with untreated controls. The results suggested that the biocontrol mechanisms employed by *P. oligandrum* against *R. solani* might involve induction of disease resistance as well as mycoparasitism (Ikeda et al. 2012).

Pythium oligandrum suppressed the development of tomato bacterial wilt disease caused by *Ralstonia solanacearum*. The rhizosphere competence of *P. oligandrum* remained doubtful because of the conflicting reports. Hence, the colonization of *P. oligandrum* in tomato rhizosphere was analysed by employing .real-time PCR assay and confocal laser-scanning microscopy. The real-time PCR could specifically quantify the BCA in the rhizosphere over a range of 0.1 pg to 1.0 ng of *P. oligandrum* DNA from 25 mg dry weight of soil. Confocal microscopic visualization also showed that hyphal development was frequent on the root surface and some hyphae penetrated into root epidermis. The results indicated that *P. oligandrum* did not seem to actively spread its propagules along roots and it did not protect the roots over the longer term from root-infecting pathogens with direct competition for infection sites and nutrients. Microscopic observations revealed that the colonization frequency of the 5×10^4 oospore treatment was only 15 %, indicating that 85 % of the visual fields on tomato root surface were free of the BCA. The ethylene-and jasmonic acid (JA) – dependent signaling pathways were significantly accelerated in tomato treated with the mycelial homogenate of *P. oligandrum*. Further, induction of defense-related genes including PR-protein P14 and class II

chitinase genes was observed in tomato roots treated with a cell wall protein fraction of *P. oligandrum* containing two elicitor proteins. The results suggested that the principal mechanism of biocontrol activity of *P. oligandrum* against *R. solanacearum* could be through induced resistance (Takahashi et al. 2006; Takenaka et al. 2008).

In order to understand the primary biocontrol mechanisms of tomato bacterial wilt disease by *Pythium oligandrum* (*Po*), tomato plants were pretreated with sterile water or preinoculated with *P. oligandrum* followed by challenge inoculation with the pathogen *Ralstonia solanacearum* (*Rs*). The interactions between *Po* and *Rs* were observed in tomato tissues using a confocal laser scanning microscope and fluorescence labeling until 14 days after inoculation with *Rs*. Horizontal and vertical movement of the bacterial pathogen was frequently visualized in the xylem vessels of roots and stems of tomato plants in untreated control plants. In contrast, in plants pretreated with *Po,* the movement of *Rs* was suppressed and the bacteria appeared to be restricted to the pit vessels, a reaction similar to that observed in resistant root stocks. *Po* colonized mainly the surfaces of taproots and lateral roots and the middle sections of the lateral roots. In addition, *Po* was present near the wound sites or root tips where the bacterial pathogens attempted to colonize. However, repression of colonization was seen at these sites in *Po*-treated plants. The results suggested that the induction of plant defense reactions might be the principal mechanism of biocontrol activity of *P. oligandrum* against the tomato bacterial wilt pathogen *R. solanacearum* (Masunaka et al. 2009).

Canola blackleg disease is a complex caused by at least two fungal species viz., *Leptosphaeria maculans* with highly virulent pathogenicity groups 2, 3 and 4 (PG2, 3 or 4) and *L. biglobosa* with weakly virulent or avirulent pathogenicity group 1 (PG-1). When *L. biglobosa* (PG-1) was either pre-or coinoculated at 0, 12, 24 and 48 h with virulent isolates of *L. maculans* (PG-2, PG-3 and PG-4), the percent lesion/percent leaf area (PLLA) on cotyledons of two canola cultivars Westar and Invigor 2153 were smaller. On six-leaf stage plants of Westar, the PLLA declined significantly, compared with control plants, when the lower leaves were treated with either PG-1 or salicylic acid, and then challenged with the virulent PG-2 isolate 24 h later. The activities of defense-related enzymes chitinase, β-1,3-glucanse, peroxidase and phenylalanine ammonia lyase were enhanced at 48 and 72 h, when cotyledons of Westar were inoculated first with PG-1 followed by PG-2, 24 h later, compared with activities of these enzymes in water treated control. The results showed that application of pycnidiospores of PG-1 prior to the natural infection by PG-2 might effectively induce resistance and significantly decrease infection by severe strains of *L. maculans* (Chen and Fernando 2006). The mechanism of biocontrol activity of *Talaromyces wortmannii* FS2 against *Colletotrichum higginsianum* causing anthracnose disease of *Brassica campestris* var. *perverdis* was studied. The BCA emitted several terpenoid-like volatiles including β-caryophyllene. Growth of seedlings and their resistance to the disease were significantly enhanced by β-caryophyllene. The results indicated the dual benefit of employing the plant growth-promoting fungus (PGPF), *T. wortmannii* as the BCA (Yamagiwa et al. 2011).

Bioprotection in mycorrhizal plants against soilborne fungal pathogens may be due to preactivation of defense responses which include structural modifications and accumulation of PR-proteins. The arbuscular mycorrhizal fungi (AMF) have been shown to reduce the adverse effects of Verticillium wilt disease of pepper caused by *V. dahliae.* Colonization of pepper roots by *Glomus deserticola* induced the appearance of new isoforms of acidic chitinases, superoxide dismutase (SOD) and peroxidases at early stages. *V. dahliae* did not stimulate either polyphenylpropanoid pathway or elicit hydrolytic activities in infected pepper roots. However, in mycorrhizal plants, challenge inoculation with *V. dahliae* enhanced both PAL and PO activities after 2 weeks. The results indicated that appearance of new isoforms of acidic chitinases and induction of SOD along with enhanced activities of PO and PAL might have significant role in the biocontrol activity of *G. deserticola* in restricting the development of *V. dahliae* in pepper plants. (Garmendia et al. 2006). The efficiency of combination of AMFs *Gigaspora margarita* and *Acaulospora tuberculata* and *Trichoderma asperellum* PR11 in inducing resistance in cocoa against black pod disease caused by *Phytophthora megakarya* was assessed. Plant growth parameters were increased in plants following inoculation with AMF or BCA alone. Dual inoculation of cocoa seedlings with PR11 and AMF did not always positively benefit the plants. Leaf inoculation showed variation among treatments with the lowest disease index (highest level of resistance) recorded in plants inoculated with either AMF or *T. asperellum* only. Synthesis of high concentration of amino acids and phenolic compounds were implicated in disease resistance (Tchameni et al. 2011).

Inducing resistance to postharvest diseases using biotic agents capable of eliciting resistance responses in fruits and vegetables holds promise as a new technology and as an alternative to the use of synthetic fungicides. Several species of yeasts have been demonstrated to be effective, since they can grow rapidly and colonize wound sites present on the fruit/vegetable surface where infections are most likely to occur and out-compete postharvest pathogens for space and nutrients. In addition, some of them may induce resistance in host tissues resulting in significant reduction in decay development. Management of diseases caused by fungal diseases by employing fungal biocontrol agents has been shown to be highly effective, feasible and particularly suitable for postharvest diseases (Narayanasamy 2006).

Antagonistic yeasts are capable of inducing resistant responses as in the case of *Pichia guilliermondii*, as evidenced by the increased production of defense-related enzymes and antimicrobial compounds (Wisiniewski and Wison 1992). *Aureobasidium pullulans,* another yeast antagonist, could reduce the decay in apples due to *Botrytis cinerea* and *Penicillium expansum* causing gray and blue mold diseases respectively. The enhanced resistance of treated apples was associated with transient increase in β-1,3-glucanase, chitinase and peroxidase activities, commencing from 24 h after treatment and reaching the maximum levels at 48–96 h after treatment (Ippolito et al. 2000). Enhancement of natural resistance in strawberry to *B. cinerea*, following treatment with *A. pullulans* was also reported by Adikaram et al. (2002). Phytoalexins produced in plant tissues in response to pathogenic infection are considered to have a role in the development of resistance to diseases. Reduction

of green mold assay (*Penicillium digitatum*) in orange due to application of yeast species *Candida famatum* was attributed to the enormous increase (12 folds) in the phytoalexins scoparone and scopoletin in the wounds at 4 days after inoculation. But the production of phytoalexins occurred at slow rate indicating that the phytoalexin production might not have a significant role in the development of resistance to green mold disease. However, the rapid colonization of fruit surface and partial lysis of the hyphae of *P. digitatum* by *C. famatum,* in addition to induction of phytoalexin production might indicate that the multiple mechanisms of biocontrol activity of the BCA may exert additive effect for successful control of green mold disease in orange fruits (Arras 1996). Mutants of *Colletotrichum gloeosporioides* with reduced pathogenicity to avocado were generated by insertional mutagenesis by restriction enzyme mediated integration (REMI) transformation. The mutant Cg-M-142 caused reduced symptom on avocado fruit pericarp and mesocarp. Preinoculation of avocado fruit with the mutant delayed symptom development by the wild-type isolate Cg-14. Induced resistance was accompanied by an increase in the levels of preformed antifungal diene from 760 to 1,200 μg/g fresh weight at 9 days after inoculation. The results indicated that the mutant with reduced-pathogenicity had the potential for use against the anthracnose disease of avocado (Yakoby et al. 2001).

Candida oleophila, another yeast species, active component of the commercial product, Aspire, induced systemic resistance in grapefruit to *Penicillium digitatum* causing green mold disease. Application of *C. oleophila* to surface wounds or intact 'Marsh Seedless' grape fruit elicited systemic resistance against *P. digitatum.* Induction of pathogen resistance in fruit was already at high levels at 24 h after elicitation. Resistance responses depended on distance, concentration and time of application and it was restricted to the peel tissue closely surrounding the yeast application site. Induction of resistance required the presence of live cells of *C. oleophila,* whereas nonviable autoclaved or boiled yeast cells or lower concentrations ($<10^8$–10^9 cells) were ineffective in enhancing disease resistance. Application of the BCA cell suspensions to grapefruit peel tissue increased ethylene biosynthesis, phenylalanine ammonia lyase (PAL) activity and phytoalexin accumulation. Increased chitinase and β-1,3-endoglucanase protein levels were detected by western immunoblotting analysis. The results suggested that induced resistance against postharvest decay of citrus fruit might be considered as an important component of the multiple modes of action of *C. oleophila.* Scanning electron microscopic (SEM) observations revealed inhibition of spore germination and germ tube elongation to a great extent on wounds made near the yeast-treated sites (Droby et al. 2002).

In studies conducted earlier, with harvested commodities, induction of resistance by microbial antagonists has been inferred, but not clearly established, because other putative modes of action of the antagonist could not be ruled out. Later, the ability of *Candida saitoana* to induce systemic resistance in apple fruit against *Botrytis cinerea* was investigated by inoculating the pathogen and the antagonist in spatially separated wounds. *C. saitoana* was effective in inducing disease resistance on fresh apples, but not on stored apples. In addition to inducing systemic resistance, *C. saitoana* increased chitinase and β-1,3-glucanase activities with higher

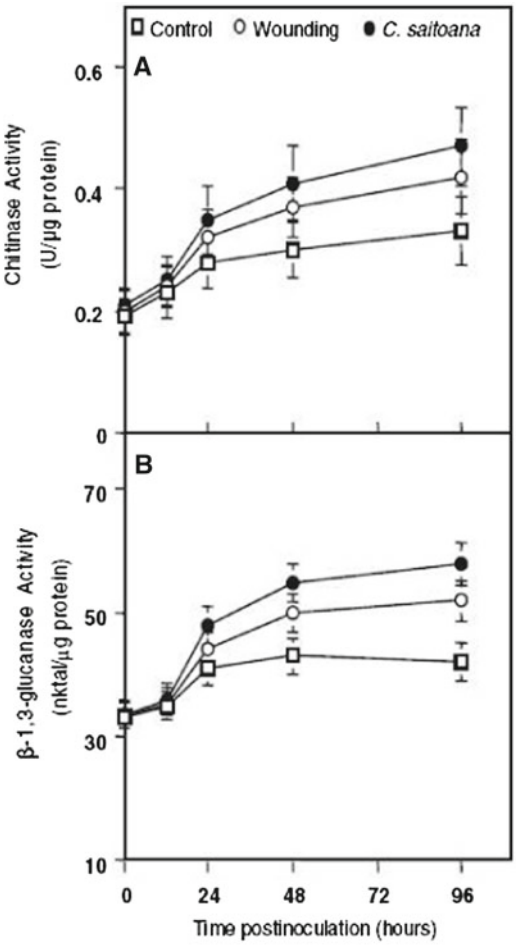

Fig. 3.8 Effect of treatment of wounds of stored apples with *Candida saitoana* on the activities of chitinase and ß-1,3-glucanase at different intervals Treatment with *C. saitoana* (●), sterile water (○) and non-wounded control fruit (□) (Courtesy of El-Ghaouth et al. 2003 and with kind permission of The American Phytopathological Society, MN, USA)

accumulation in fresh than in stored apples. In fresh apples, the onset of systemic resistance to *B. cinerea* coincided with the increase in chitinase and β-1,3-glucanase activity in systemically protected tissue (Fig. 3.8). The results indicated that the fruit mediated-resistance induced by *C. saitoana* can be exploited for effective management of postharvest diseases (El-Ghaouth et al. 2003). The effect of combined treatment of the yeast *Cryptococcus laurentii* and plant growth regulator indole acetic acid (IAA) on the suppression of gray mold disease of harvested fruit caused by *Botrytis cinerea* was assessed. Gray mold incidence in the combined treatment was reduced to 50 % of the disease incidence recorded in apple wounds treated with *C. laurentii* alone. Although IAA had no direct anti-fungal effect on the pathogen, application of IAA strongly reduced gray mold infection, when IAA was applied 24 h prior to inoculation with *B. cinerea* in apple fruit wounds. The activities of defense-related enzymes such as catalase, peroxidase and superoxide dismutase were stimulated to a greater level by the application of *C. laurentii* and IAA in the apple wounds than

by treatment with the yeast alone. The results indicated the advantage of combining the yeast BCA and IAA to achieve more effective disease control, because of the integration of the dual biological activity of the biotic and abiotic agents (Yu et al. 2008).

Verticillium lecanii has been found to be effective against *Penicillium digitatum* causing green mold disease in citrus. The interaction between the BCA and the pathogen was studied by applying cytochemical methods to determine the changes in the exocarp tissues of citrus fruits treated with *V. lecanii*. Accumulation of callose and lignin-like compound was seen at sites of colonization by the pathogen and this resulted in the restriction of decay development in the treated fruits compared with untreated control fruits. The rate and extent of colonization, of citrus fruits by *P. digitum,* in addition to cell viability, were significantly reduced by treatment with *V. lecanii*. Further, *V. lecanii* and chitosan, a known inducer of disease resistance, elicited similar transcriptional activation of defense genes in treated citrus fruits, leading to the accumulation of structural and biochemical compounds at strategic sites (Benhamou 2004). Peach fruits are infected by *Monilinia fructicola* causing brown rot disease and *Penicillium expansum* causing blue mold disease. Application of *Cryptococcus laurentii* either alone or in combination with methyl jasmoante (MeJA) significantly reduced the severity of the diseases. In addition, the treatments induced a higher level of activities of defense-related enzymes, chitinase, β-1,3-glucanase, phenylalanine ammonia lyase (PAL) and peroxidase, resulting in enhancement of resistance in treated peaches to these diseases (Yao and Tian 2005). Induction of chitinase and β-1,3-glucanase activity in the fruits treated with biocontrol agents has been reported in many pathosystem. However, little information is available to indicate whether β-1,3-glucanase activity present in fruit tissues actually exhibits sufficient antifungal activity or accumulates to a level high enough to block fungal pathogen infection. Two β-1,3-glucanase genes were cloned from jujube (*Ziziphus jujube*) fruit and designated *Glu-1* and *Glu-2*. A semi-quantitative RT-PCR assay was employed to monitor the expression of these genes in jujube fruits in response to wounding and inoculation with *Cryptococcus laurentii*. Both treatments stimulated an increase in β-1,3-glucanase (EC 3.2.1.39) activity in jujube fruit. *Glu-1* was induced highly by wounding and *C. laurentii*, whereas *Glu-2* was broadly not responsive to the BCA. The expression of *Glu-1* was substantially enhanced with increased concentration of *C. laurentii*, suggesting that *Glu-1* might play a role in defense responses to fungal pathogens. A significant decrease in disease incidence and lesion diameter appeared to provide evidence that changes in β-1,3-glucanase activity are possibly related to expression of the genes encoding the enzyme (Tian et al. 2007).

The ability of biocontrol agents *Metschnikowia fructicola* (strain 277) and *Candida oleophila* (strain 182) to induce resistance in apple and citrus was assessed. The two yeast biocontrol agents were able to induce defense-related oxidative responses in apple fruits, as shown by their capacity to generate greater levels of superoxide anion on intact fruit surfaces (poor in nutrients) than those applied on a nutrient-poor agar medium. Although yeast antagonists liberated a high level of O_2 on nutrient-rich media, when applied on fruits around wounds (areas abound in

nutrients) accumulation of super oxide anion as detected by nitroblue tetrazolium staining, occurred much more rapidly on the latter. Using laser scanning confocal microscopy, it was observed that application of *M. fructicola* and *C. oleophila* into citrus and apple fruit wounds correlated with an increase in H_2O_2 accumulation in host tissue. In citrus fruit, the level of H_2O_2 around inoculated wounds increased by 4-fold, compared to controls as early as 18 h after inoculation. Similar increase in H_2O_2 accumulation around yeast-inoculated wounds was observed in apple fruit exocarp. The results indicated that the yeast-induced oxidative response in fruit exocarp might be associated with the ability of specific yeast species to function as effective biocontrol agents of postharvest diseases (Macarisin et al. 2010). In order to have an insight into the mechanism of action of the yeast BCA *Cryptococcus laurentii,* a forward subtractive suppression hybridization (SSH) cDNA library was constructed. The SSH was carried out with cDNA from cherry tomato fruit (*Lycopersicon esculentum*) inoculated with water as the "driver" and cDNA from tomato fruit inoculated with the BCA as the "tester". By sequencing a total of 150 clones in the SSH library, 50 unigenes were identified. Of these genes, 35 cDNAs showed significant sequence homologies with known sequences in the NCB1 database. The identified cDNAs encoded proteins involved in the cellular process such as the primary metabolism, signal transduction and defense responses to pathogens. Several transcripts encoding proteins/enzymes known to be up-regulated under some biotic and abiotic stresses were also up-regulated, following application of the BCA to cherry tomato fruit. It is possible that these proteins encoded by the transcripts may have a role in enhancing resistance of fruits to infection by pathogens during storage (Jiang et al. 2009).

Effects of treatment of sweet cherry fruit with *Pichia membranifaciens* (5×10^7 cells/ml) or salicylic acid (SA) (0.5 mM) on activities of enzymes considered to have a role in the development of resistance to the postharvest blue mold disease caused by *Penicillium expansum* were investigated. Immersion of fruits for 10 min in BCA cell suspension or SA solution reduced the incidence of decay as well as the size of the lesion caused by *P. expansum.* In the absence of the pathogen, yeast-treated fruit showed increased peroxidase (PO) activity and decrease in the activities of catalase (CAT) and superoxide dismutase (SOD). In fruits inoculated with the pathogen, CAT activity and SOD activity increased due to treatment with yeast or SA. Activity of PO did not show any variation due to treatment. However, treatments with yeast and SA changed the expression of PO isozymes. Furthermore, treatment with the BCA and SA increased total protein content of the sweet cherry fruit and up-regulated 33- and 47-kDa protein bands as revealed by SDS-PAGE analysis. The results indicated that treatment with the BCA or SA induced synthesis of anti-oxidant enzymes and specific proteins which might be involved in inducing resistance in sweet cherry fruit against the blue mold pathogen *P. expansum* (Chan and Tian 2006). The biocontrol potential of *Pichia guilliermondii* to suppress the development of *Rhizopus nigricans* infecting tomatoes during storage and the mode of its action of biocontrol activity were studied. The autoclaved BCA culture or the culture filtrate did not exert any adverse effect on disease development, indicating that the yeast did not produce any metabolites inhibitory to the pathogen.

However, treatment of tomatoes prior to inoculation with *R. nigricans* showed greater biocontrol efficacy. The yeast rapidly colonized the wound sites during the initial 3 days at 20 °C and the population stabilized during the next 4 days, suggesting the possible competition for nutrients and space on the wounds. In addition, the tomatoes inoculated with the yeast showed changes in peroxidase (PO), polyphenoloxidase (PPO), superoxide dismutase (SOD), catalase (CAT), phenylalanine ammonia lyase (PAL), chitinase (CHI) and β-1,3-glucanase activities which had a bearing on the development of induced resistance. It is likely that *P. guilliermondii* might activate defense mechanisms operating in the tomatoes, leading to higher level of resistance to the postharvest pathogens (Zhao et al. 2008).

Molecular mechanisms of biocontrol activity of the epiphytic yeast *Pichia guilliermondii* in citrus fruits against *Penicillium digitatum* were studied. Assays of antagonistic activity of the BCA in vitro indicated a strong inhibitory effect on pathogen growth and spore germination. Antagonist gene expression was determined in induced condition as well as direct interaction using differentially expressed sequence tags (ESTs) obtained by suppression subtractive hybridization (SSH) and differential display procedures. Three different specific metabolic conditions viz., starvation by carbon source competence, sensing of extracellular metabolites produced by active mycelium of *P. digitatum* (membrane system) and induction by fungal cell walls were created to determine the genetic responses of the BCA. The assessment revealed just one EST associated, as expected to energy metabolism in starvation conditions; seven ESTs for the membrane system were identified by SSH technique and all related with some of the metabolic processes such as energy, nitrogen, cell cycle, ABC transporters, response to stress and one unknown sequence. The induced system involving fungal cell walls produced the highest number of ESTs, with a total of 22, including all the metabolic networks mentioned above for the membrane system along with ESTs associated with signal transduction. The results revealed the functioning of multiple mechanism operating in *P. guilliermondii* leading to the effective suppression of the development of the postharvest pathogen of citrus (Larralde-Corona et al. 2011).

Biocontrol potential of *Pichia guilliermondii* strain M8 against the gray mold pathogens *Botrytis cinerea* was assessed under storage conditions. The strain M8 reduced gray mold infection of apples to 20.0 % as against 45.3 % in untreated control fruits. In apple juice medium (AJM) and in wound-inoculated apples, M8 strain inhibited spore germination of *B. cinerea* and the gray mold development. When the pathogen and the yeast were coincubated in apple wounds with addition of the nutrients, the inhibition of the rots was significantly reduced by the supplemented nutrients. Observations under light microscope showed the yeast cells firmly adhering to the hyphae and conidia of *B. cinerea*. The BCA strain produced hydrolytic enzymes including β-1,3-glucanase and chitinases in minimal salt media with different carbon sources. *Pichia guilliermondii* strain M8 was highly efficient in suppressing the development of gray mold disease caused by *Botrytis cinerea* infecting apples under semi-commercial conditions. The strain M8 produced high quantities of active exo-1,3-β-glucanase in Lilly-Barnett minimal salt medium with different carbon sources. This enzyme inhibited strongly in vitro and in vivo assays.

Hence, an exo-1,3-glucanase gene, *PgExg1* was cloned in the genomic DNA of strain M8 by genome walking. An open reading frame (ORF) of 1,224-bp encoding a 408-amino acid (aa) protein (MW 46.9-kDa) and an iso-electric point (pI) of 4.5 was characterized. With an optimal pH of 5.0 and temperature of 40 °C, the recombinant protein showed the highest activity towards laminarin and it was highly stable, when stored at a pH 7.0 and temperature of 4 °C. Pretreatment of apples with M8 cells (10^8/ml) followed by washing, reduced the infection by *B. cinerea* significantly, suggesting the possibility of induction of defense responses, as one of the mechanisms of biocontrol activity of *P. guilliermondii* against *B. cinerea* (Zhang et al. 2011a, b).

Pichia guilliermondii strain R13 was evaluated for its ability to induce resistance in harvested chilli against the fruit rot pathogen *Colletotrichum capsici*, in addition to its other modes of mechanism of biocontrol activity. The pretreatment of chilli (pepper) with the strain R13, physically separated from the pathogen by predetermined distances, significantly reduced the disease incidence and lesion diameter caused by *C. capsici*. The activities of phenylalanine ammonia lyase (PAL), chitinase and β-1,3-glucanase were enhanced in the yeast-treated chilli fruits, in addition to accumulation of capsidol phytoalexin in chilli tissue. Abnormality in the morphology of spores and hyphae and restriction of hyphal growth were revealed by observations under scanning electron microscope (SEM), following treatment of pathogen conidia with the BCA. The results provided evidence to indicate that the yeast strain R13 might induce resistance to the fruit rot disease of chilli, in addition to other modes of action such as nutrient competition and hydrolytic enzyme secretion (Nantawanit et al. 2010).

The constituents or secretory compounds of fungi have been shown to induce systemic acquired resistance (SAR) in plants against microbial plant pathogens. A protein elicitor PeaT1 from the mycelium of *Alternaria tenuissima* was purified by column chromatography. PeaT1 was an acidic protein and heat-stable. It induced SAR in tobacco to *Tobacco mosaic virus* (TMV), but it did not induce hypersensitive response (HR). The gene encoding the protein elicitor was cloned and sequenced. Sequence analysis showed that the cDNA had 624-bp and the open reading frame (ORF) encoded for a polypeptide of 207 amino acids. The recombinant elicitor obtained from the transformed *Escherichia coli* BL21 (DE3) could also trigger defense responses in intact tobacco plants. The possibility of obtaining pure elicitor protein opens up the avenue for developing an effective strategy for the management of a universal virus disease (Mao et al. 2010).

The influence of arbuscular mycorrhizal (AM) symbiosis on the health of linseed (*Linum usitatissimum*) infected by the wilt pathogen *Fusarium oxysporum* f.sp. *lini* and powdery mildew pathogen *Oidium lini* was investigated. Level of resistance to wilt disease was increased in AM plants. But the extent of resistance enhancement depended on linseed cultivars which, however, exhibited the same level of root colonization by AM fungi. On the other hand, the susceptibility of AM plants to powdery mildew was at higher level compared with non-mycorrhizal plants in terms of shoot fresh weight, CO_2 assimilation and sucrose content on the shoot apex. The results indicated that AMF could activate resistance mechanisms in symbiotic plants

against certain fungal pathogens and also enhance tolerance to other pathogens infecting the same plant species. The AMF association resulted in increased concentrations of phytohormones content and composition of free sterols and respiratory activity, while degree of DNA methylation registered a reduction following AMF infection (Dugassa et al. 1996).

3.1.6 Natural Host Plant Resistance

Natural plant resistance to microbial plant pathogens varies widely due to interplay of several factors like environment, availability of nutrients required for robust growth and nature of microorganisms present in spermosphere, rhizosphere and phyllosphere. Nature and composition of communities of microorganisms may be significantly altered by the exudates from roots and leaf surfaces and other organs of plants. Most of the microorganisms may remain inactive in the soil, because of the environmental limitations which include temperature, water availability, aeration and available substrates for metabolism and growth. However, the rhizosphere region is not affected by nutrient limitation in general. Several simple sugars, amino acids and many other compounds are exuded by plant roots. These compounds differentially favour different kinds of microorganisms. When the pathogens find these compounds suitable for their prolification, chances for infection of the plant species/varieties producing such substances may increase, if the plants are susceptible to the pathogen(s) concerned. Root exudates exert definite influence on the biocontrol agents present in the rhizosphere region. Variations in the nature and concentrations of substances present in the susceptible and resistant cultivars have been observed in some pathosystems (Narayanasamy 2002).

The biocontrol agents *Trichoderma* spp. have been shown to act on the cotton preemergence damping-off disease pathogens, *Pythium ultimum* and *Rhizopus oryzae* through an uncommon mechanism. Cotton seeds during germination produce germination stimulants that induce germination of propagules of the pathogens. *Trichoderma virens* strains (G6, G6-5) or protoplast fusants obtained by protoplast fusion of cells of *T. virens*/*T. longibrachiatum* (Tvl-30, Tvl-35) were able to metabolize the germination stimulants, preventing the germination of pathogen propagules. Disease control could be achieved by the wild-type strains and genetically modified strains that were deficient for mycoparasitism, antibiotic production and induction of terpenoid synthesis in cotton roots. Cotton cultivars that did not exude germination stimulants through roots, were completely resistant to the damping-off disease. However, when the pathogen propagules were artificially induced to germinate, these cotton cultivars became susceptible to the disease. The results indicated that the mechanism of biocontrol activity of *Trichoderma* spp. on *Pythium ultimum* and *R. oryzae* was through metabolism of germination stimulants by the BCAs (Howell 2002).

3.1.7 Factors Influencing Activities of Biocontrol Agents

Activities of biocontrol agents (BCAs) are likely to be influenced by several factors including requirements of BCAs for growth and reproduction, their survival and perpetuation in the field conditions, nature of pathogen, requirements of host plants for high yields, soil conditions, microclimate of crop canopy, agricultural inputs and interactions with other rhizosphere and phyllosphere organisms. Of these factors, environmental conditions can influence all the three interacting components viz., host plant, pathogen and biocontrol agents. The expected target of having healthy plants capable of providing disease-free produce depends on the ability of host plant to overcome the ill-effects of the pathogen(s) with the active assistance of the biocontrol agents. The influence of environmental factors on biocontrol agents applied against soilborne diseases, foliage diseases and postharvest diseases caused by fungal pathogens are discussed below:

3.1.7.1 Soilborne Diseases

Disease suppression appears to be more due to soil support to biocontrol activity than to suppression of pathogen activity, as observed in the case of wheat take-all disease. *Trichoderma koningii,* isolated originally from a take-all suppressive soil in Western Australia, effectively controlled the disease in various field trials conducted in Australia, China and United States and it increased the yield as well (Duffy et al. 1997). However wide variations were observed in the levels of protection provided by *T. koningii* isolates in different fields in the same country. Negative correlation was observed between biocontrol activity and contents of iron, nitrogen, boron, copper, soluble magnesium and clay percentage. The results suggested that soil amendments or BCAs with beneficial effects suitable to the soil concerned have to be carefully selected (Duffy et al. 1997). Treatment of seeds of maize inbred line Mo17 with *T. harzianum* T22 had dramatic effects on root and shoot growth. The beneficial effects might be due to both control of deleterious soil micro flora and direct stimulation of plant growth by *T. harzianum* T22. Experiments conducted in over 500 fields on maize across the United States indicated that T22 applied as seed treatment provided a general grain yield increase averaging ~5 %. The experiments also revealed involvement of a strong genetic component in the response to *T. harzianum* T22 (Harman et al. 2004a, b, c).

Isolates of *Trichoderma* require exogenous nutrients for germination. Under nutrient poor conditions, germination percentage, rate of hyphal extension and sporulation are considerably reduced. Preactivated conidia with sufficient nutrients could inhibit spore germination and blossom infection by *Botrytis cinerea* to a greater extent than the quiescent conidia (Hjeljord et al. 2001). In a nutrient-rich medium almost all conidia of *T. atroviride* P1 conidia initiated germination processes and increased respiration. On nutrient-poor media P1 conidial germination was drastically reduced. When P1 conidia were nutrient-activated, oxygen consumption

by the inoculum and inhibition of *B. cinerea* were increased. Pre-germination respiration also affected competitive capacity of the antagonist on solid substrates, where respiratory CO_2 stimulated germination rate and initial colony growth. Conidia of *T. atroviride* became more sensitive to temperature at 23 °C and killed by desiccation in about 2 h. The results showed that nutrient-induced changes preceding conidial germination of P1 might either enhance or decrease the biocontrol potential, depending on the environmental conditions prevailing in the microhabitat (Hjeljord and Tronsmo 2003).

The effectiveness of biocontrol may be influenced by soil type, origin of both biological control agents and the pathogen and environmental conditions. Two isolates of *Trichoderma viride* and one isolate of *T. koningii* were evaluated for their biocontrol potential on the sclerotia of four isolates of *Sclerotium cepivorum,* causing *Allium* white rot disease. All three *Trichoderma* isolates degraded *S. cepivorum* in four soil types and against four isolates of *S. cepivorum* under controlled conditions. Sclerotial degradation did not significantly vary greatly between soils or pathogen isolates. However, the ability of the BCA isolates did show variation to reduce white rot in the seedling bioassays. When tested in different soils, none of the *Trichoderma* isolates was effective in all soil types. The results indicated that experiments should be conducted to study the effects of the BCA using different assay systems. Mechanisms other than sclerotial degradation might be involved in reducing white rot disease incidence. The relationship between the efficacy of *T. viride* isolates to degrade the sclerotia of *S. cepivorum* and soil water potentials was studied. Over 90 % of *S. cepivorum* sclerotia were degraded at high water potentials (>−0.022 MPa), when the soil was nearly saturated, even when the sclerotia were not treated with the BCA. Degradation of sclerotia by both isolates of *T. viride* increased with temperature from 5 to 25 °C and soil water potential of −0.022 MPa with most degradation occurring at 10 °C. The results indicated the importance of determining the effects of environment, pathogen and BCA characteristics for enhancing the effectiveness of biocontrol against crop diseases (Clarkson et al. 2004).

Trichoderma koningii Tr5 was reported to suppress the development of *Allium* white rot disease caused by *Sclerotium cepivorum* under field conditions. The reason for lack of complete control of white rot by *T. koningii* was investigated to understand the interaction between the BCA and pathogen. The relationship between inoculum density of *S. cepivorum* and the biocontrol efficacy of *T. koningii* in suppressing infection was studied. The biocontrol efficacy of the BCA remained relatively constant at 63.2–79 %, irrespective of the quantum of pathogen inoculum (10–100 sclerotia/kg of soil). Root colonization by *T. koningii* Tr5 averaged 97 % in pots amended at the lowest rate of *T. koningii* Tr5-colonized millet (1,590 kg/ha). The failure of *T. koningii* to offer greater level of protection (>75 % suppression) indicated that *T. koningii* was unable to challenge and successfully suppress approximately one in five *S. cepivorum* infections in roots. The results suggested that the biocontrol potential of a BCA is genetically controlled and it may not be possible to enhance effectiveness of protection beyond certain level, unless other approaches such as using mixture of strains or species of BCAs or manipulation of the genetic structure of the antagonist concerned, are also integrated (Metcalf

et al. 2004). Direct interactions in the rhizosphere between *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) and the non-pathogenic strain Fo47 of *F. oxysporum* were investigated. Carbon sources are the growth limiting factor for fungi in soil. Glucose at concentrations 50 folds higher than that estimated to be present in tomato root exudates could be more efficiently consumed by Fo47 than FORL (Couteaudier and Alabouvette 1990). In a later study, spore germination was determined in tomato root exudates and its major sugar (glucose) and organic acid (citric acid) at concentrations estimated to be present in tomato root exudates. A higher percentage of Fo47 spores germinated on these three components over a period of 7 days. In addition, the inoculum concentration of Fo47 in the tomato rhizosphere was 50 times greater than that of FORL. Consequently these two factors in combination may be expected to reduce the availability of nutrients for spore germination and subsequent growth of FORL, resulting in significant reduction in the number of hyphae of FORL reaching the root surface to attack and colonize tomato roots (Lugtenberg and Bloemberg 2004; Bolwerk et al. 2005).

A major constraint of biocontrol approach for root diseases is that a single application of a BCA may not provide effective protection during the entire season, particularly if the BCA is not rhizosphere competent. It is essential to maintain adequate BCA population level for successful disease management. Further, the timing of application and type of plant growth media (soil mix in greenhouse) and soil to which the BCA is applied. Application of binucleate *Rhizoctonia* (BNR) isolate after transplanting rooted poinsettias was effective, in suppressing stem rot disease caused by *R. solani*. But it was not effective when applied, during propagation stage. In contrast, *Burkholderia cepacia*, a bacterial BCA was effective, only if it was applied at propagation, but not after transplantation of poinsettia. Population dynamics of BNR isolates showed that colonization of poinsettia roots by the BCA was closely related to biocontrol activity and affected by application strategy (Hwang and Benson 2002). The biocontrol efficacy of different strains/isolates of a fungal species may vary, depending on the aggressiveness (virulence) of the fungal pathogen. Likewise, the level of susceptibility of cultivars may also influence the effectiveness of bioprotection offered by a BCA. The isolates of binucleate *Rhizoctonia* (BNR) differed in their efficacy against two anastomosis group (AG) AG-4 and AG2-2 of *Rhizoctonia solani* causing root rot/seedling disease complex of soybean. Three out of nine isolates BNR-4, BNR-8-2 and BNR-8-3 consistently reduced disease induced by both AGs of *R. solani* in soybean depending on the production area. Further, these three BNRs effectively protected all seven soybean cultivars against *R. solani*. In addition to the bioprotection, the BNR isolates significantly increased plant height compared with untreated control plants, enhancing the desirability of using the BNRs for the control of soybean root disease (Khan et al. 2005).

Rhizoctonia solani AG 2-2 is known to cause severe damage to sugar beet by inducing root rot and damping-off disease which occurs usually in patches. The patches of disease are highly mobile. It may be attributed generally to water movement and mainly to mechanical dispersal of inoculum during harvest and cultivation practices. Soil inoculum potential is the pathogenic energy present in the soil and is assessed by growing susceptible host plants in the soil under environmental

conditions favorable for disease expression. Mycoparasitism, as a natural means of control of *R. solani* is expressed by *Trichoderma* (Verma et al. 2007). Temporary out-competition of *R. solani* by indigenous microorganisms was suggested as a possible reason for the presence of disease patches caused by *R. solani* AG-2 in flower bulbs (Schneider et al. 2001). The relationship between biotic changes and local decrease in soil conduciveness in disease patches towards the disease incited by *R. solani* AG 2-2 in sugar beet field in France was investigated. Samples from healthy and diseased areas were analysed for fungal and bacterial densities, molecular and physiological microbial community structures and antagonistic potential of *Trichoderma* isolates collected from diseased and healthy areas. Although the inoculum potential was higher inside the disease patches, the respective soil was less conducive towards disease caused by *R. solani* AG 2-2. The results indicated that though the pathogen was present in healthy areas, it did not incite disease under field condition. In contrast, the response of the microflora to previous development of *R. solani* in diseased areas prevented further pathogenic activity. The genetic and physiological structures of fungal communities were modified, in disease patches compared to healthy areas (Anees et al. 2010).

The spectrum of activity of biocontrol agent (BCA) is important. The BCA that can establish on a range of host plant species, may be preferable to a BCA with restricted activity on a single plant species. At the same time the BCA should not induce any deleterious effect on any one host that is colonized by the BCA. Endophytes were isolated from the roots of eggplant, melon, barley and Chinese cabbage. Two isolates of *Phialocephala fortinii* and one isolate of a dark septate endophytic (DSE) fungus were effective against *Verticillium longisporum* causing yellows disease of Chinese cabbage. However, under certain conditions isolates of *P. fortinii* caused severe damage to Chinese cabbage plants (Narisawa et al. 2004). On the other hand, the endophytic fungus *Piriformospora indica* has been shown to colonize many vegetable and cereal crops and no detectable deleterious effect was noted on any of the plant species colonized by *P. indica* (Serfling et al. 2007). The endophyte *Piriformospora indica* is able to colonize the roots of a wide range of crop plants such as mustard, cabbage, spinach and cereals. It enhances the biomass of the host plant species apparently due to increased nutrient supply. *P. indica* seems to mediate particularly phosphorus and nitrogen uptake from the soil, in addition to the protection offered against soilborne fungal pathogens. The performance of *P. indica* was evaluated in different substrata under greenhouse and field conditions. Roots of winter wheat were colonized by *P. indica* efficiently and the plant biomass increased especially on poor substrata. Colonization of wheat occurred consistently over the years and conditions tested, even in the field. *P. indica* has been recommended as a putative biofertilizer and biocontrol agent, because of its ability to colonize and benefit a variety of unrelated plant species (Waller et al. 2005). *P. indica* has the potential to protect its host even better than arbuscular mycorrhizal fungi (AMF) that are known to be effective against many soilborne pathogens, but not against leaf pathogens (Serfling et al. 2007). Hence, caution has to be exercised, while selecting the isolates for wider application.

Population dynamics were investigated following the introduction of the BCA, *Pythium oligandrum* into the rhizosphere of tomato plants. The BCA colonized roots without damaging the host plant cells and survived in the rhizosphere where it exerted its biocontrol activity (Le Floch et al. 2005). Effective biocontrol by *P. oligandrum* may be limited by its heterogenous implantation and persistence in the rhizosphere. Bioprotection level may be increased by enhanced implantation and persistence of *P. oligandrum* in the rhizosphere. Strains of *P. oligandrum* with required characteristics viz., ability to produce oospores to allow root colonization, to favor persistence, to synthesize tryptamine (a plant growth enhancer) and to produce oligandrin (a plant-protective elicitor) were selected for application. Real-time PCR assay and plate counting demonstrated the persistence of large amounts of the antagonist in the rhizosphere throughout the cropping season. Careful selection of the isolate/strain of the BCA that can adapt to conditions existing in rhizosphere is a crucial factor for successful biocontrol of soilborne diseases (Vallance et al. 2009).

Phytophthora cinnamomi, infecting avocado, produces zoospores and chlamydospores as asexual spores and oospores as sexual spores. Chlamydospores and oospores are thick-walled spores that aid in pathogen survival. The biocontrol agent has to effectively attack these spore forms. Lysis of the mycelium results in an increase in the formation of zoospoarangia containing zoospores which are the most common infective propagule of *P. cinnamomi.* Thick-walled chlamydospores formed inside plant roots may require substantial enzymatic digestion to cause lysis of their spore walls. Continuous addition of cellulosic mulches in avocado groves may be required to maintain high substrate availability for the saprophytic antagonists and their enzyme systems may be stimulated for effective biocontrol activity (Downer et al. 2001). The biocontrol potential of two isolates of nonpathogenic BCA *Fusarium oxysporum* (CS-20 and CS-24) and one isolate of *F. solani* (CS-1) were assessed against tomato wilt disease at different temperatures, light soil-types, pathogen isolate and race and tomato cultivar. Liquid spore suspensions (10^6/ml) of the BCA isolates were applied to soilless potting mix at the time of sowing followed by transplanting of seedlings 2 weeks later. The isolate CS-20 significantly reduced wilt disease to a greater extent at all temperature regime (22–32 °C) tested, the reduction in disease incidence being 69–100 %, compared with controls and other isolates. The biocontrol efficacy of the isolates was not affected by light/shade, though the growth of plants was affected. Disease incidence was reduced by 56–79 % in all four different soil types with varying organic matter content, when isolate CS-20 was applied. The other isolates were less effective. The isolates CS-1 and CS-20 were equally effective in reducing disease incidence by 66–80 % due to pathogen races 1, 2 and 3 on eight cultivars of tomato (Fig. 3.9). Overall the isolate CS-20 could offer better protection to tomato cultivars with different levels of resistance under different environmental conditions to Fusarium wilt disease caused by *F. oxysporum* f.sp. *lycopersici* (Larkin and Fravel 2002).

Sclerotinia sclerotiorum survives in the soil as sclerotia which germinate myceliogenically infecting plants directly or carpogenically producing apothecia which release ascopsores. Glasshouse crops like lettuce are infected by ascospores carried by wind. The effects of different inocula of the mycoparasite *Coniothyrium minitans*

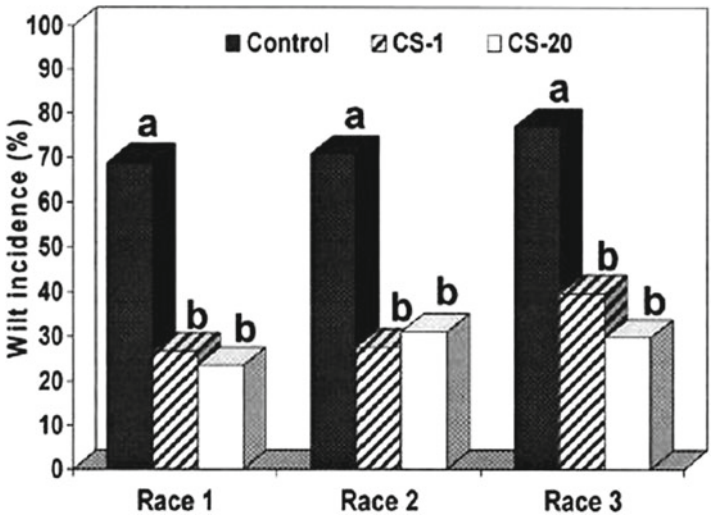

Fig. 3.9 Effect of application of non-pathogenic fungal biocontrol agents *Fusarium oxysporum* CS-20 and *F. solani* CS-1 on the incidence of Fusarium wilt disease of tomato caused by race 1, race 2 and race 3 of *F. oxysporum* f.sp. *lycopersici*. *Bars topped by the same letter* within each race designation are not significantly different according to Fisher's protected least significant difference test at P = 0.05 (Courtesy of Larkin and Fravel 2002 and with kind permission of The American Phytopathological Society, MN, USA)

on carpogenic germination of sclerotia of *S. sclerotiorum* were assessed. *C. minitans* (isolate Conio) applied as maize meal-perlite inoculum reduced sclerotial germination and apothecial production in three types of box assays, decreasing sclerotial recovery and viability and increasing infection of sclerotia by *C. minitans*. The isolate Conio applied as maize meal-perlite inoculum survived in the soil throughout the experiments, being recovered at the end of each of the three bioassays at levels similar to those applied. Apothecial production by *S. sclerotiorum* was delayed or inhibited by temperatures >26 °C. Another major factor in reducing apothecial production by sclerotia was the inoculum level of *C. minitans* applied. Interactions between the effect of temperature on apothecial production by sclerotia and on parasitism of sclerotia by *C. minitans* appeared to be a crucial factor determining the control of *S. sclerotiorum* by *C. minitans* (Jones et al. 2004). In a later study, the development and survival of *C. minitans* in pasteurized and natural soil were monitored using SEM. The pycinidia of the BCA were seen within the sclerotia of *S. sclerotiorum* at 7 days post-inoculation (pi) in pasteurized soil and at 14 days pi in natural soil. Conidial droplets were exuded onto the outer surface of infected sclerotia. The conidia in dried droplets could germinate even at 10 months pi, indicating the possibility of infected sclerotia of the pathogen being a unique reservoir for the survival of *C. minitans* (Bennett et al. 2006). The compatibility of 18 isolates of *Clonostachys rosea* (syn. *Gliocladium roseum*) effective against many pathogens including *Phytophthroa palmivora* was investigated with a view to identifying the combination

of isolates for improving the effectiveness of protection. An intra- or inter-isolate pairings (dual cultures) on water agar plates, a hyphal interaction experiment and a modified host range experiment were applied to select compatible pairs of isolates. Growth inhibition was seen in all combinations of isolates, as none showed free hyphal intermingling. The level of aggressiveness and/or susceptibility of an isolate to mycoparasitism by other isolate was largely dependent on the isolate with which it was challenged. The primary host *P. palmivora* did not affect antagonistic capabilities of *C. rosea* isolates. The competitive ability of *Clonostachys* isolates depended on the partner with which they were applied and less on the resource availability (ten Hoopen et al. 2010).

Seed treatment with biocontrol agents is the preferred method of BCA application to tackle the pathogens present in/on the seeds as well as those existing in the soil as saprophytes. Biological control of *Pythium ultimum* causing preemergence damping-off disease of cotton seedlings was achieved by applying *Trichoderma virens*. Disease control by the BCA was attributed to metabolism of germination stimulants released by the cotton seed, thus preventing germination of the pathogen propagules. Hence, the presence of *T. virens* in the spermosphere in an active and viable state is necessary for successful biocontrol of the cotton seedling disease. Spermosphere and rhizosphere competence of the BCA is a vital attribute for effective disease management (Howell 2002). Organic production of crops is gaining importance in developed countries, because of certain perceived advantages of using organically produced products. Hence, the biocontrol agents to be used against crop diseases have to satisfy stringent conditions like absence of antibiotics or other toxic materials that are produced by the BCAs present in commercial products. Treatment of seeds with BCAs can protect the emerging seedlings against seedborne as well as soilborne pathogens. Seeds of spinach were treated with the proprietary products GTGI and GTGII (each containing a proprietary organic disinfectant and the latter also included *Trichoderma harzianum* T22). Treated seeds showed early germination and emergence which could reduce the duration of susceptibility of spinach seedlings to infection by *Pythium ultimum* and *Rhizoctonia solani* in organic spinach crops. Suitability of the BCAs for use for the control of soilborne pathogens will be an advantage for promoting the BCA for commercial development (Cummings et al. 2009). The fungal endophytes *Epicoccum nigrum* and *T. atroviride* could effectively suppress *R. solani,* in addition to improving the potato yield significantly. However, their rhizosphere competence, adaptability to conditions in different fields and absence of toxicity to humans and animals of formulated products will determine the possibility for wider application (Lahlali and Hijri 2010).

Combination of *Trichoderma* isolates and *Brassica carinata* seed meal (BCSM) was evaluated for their effectiveness in suppressing the development of sugar beet damping-off disease caused by *Pythium ultimum.* Forty isolates of *Trichoderma* were found to be generally less sensitive to the toxic volatiles (glucosinolate-derived compounds) than the soilborne pathogens such as *Rhizoctonia solani, Fusarium oxysporum* and *Pythium ultimum*. *Trichoderma* isolates were able to grow on BCSM and over the pathogens tested. BCSM incorporation increased pathogen population, but reduced disease incidence, probably due to indirect mechanisms. Disease

suppression was maximum, when BCSM was combined with *Trichoderma,* regardless of the ability of BCSM to release the volatile isothiocyanate. A reduction of allyl-isothiocyanate concentration in soil might occur due to the activity of some *Trichoderma* isolates, facilitating establishment of introduced *Trichoderma* isolates, but reducing the efficacy of biofumigation against pathogens (Galletti et al. 2008). *Coniothyrium minitans* produced the macrolide antibiotic macrosphelide A in modified Czapek Dox broth (MCD). The antibiotic was produced by all isolates (conio, contans and IVT1) at 10–30 °C and the culture filtrates (CFs) from all isolates inhibited the growth of the pathogen *Sclerotinia sclerotiorum* by more than 50 %. Antibiotics were produced by the isolates at a pH range of 3–5 the maximum inhibition being at pH 3.0. Culture filtrates from conio grown at pH 3.0 inhibited *S. sclerotiorum* to a greater extent than IVT1 at the same pH. The results indicated that biocontrol efficacy of the isolates of *C. minitans* might vary depending on variations in soil conditions (Tomprefa et al. 2011).

Biotic and abiotic stresses affect the development of pathogen and biocontrol agents. Chlamydospores are vital asexual resting cells aiding the survival of most of the pathogenic *Fusarium* spp. in the soil. The mycoparasite *Acremonium strictum* SMCD 504 and antagonistic *Bacillus amyloliquefaciens* posed minimal effects on the chlamydospore formation by *Fusarium graminearum* and *F. sporotrichoides.* In contrast, manitol supplement to minimal conversion media (MCM) induced high chlamydospore size and chain abundance at optimal 21 °C and extreme 37 °C respectively in *F. sporotrichoides. F. graminearum* showed low chlamydospore formation even at 37 °C on MCM-manitol media. The results indicated that the BCA suppressed the pathogen development without triggering the chlamydospore formation (Goh et al. 2009). Colonization of roots of cucumber by *Clonostachys rosea* f. *catenulata* (*Gliocladium catenulata*) is significantly influenced by environmental and host factors. Conidia of a GUS-transformed strain of *C. rosea* f. *catenulata* were used to inoculate roots of cucumber grown in nutrient solution in containers. Population levels of the BCA associated with roots over time were assessed by colony-plate counts, GUS staining and enzymatic assays to determine GUS activity. The pH, temperature and growing medium exerted appreciable influence on BCA populations, whereas the cucumber cultivar, addition of nutrients or wounding of roots did not seem to affect colonization of roots. The BCA population was the highest at pH 5–7 and at temperatures 18–22 °C. More accurate assessment of root colonization levels could be obtained by employing GUS activity measurement than by colony-plate counts. The results indicated the need for providing optimal conditions to ensure maximum root colonization by *C. rosea* f. *catenulata* and consequent increase in the effectiveness of biocontrol activity against the target pathogen (Chatterton and Punja 2010).

Cylindrocladium spathiphylli causing root rot disease of banana has been reported to cause serious losses (Risede and Simoneau 2001). Lack of effective and economical control by application of chemicals necessitated the search for alternative strategies including biocontrol methods. Bananas are commonly associated with arbuscular mycorrhizal fungi (AMF). The biocontrol potential of four AMF *Glomus* spp., *G. proliferatum, G. intraradices* and *G. versiforme* was assessed in reducing

incidence of root rot disease and improving growth of banana plants. Root infection of banana by *C. spathiphylli* reduced the growth of plants. Preinoculation of plants with AM fungi lessened this adverse effect on plant growth. The mean root necrosis index (RNI) representing disease severity was reduced to 40 % in plants colonized by *G. versiforme* and to 29 % in plants colonized by *Glomus* sp. as against 57 % in nonmycorrhizal plants. The reduction in disease severity in mycorrhizal plants was associated with improved growth of plants. The relative mycorrhizal dependency (RMD) of plants inoculated with *C. spathiphylli* increased to 59–74 %, while the RMD was between 39 and 46 % in the absence of infection. It appeared that under stressed conditions caused by *C. spathiphylli*, the positive effects of AM fungi on growth of banana were more pronounced than in the absence of the stress. *Glomus* sp. and *G. proliferatum* enhanced the plant growth to the maximum extent with corresponding reduction in infection by *C. spathiphylli* (Declerck et al. 2002). Information on the extent of spread of the biocontrol agents from the treated soil or plant organs to other locations or plants is not known in most cases of plant-. BCA interactions. It is essential to monitor the mode of dissemination and survival of the BCAs under natural conditions to determine the suitability of the BCA for commercialization. Water-assisted dissemination of conidia of *Coniothyrium minitans* (*Cm*), the mycoparasite of *Sclerotinia sclerotiorum* (*ss*) in four types of soils was investigated. The conidial concentration of *Cm* was logarithmically reduced with increase in depth of vertical dissemination (VD) or the distance in horizontal dissemination (HD). Dissemination of *Cm* was at greater rate in sandy soil than other types of soils. The minimum *Cm* concentration for suppression of *Ss* carpogenic germination was 1,000 conidia/g of soil. The results indicated that water-assisted application of *Cm* could be adopted at the time of transplanting oilseed rape seedlings to suppress *Ss* carpogenic germination. This strategy might result in reduction of primary infection source for Sclerotinia diseases of oilseed rape (Yang et al. 2009).

3.1.7.2 Aerial Diseases

Mycoparasitism as the mechanism of biocontrol activity has been reported in many pathosystems. *Ampelomyces quisqualis* was shown to be an effective biocontrol agent against biotrophic powdery mildew pathogen *Sphaerotheca fuliginea* infecting cucumber. The BCA produced, within 24 h after application, germ tubes which formed appressorium-like structures at the point of contact with the pathogen hyphae. Pycnidial formation with conidiophores and conidia on the hyphae of *S. fuliginea* was seen within 5 days after BCA application (Sundheim and Krekling 1982). *A. quisqualis* exhibited similar biocontrol activity against grapevine powdery mildew disease caused by *Erysiphe* (*Uncinula*) *necator* (Daoust and Hofstein 1996). Spread of *Ampelomyces* from plant to plant may occur through transportation of hyphae within infected powdery mildew conidia by wind to long distances (Sundheim 1982). *A. quisqualis* has a wide host range that includes more than 66 species of the family Erysiphaceae enclosing different powdery mildew pathogens occurring all over the world (Kiss 2003). *A. quisqualis* was able to overwinter in the host fungal

structures including mycelium, ascomata and conidiophores. On apple trees *Ampelomyces* overwintered as resting hyphae in the dried powdery mildew mycelia covering the shoots and in the parasitized ascocarp of *Podosphaera leucotricha* on the bark and scales of apple flower buds. *Ampelomyces* could survive the winter in the field as pycnidia and as resting hyphae in the dried mycelia of the fungal pathogen (Szentiványi and Kiss 2003).

Powdery mildew pathogens are parasitized by other fungal biocontrol agents such as *Verticillium lecanii* (Verhaar et al. 1993), *Acremonium alternatum*, *Cladosporium cladosporioides* (Malathrakis 1985) and *Lecanicillium* (=*Verticillium) lecanii* (Romero et al. 2007). These mycoparasites have been reported to be favored by high humidity (low vapour pressure deficit) conditions for their activity. *Fusarium proliferatum* has also been shown to be a mycoparasite of another biotrophic fungal pathogen *Plasmopara viticola* causing downy mildew disease of grapevine (Falk et al. 1996). Biocontrol efficiency of the fungal BCAs depends on several factors such as characteristics of the BCA itself, epidemiology of the target pathogen and the environment conditions in which the relationship has to be established. The mycoparasites *Ampelomyces quisqualis* (as AQ10®), *Lecanicillium lecanii* (as Mycotal®) were more effective against cucurbit powdery mildew pathogen *Podosphaera fusca,* when the relative humidity was above 80 %. Further, only in combination with the mineral oil ADDIT, the mycoparasite-based products AQ10 or Mycotal were most effective providing percentage reduction in disease up to 80–95 %. In the absence of mineral oil, the disease severity was not significantly reduced to a level below that of untreated or water controls. Hence, the high relative humidity and presence of the mineral oil were found to be crucial factors for effective biocontrol activity of the mycoparasites against cucurbit powdery mildew pathogen *P. fusca* under greenhouse conditions (Romero et al. 2007).

Gliocladium roseum, the fungal biocontrol agent has been demonstrated to be versatile in its antagonistic activity on the gray mold pathogen *B. cinerea,* infecting several crops such as strawberry, raspberry, conifer seedlings, and vegetable and flower crops grown in the greenhouses (Sutton et al.1997). *G. roseum* has to be applied at appropriate time to obtain effective control of the target disease. The BCA was applied weekly to protect flowers through which infection spreads to strawberry fruits. The BCA reduced the infection of stamens and fruits by 48–96 % depending on the experimental conditions. *G. roseum* was highly efficient in suppressing foliage infection which forms the primary source of inoculum. In six tests, *G. roseum* suppressed the conidial production by *B. cinerea* on leaves by 90–100 % and it was as effective as the fungicide chlorothalonil in all tests. On the other hand, *T. viride* and *Penicillium* spp. provided protection equal to that of chlorothalonil only in three of the six tests. The results indicated that *G. roseum* had the potential to provide effective protection to many crops grown under varied climatic conditions and it could be an alternative to the fungicide commonly applied against gray mold disease (Sutton 1995; Sutton et al. 1997).

Microbial biocontrol agents are known to act on the phytopathogens through many mechanisms of biocontrol activity. It may be desirable to have BCAs with two or more mechanisms of bicontrol activity so that the pathogen development may be

more effectively suppressed. Management of gray mold diseases caused by *B. cinerea* is challenging, because of its abilities to survive as a saprophyte, rapidly invading host tissue and quickly producing abundant conidia that are easily disseminated by air currents even to distant locations. Moreover, this pathogen is capable of growing within a wide range of temperatures (0–35 °C), the optimum being 24–28 °C for growth. The biocontrol agents *Pichia guilliermondii* (yeast) and *Bacillus mycoides* were evaluated for their biocontrol potential individually and in combination for suppressing the development of *Botrytis cinerea* on the leaves of strawberry. The control efficacy achieved by individual BCA varied from 38 to 98 % and it was highly variable and under certain combinations of temperatures (10–30 °C) and humidities (78–100 %). In contrast, the mixture of *B. mycoides* and *P. guilliermondii* suppressed the development of *B. cinerea* by 80–99.8 % under all conditions tested. The results showed that simultaneous application of both yeast and bacterial antagonists provided more effective protection against *B. cinerea* and also reduced the variability of disease control. The results lend support to the hypothesis that use of combination of BCAs would broaden the environmental conditions under which biological control could be effective by reducing the variability of control efficacy under diverse conditions (Guetsky et al. 2001). *Pichia guilliermondii* could compete with gray mold pathogen *Botrytis cinerea* infecting strawberry for nutrients available in the substrate. In addition, it produces an inhibitory compound cable of inhibiting conidial germination of the pathogen. Furthermore, application of *P. guilliermondii* to the root zone of plants had some suppressive effect on the pathogen on the foliage implying that this yeast antagonist might activate the defense mechanism of treated plants. Another advantage of using *P. guilliermondii* against *B. cinerea* was the additive effect in improving the effectiveness of bicontrol, when it was combined with the bacterial BCA *Bacillus mycoides.* Furthermore, germination of *B. cinerea* conidia could be further reduced by combining the live cells of both BCAs, as revealed by observations under scanning electron microscope (SEM) (Guetsky et al. 2002).

Significant differences were observed in the structure and above-ground grapevine-associated microorganisms from organically and conventionally managed vineyards. *Aureobasidium pullulans*, a copper detoxifying BCA appeared to have a key role in the microbial community structures. This fungal BCA was strongly enriched in the communities of organically managed plants and yielded a greater indigenous antipathogenic potential (Schmid et al. 2011). The yeast species *Pseudozyma fusiformata* strain AP6, *Metschnikowia* sp. strain AP6 and *Aureobasidium pullulans* strain PL5 were effective against *Monilinia laxa* causing brown rot disease of peaches. The antagonistic activity of *A. pullulans* and *P. fusiformata* depended on the cell concentration. The effectiveness of the three strains was higher at 1 °C than at higher temperatures (8 or 20 °C). In semi-commercial conditions (at 1 °C and 96 % RH) the strains AP6 and PL5 were equally effective at 1×10^7 cells/ml as at 1×10^8 cell/ml, indicating that the BCA strains could be employed at lower concentration in formulations. The BCA strains did not impair any of the postharvest quality parameters, including firmness, total soluble solids, ascorbic acid content and titrable acidity. The results indicated that the BCA strains had the potential for large scale application under specified conditions (Zhang et al. 2010).

The epidemiological factors favoring disease development have to be carefully considered to select the fungal biocontrol agent that may be most effective against the target pathogen. Factors influencing development of BCA, *Clonostachys rosea* and the pathogen, *Botrytis cinerea* on leaves and petals were investigated. *C. rosea* germinated, established endophytic growth and sporulated abundantly, whether the plant tissues were mature, senescent or dead when inoculated. When rose leaves were wound inoculated, germination of *C. rosea* increased from 45 to 92 %, but sporulation was high (≥75 %) regardless of wounding. When leaves were inoculated serially, with the BCA and *B. cinerea*, after wounding, pathogen germination was reduced by 25–41 % and sporulation by ≥99 %. A humid period prior to inoculation of senescent or dead leaves promoted communities of resident fungi reducing the sporulation of both the BCA and the pathogen. But in dead leaves the effectiveness of disease suppression by *C. rosea* was increased. The results showed that *C. rosea* could appreciably suppress sporulation of *B. cinerea* in rose leaves and petals, regardless of developmental stage, minor wounds and natural densities of microorganisms (Morandi et al. 2000). Senescent petals of alfalfa generally remain attached to pods during pod development, favoring infection of pods and seeds by *Botrytis cinerea*. *Clonostachys rosea* and *Gliocladium catenulatum* could colonize both young and senescent petals in vivo as evidenced by production of conidiophores and conidia by the BCAs. These two BCAs might have higher saprophytic colonization ability on senescent petals of alfalfa compared with *T. atroviride* tested. Further, *C. rosea* and *G. catenulatum* might be less sensitive to scarcity of available water than *T. atroviride* during colonization of alfalfa petals. *C. rosea* has been demonstrated to be very effective in suppressing alfalfa pod and seed rots under varied field conditions. These characteristics of *C. rosea* in addition to its wide spectrum biocontrol activity against other fungal pathogens such as *Fusarium culmorum, Bipolaris sorokiniana* and *Phomopsis sclerotioides* infecting crop hosts, indicated that *C. rosea* could be a suitable candidate for commercial development of a biocontrol product (Li et al. 2004).

Suppression of pathogen sporulation is considered as a potential strategy of biocontrol of microbial plant pathogens like *Botrytis cinerea*, since abundant sporulation of *B. cinerea* on dead and senescent plant tissues contributes to the development and maintenance of an epidemic within crops like strawberry. The ability of suppressing sporulation of *B. cinerea* by three strains of *Ulocladium atrum* differed significantly. The strain 385 reduced the sporulation of *B. cinerea* and it could contribute to the slow-down of the gray mold spread and prevent the development of an epidemic. In addition, strain 385 colonized the necrotic strawberry leaves more efficiently than other two strains. This competitive colonization was regarded as an important mechanism in the biocontrol strategy of *U. atrum*. It is essential to assess the genetic diversity of the BCAs and the most effective isolate or strain within the morphologic species with reference to efficacy of biocontrol activity against the target pathogen has to be precisely identified (Berto et al. 2001). Inhibition of spore germination by the BCA may be an effective biocontrol mechanism in the case of rust pathogens (heterocyclic), requiring two plant species for the completion of life cycle of the pathogen *Cronartium flaccidum,* causing needle

pine stem rust disease. The biocontrol agent *Cladosporium tenuissimum* inhibited germination of aeciospores of *C. flaccidum* and *Peridermium pini*. Cladosporal isolated from the culture filtrate of *C. tenuissimum* was considered to be the inhibitory compound. Mycoparasitic ability of *C. tenuissimum* added to the arsenals that could be directed against the pine stem rust pathogens (Moricca et al. 2001).

The survival and spread of the BCA *Trichoderma atroviride* C65 on kiwi fruit leaves in the shadehouse and flowers/fruit in the orchard by employing a modified dot blot assay. The isolate C65 could survive on both leaves and flowers/fruit over an entire growing season. The BCA applied once in November/December to coincide with bud burst was detected on both leaves and fruit till harvest in March. Further, the isolate C65 was able to spread to uninoculated leaves and fruit on the same plant and also plants at least 3 m away. The involvement of thrips present at flowering in the spread of the BCA isolate within the orchard was postulated. The ability of the isolate C65 to survive and spread in the phylloplane and fructoplane of kiwifruit vines over an entire growing season is a desirable attribute for an ideal biocontrol agent (Dodd et al. 2004). The yeast species have the ability of proliferate rapidly on the leaf, fruit and flower surfaces especially in the presence of sugar. They dominate the phyllosphere environment by inhibiting the development of other microorganisms including phytopathogens, through competition for space and/or nutrition (Saligkarias et al. 2002). The yeast *Torulaspora globosa* was effective against *Colletotrichum sublineolum* causing anthracnose disease of sorghum. The antagonistic activity of *T. globosa* was ascribed to the competition for space and nutrients with the pathogen as well as the action of killer toxin produced by the BCA. *T. globosa* was effective against the anthracnose pathogen under field conditions also (Rosa et al. 2010). Such a demonstration of effectiveness of the BCA against the target pathogen(s) is available only in a limited number of pathosystems

Trichoderma spp. have been detected on sunflower heads and they have been demonstrated to be effective against several fungal pathogens. A composite mixture of *Trichoderma* spp. was tested as a biocontrol product against *Sclerotinia sclerotiorum* causing sunflower head rot disease. Honey bees were employed as vectors of *Trichoderma* spp. to disseminate the BCAs, based on their ability as pollinators of sunflower and vectors. The efficacy of a mixture of six isolates including *Trichoderma koningii*, *T. aureoviride* and *T. longibrachiatum*, was evaluated under field conditions. *Trichoderma* formulation (TF) contained the conidia and viable hyphal fragments of the BCAs, industrial talc and milled corn kernels. Honey bees (*Apis mellifera*) were employed for disseminating TF for 6 weeks from the onset of flowering. Sunflower heads were inoculated with ascospores of *S. sclerotiorum* after the first TF delivery through honey bees. A delay of disease incidence was observed following TF dissemination by honey bees. The dispersion of *Trichoderma* spp. for half an hour per day with high bee load proved to be effective, when disease incidence was lower than 80 %. With free-ranging bees removing 100 g TF per day, head rot incidence was significantly reduced. The efficacy of TF was not affected by the cultivar or environment. By combining TF delivery with partially resistant sunflower genotypes, the disease incidence was reduced from 75 to 15 % or from 90 to 23 %. The approaches of employing honey bees for delivering BCAs holds promise

for further exploitation (Escande et al. 2002). Vectoring of biocontrol agents for their rapid and timely dispersal for suppressing disease development is a novel approach. Attempt was made to use the pathogen itself as a vector for the biocontrol of *Botrytis cinerea*. An isolate of the yeast *Trichosporon pullulan,* by the laboratory simulation, was employed as a potentially effective BCA that could be vectored by the conidia of *B. cinerea*, the gray mold pathogen. Yeast isolates capable of binding to *B. cinerea* were formulated with a cellulose carrier and applied to sporulating colonies of the pathogen. Inoculum from treated colonies was harvested and applied to tomato stem tissue for pathogenicity tests. Disease development was significantly reduced relative to that in cellulose-only controls. *T. pullulans* was more effective, since it was able to multiply and grow on *B. cinerea* hyphae during pathogen germination. Application of freeze-dried yeast cells resulted in strong attachment to conidiophores and conidia of *B. cinerea.* The use of yeasts such as *T. pullulans* employed as a vectored BCA has opened up a new route for the spread of BCA rapidly in the field (Cook 2002a, b).

The biocontrol agents (BCAs) applied against microbial plant pathogens are expected to have no adverse effects on nontarget organisms that are useful for crop production. Honey bees and bumble bees are known to be important pollinators of agricultural and horticultural crops. The need to determine the potential risks of biocontrol agents against these beneficial insects was recognized. Two commercial biofungicides Binab-TF-WP and Binab-TF-WP-Konc containing combination of *Trichoderma harzianum* and *T. polysporum* were evaluated for their effects on bumble bees. The BCA products were applied on the bumble bees at their respective maximum concentration in the field (MFRC) through three routes viz., dermal contact, orally via the drinking of treated sugar water and via treated pollen. The tests showed that the two products did not cause worker mortality or deleterious effect on reproduction. Further, the BCAs were unable to either survive or grow on the bodies of adult worker bees and no adverse effects on the bumble bee larvae (third and fourth instars) could be observed. The results indicated that under the conditions tested, the bioproducts appear to be safe for use for the control of the gray mold pathogen *Botrytis cinerea* (Mommaerts et al. 2008).

3.1.7.3 Postharvest Diseases

Biocontrol agents may be applied prior to harvest in the field and/or under controlled storage conditions. Investigations have been conducted predominantly on fruits and vegetables under storage. The BCAs have to survive the conditions existing in the field as well as during storage. For example the yeast *Candida sake* was able to provide protection to apple (cv. Golden Delicious) against *Penicillium expansum,* when applied as preharvest sprays and later applied on fruits under commercial storage conditions (Teixidó et al. 1998). *Candida sake* was able to multiply and its populations increase by more than 50-folds at 20 °C. Population of *C. sake* increased in 3 days at 20 °C to a level that could be reached only after 20 days at 1 °C, indicating the temperature effect on population buildup. Availability of optimal temperature is

a requirement for biocontrol activity of the BCAs against postharvest pathogens (Viñas et al. 1998). In a later study, the ability of *C. sake* strain CPA-1 to colonize the surface of apples under various storage conditions and its greater capacity to colonize apple wounds were considered as desirable attributes to be preferred for the biocontrol of apple blue mold disease (Usall et al. 2001). The efficiency and rapidity of colonization of wound sites and fruit surface by BCAs are important factors for successful biocontrol of postharvest diseases. *Candida reukaufi* and *C. pulcherrima* were evaluated for their efficacy in protecting strawberry fruits against the gray mold pathogen *Botrytis cinerea,* when applied at 10^3 CFU/wound. These BCAs effectively colonized the fruits and strongly inhibited spore germination of *B. cinerea* in vitro (Guinebretiere et al. 2000).

The yeast *Metschnikowia fructicola* applied at pre-and post-harvest stages was effective against *Botrytis cinerea* and *Rhizopus stolonifer* causing gray mold and storage rot diseases of strawberry. *M. fructicola* application was as effective as the fungicide fenhexamid (Karabulut et al. 2004). The period of survival of the BCA may vary, when they are applied at preharvest stage. The yeasts *Cryptococcus laurentii*, *Rhodotorula glutinis* and *Trichosporon pullulans* were applied as preharvest sprays to protect sweet cherries and they could colonize the surface of the fruits. However, the period of their survival varied significantly under field conditions. *C. laurentii* exhibited strong survival ability on fruit surfaces as well as adaptability to postharvest storage conditions of low temperature, low oxygen and high CO_2 concentrations (Tian et al. 2004). The biocontrol efficacy of *Cryptococcus laurentii* against the pear gray mold pathogen *Botrytis cinerea* was assessed. The interval between the pathogen inoculation and BCA application adversely affected the effectiveness of biocontrol significantly. The longer interval, the least was the effectiveness. Higher level of effectiveness was observed, when *C. laurentii* was applied simultaneously or prior to inoculation with *B. cinerea*. Higher concentrations of *C. laurentii* were more effective in reducing the disease incidence as well as lesion diameter regardless of the storage duration and temperature. Addition of $CaCl_2$ (2 %) enhanced the efficacy of the BCA. Natural development of decay was significantly reduced. In addition, treatment with the BCA did not impair the fruit quality parameters, when the pear fruit was stored at 2 °C for 60 days followed by storage at 20 °C for 15 days (Zhang et al. 2005).

Candida sake was able to multiply and its populations increased by more than 50-folds at 20 °C. Population of *C. sake* increased in 3 days at 20 °C to a level that could be reached only after 20 days at 1 °C, indicating the temperature effect on population buildup. Availability of optimal temperature is a requirement for biocontrol activity of the BCAs against postharvest pathogens (Viñas et al. 1998). In a later study, the ability of *C. sake* strain CPA-1 to colonize the surface of apples under various storage conditions and its greater capacity to colonize apple wounds were considered as desirable attributes to be preferred for the biocontrol of apple blue mold disease (Usall et al. 2001). The efficiency and rapidity of colonization of wound sites and fruit surface by BCAs are important factors for successful biocontrol of postharvest diseases. *Candida reukaufi* and *C. pulcherrima* were evaluated for their efficacy in protecting strawberry fruits against the gray mold

pathogen *Botrytis cinerea,* when applied at 10^3 CFU/wound. These BCAs effectively colonized the fruits and strongly inhibited spore germination of *B. cinerea* in vitro (Guinebretiere et al. 2000).

Production of volatile compounds by some fungi has been exploited for the reduction of disease intensity/incidence. *Muscodor albus* produces about 28 different volatile compounds which could inhibit or kill several species of fungi, oomycetes and bacteria. Application of fumigants is an ideal method of managing postharvest diseases. Fumigation of apples for 7 days with a culture of *M. albus* grown on autoclaved grain protected the apples completely against infection by blue mold pathogen (*Penicillium expansum*) and gray mold pathogen (*Botrytis cinerea*) in wound-inoculated fruits. Two major volatile compounds produced by *M. albus* were identified as 2-methyl-1-butanol and isobutyric acid. As the BCA *M. albus* did not require direct contact with the pathogens, the BCA can be a potential candidate for development of commercial products for use against postharvest pathogens (Mercier and Jiménez 2004). The effect of volatile compounds of *M. albus* on dormant and physiologically active teliospores of the smut fungi *Tilletia horrida, T. indica* and *T. tritici* causing rice kernel smut, wheat Karnal bunt and wheat common bunt diseases respectively. In vitro tests in petridishes showed that the teliospores of all three smut pathogens lost their capacity for germination, when biofumigated with *M. albus* for 5 days. The teliospores of *T. tritici* within intact sori and dormant spores of *T. horrida* and *T. indica* were not affected by the volatiles of *M. albus*. The results suggested that *M. albus* may have potential as seed or soil treatment for preventing infection of seedlings by germinating teliospores prior to seedling emergence (Goates and Mercier 2009).

Postharvest diseases are managed conventionally by applying different chemicals. The use of chemicals has to be restricted, because of possible adverse effects on consumers and environment. Hence, search for the biocontrol agents that have potential to replace or reduce the use of the chemicals became necessary. A combination of yeasts *Cryptococcus laurentii* and *C. infirmo-miniatus* showed effectiveness equal to that of thiabendazole (TBZ) for the control of pear blue mold disease caused by *Penicillium expansum.* The BCA combination reduced the infection by 91 % as against 88 % obtained using TBZ at a high dose of 528 µg/ml (Spotts et al. 1998). The fungal pathogens are known to develop resistance to certain chemicals that are frequently and repeatedly applied over several seasons/years. In such cases, the BCAs have been reported to offer effective protection against fungicide-resistant strains of the pathogens. Control of wound infection by TBZ-resistant strains of *P. expansum* could be achieved by applying yeast species *Rhodotorula glutinis, C. infirmo-miniatus* and *C. laurentii* (Sugar and Spotts 1999).

Candida oleophila, primary component of the commercial bioproduct Aspire, has been shown to suppress the citrus green mold pathogen *Penicillium digitatum* through different mechanisms such as direct parasitism, nutrient competition, and site exclusion by colonizing the wounds rapidly. Later application of *C. oleophila* to surface wounds or intact "Marsh Seedless" grapefruit elicited systemic resistance against *P. digitatum.* Various resistant responses were activated in the vicinity of wounds and in whole intact fruits following treatment with *C. oleophila*. Activation

of resistance mechanisms at points beyond the wound site is important for protection against possible infections that may occur later. Further, the restriction of fungal growth and sporulation in the case of decay developing from *C. oleophila*-treated or untreated surface wounds may add to the effectiveness of bioprotection (Droby et al. 2002). The ability of the BCA to act through different mechanisms simultaneously at spatial and temporal intervals may reinforce the extent of biocontrol offered by the biocontrol agent employed. *Candida guilliermondii* was demonstrated to be effective against apple blue mold disease caused by *Penicillium expansum.* However, cases of onychomycosis and fungaemia caused by *C. guilliermondii* in humans have been reported (Krcmery and Barnes 2002). Hence, the pathogenic behavior and genetic structure of *C. guilliermondii* isolates selected as antagonists of fungal plant pathogens have to be clearly studied and compared with clinical specimens to bring out the differences between the antagonists and human pathogenic strains before initiation of commercialization process.

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Chapter 4
Detection and Identification of Bacterial Biological Control Agents

Bacteria, classified as prokaryotes, are much simple in structure and smaller in size. They have less complex structural features compared to the fungi (placed under eukaryotes) which have well defined thallus composed of different asexual and sexual structures bearing various spore forms at different stages of their life cycle. Most of the bacterial species are strictly saprophytic and they are involved in the decomposition organic materials present in the soil and water, dead plants and animals. They are useful in denaturing large quantities of organic wastes from industries and oil spill occurring in the sea water. Some of them cause diseases in plants, humans and animals. Many bacterial species are beneficial to human beings, as they help build up nutritional levels of the soil and have potential for reducing the activity of or inhibiting the development of bacterial plant pathogens. The morphological characteristics of bacterial cells are less variable and hence biological, biochemical, physiological, immunological and genomic characteristics have to be determined for reliable and precise identification and meaningful classification of the bacterial biocontrol agents that are to be employed for the management of crop diseases caused by microbial plant pathogens (Narayanasamy 2002, 2011).

Taxonomic characteristics and guidelines for identification of bacterial species are described in the *Bergey's Manual of Systematic Bacteriology* (Kreig and Holt 1984). Bacterial species obtained from different habitats are cultivated in appropriate media that favor their development. Cultural characteristics such as colony morphology, production of enzymes, toxins and other metabolites, pattern of utilization of carbon sources, pH and temperature optima for growth and other nutrient requirements are determined. As the variations in the cultural characteristics are inadequate for discriminating closely related species/strains of a bacterial species, immunological properties of bacterial cell surface proteins have been used as the basis for detection, differentiation and quantification of bacteria. Nucleic acid-based techniques have been shown to be more sensitive, precise, specific and reliable, in addition to their ability to provide results rapidly. Various methods that have been employed for the detection and identification of the bacterial biocontrol agents are discussed, indicating the advantages and disadvantages of different procedures applied.

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It is desirable to know the basic information about the bacteria and their reproduction, since the bacteria are used as model organism to investigate different physiologic and genetic aspects of living organisms. The bacteria are structurally simple and much smaller in size measuring about 1 μm in diameter, while the fungal spores may have diameters varying from 50 to 200 μm. The bacterial cells may be spherical, ellipsoidal, rod-shaped, spiral, filamentous or comma-shaped. A slime layer (capsule) may be present as a large mass around the cells. The flagella, organs of locomotion, may be located at one or both ends of the bacterial cells either singly or in groups. In some species, they may be distributed over the entire cell surface. The bacteria are divided into two biologically different groups, based on the ability to retain the stain developed by Hans Christian Gram. The Gram stain is retained by one group which is named Gram positive (+) and the group that loses the stain is designated Gram negative (−). Gram positive bacteria have thick uniform cell wall, while a thin cell wall with an additional outer layer of polysaccharides and lipids is present in Gram negative bacteria. Bacteria have a simple life cycle with an asexual stage in which the bacterial cells multiply by a process known a binary fission. At maturity, the bacterial cell divides into two daughter cells, each one inheriting a complete set of nuclear material and other cytoplasmic materials. Conjugation, a process similar to sexual reproduction in eukaryotes, involves the contact between two physiologically opposite bacterial cells and transfer of a small fragment of DNA from the male cell (donor) to the female cell (receptor). The female cell multiplies by binary fission, resulting in a population of bacterial cells possessing the characteristics governed by the DNA fragment of the donor cell.

Changes in the genetic constitution of the bacteria may occur in three different ways. Mutation in the genetic element of cells of a bacterial species may lead to permanent changes in the characteristics of the mutant as in other microorganism. Transformation is the process in which the genetic material in some bacterial cells is liberated either by secretion or rupture of the cell wall. A fragment of the DNA so released gains entry into a genetically compatible bacterial cell of the same or closely related species. The recipient bacterial cell exhibits the characteristics governed by the DNA fragment that was integrated into the recipient cell. Transduction is the third process leading to changes in the genetic constitution of the bacteria. A bacteriophage is involved as a vector carrying the DNA fragment from one bacterial cell to another bacterial cell. The bacterial virus acquires a fragment of the infected bacterial cell which is lysed later. This DNA fragment is integrated into the other bacterial cell, when it is infected by the virus. It is possible to transfer different characters from one cell to another using vector (virus/plasmid), leading to changes in the genetic constitution of bacteria. As the bacteria are able to multiply very rapidly reaching high population levels within a short period, they are considered as an important factor in all ecosystems in general and particularly as pathogens of plants and animals as well as the biocontrol agents capable of providing protection against plant diseases (Narayanasamy 2002, 2008).

4.1 Methods of Detection and Identification

Bacteria are detected in various habitats by employing different methods based on the morphologic, physiologic and genetic characteristics. These methods are similar whether the bacterial species exists as a pathogen or saprophyte.

4.1.1 Cultural Methods

The bacterial biocontrol agents (BCAs) have to be isolated and brought into pure culture using appropriate media that favor their development. Various kinds of media – general or selective (specific) – have been developed to isolate the bacterial BCAs from soil, plants, seeds or planting materials or harvested produce (fruits and vegetables) (Appendix 4.1). The knowledge of basic characteristics of bacterial colonies may be useful in preliminary identification and differentiation of the target bacterial species from other bacteria. Media like King's medium B and nutrient yeast dextrose agar (NYDA) are commonly used for isolating the bacterial BCA and Pseudomonas agar F (Difco Laboratories) has been used for strains of *Pseudomonas* spp. (Mavrodi et al. 2001; Pusey 2002; Yoshida et al. 2001). Special methods have to be followed for isolation of bacteria from natural soils. Serial dilutions of suspensions are prepared and aliquots are spread onto plates containing CY agar for the isolation of myxobacteria (Bull et al. 2002). *Pseudomonas putida* was multiplied on MGY agar medium and applied for the control of *Phytophthora citrophthora* (Steddom and Menge 2001). The colony characteristics of the bacterial species, after isolation on suitable media, are used for tentative identification of the bacteria isolated.

4.1.2 Biochemical Methods

4.1.2.1 Gram Staining

Determination of response of bacterial species to Gram stain is the first step in the identification of the bacteria. Several species of the bacterial BCAs are Gram negative. The Gram complex consisting of magnesium ribonucleoprotein retains the stains. Bacterial species exhibiting biocontrol potential belonging to the genera *Agrobacterium, Erwinia* and *Pseudomonas* are Gram negative. Gram positive bacteria, capable of retaining the stain also possess biocontrol activity as in the case of *Bacillus* spp. (Cook and Baker 1983). Other characteristics useful for identification of the bacteria are the size of bacterial cells, location of flagella, presence of food reserve materials such as volutin, fat, glycogen and iogen, ability to reduce nitrates, production of hydrogen sulfide, ammonia, and indole, utilization of carbon and

nitrogen compounds, starch hydrolysis, lipolytic activity, action on litmus milk and gelatin liquefaction (Narayanasamy 2001).

The isolates of streptomycetes (94) were obtained from field soils cropped to lettuce. They were identified based on Gram staining, general morphology, spore formation, colony morphology and biochemical tests. *Streptomyces viridodiasticus* strains produced higher amounts of chitinase and β1,3-glucanase which could significantly inhibit the growth of *Sclerotinia minor* in vitro and colonize roots of lettuce seedlings (El-Tarabily et al. 2000). Population of *Pseudomonas fluorescens* A506 effective against fire blight pathogen *Erwinia amylovora* were determined by using KBR medium for isolating the BCA. King's medium amended with rifampicin (100 µg/ml) was highly selective for the antagonist. Populations of *P. fluorescens* and *E. amylovora* were determined at different periods after inoculation onto apple flowers. Large total bacterial population of A 506 treated trees were associated with significant reductions in population of *E. amylovora* and reduced incidence of fire blight and severity of fruit russet (Lindow and Suslow 2003). *P. putida* and *Bacillus pumilus* were cultivated on trypticase soy agar (TSA) and Pseudomonas Agar F for evaluating their potential of suppression of tomato bacterial wilt disease caused by *Ralstonia solanacearum* (Anith et al. 2004). *Pseudomonas fluorescens* strain EPS 62e effective against apple fire blight pathogen *Erwinia amylovora* was cultured on Luria–Bertani agar (LBA) at 25 °C. A spontaneous mutant of the wild type resistant to nalidixic acid was selected on LBA supplemented with 50 mg/l of nalidixic acid which was used to monitor the BCA in plant tissues (Pujol et al. 2006). King's medium B amended with rifampicin (100 µg/ml) was found to be suitable for the multiplication of *P. fluorescens* strain SS101 to assess the potential of the BCA to produce cyclic lipopeptide (CLP) (Mazzola et al. 2007).

Bacteria are classified based on the taxonomic characteristics and guidelines provided in *Bergey's Manual of Systematic Bacteriology* (Kreig and Holt 1984). All names proposed for new species of bacteria discovered, should be in accordance with the International Code of Nomenclature of Bacteria. Bacterial species do not exhibit variations in morphological characteristics adequate enough to differentiate related bacterial species. Hence, several physiological and biochemical properties are taken into consideration for classification of bacterial species. Results of polyphasic tests including nucleic acid analyses such as DNA-DNA and DNA–rDNA hybridization, chemotaxonomic comparisons such as cell wall composition, lipid composition, isoprenoid quinones, soluble and total proteins, fatty acid profiles, enzyme characterization and biochemical and nutritional tests form the bases of naming new species of bacteria. Determinative keys are formulated using the variations in the characteristics mentioned above (Young et al. 1992). Classification of bacteria up to genera that include the biocontrol agents (Agrios 2005) is provided below:

Kingdom	:	Procaryotae
Division	:	Gracilicutes (Gram-negative)
Class	:	Proteobacteria (mostly single-celled)
Family	:	Enterobacteria

Genus	:	*Erwinia*
		Serratia
Family	:	Pseudomonadaceae
Genus	:	*Pseudomonas*
		Rhizobacter
Family	:	Rhizobiaceae
Genus	:	*Agrobacterium*
Division	:	Firmicutes (Gram-positive)
Class	:	Firmibacteria
Genus	:	*Bacillus*
Class	:	Thallobacteria (branching bacteria)
Genus	:	*Streptomyces*

The distinguishing characteristics of some of the genera of bacteria, including the biocontrol agents more frequently employed for reducing the disease incidence, are described (Cook and Baker 1983).

1. *Agrobacterium radiobacter* K 84 is effective against crown gall disease. The bacterial cells are small short rods non-spore-forming, motile by polar flagellae, not acid-fast, gelatin not liquefied, starch not hydrolyzed, milk not coagulated, but not peptonized.
2. *Bacillus subtilis* has been shown to be effective in suppressing the diseases caused by soilborne fungal pathogens. *B. subtilis* has slime cells produces oval endospores, strictly aerobic, not requiring growth factors; produces extracellular amylases and proteases.
3. *Erwinia herbicola* has been applied for the control of fire blight diseases caused by *E. amylovora.* This species has Gram-negative rod shaped slime cells with peritrichous flagella; facultative anaerobe; does not produce indole, but produces phenylalanine deaminase and hydrogen sulfide.
4. *Pseudomonas fluorescens* has been reported to be effective in suppressing several soilborne, seedborne and airborne pathogens. The bacterial cells are rod-shaped, Gram-negative and have multitrichous flagella; gelatin liquefied, oxidase positive, arginine dihydrolase positive; produces soluble fluorescent pigments.
5. *Streptomyces* includes soilborne saprophytic species like *S. griseus*. They have filamentous cells usually producing mycelium-like structure forming spores by fragmentation of hyphal cells; sporophores are straight, flexuous or fascicled; produces antibiotics effective against microorganisms.

4.1.2.2 Metabolic Fingerprinting

Bacterial species may be rapidly identified, based on the differential utilization of 147 carbon sources. This technique was developed by API (API system, Monatiew, Vercelu, France). A biochemical kit API 50CH in combination with API 50 CHB IE (bio-Merieux, France) medium was used to identify the isolates of *Bacillus* spp. This is a standardized system consisting of 50 biochemical tests for determining

carbohydrates utilization patterns for each isolate/species of bacteria. *B. subtilis, B. pumilus, B. amyloliquefaciens* and *B. stearothermophilus* were identified (Gacitúa et al. 2009). A system similar to the API was developed by Biolog Inc, Haryward, California. The Biolog automated identification is based on the utilization of 95 carbon sources by different bacterial species/isolates/strains. A redox dye, tetrazolium violet is used to visualize the increased respiration of bacteria, while utilizing a carbon source. Each bacterial species produces a metabolic fingerprint that can be compared to those of known bacteria whose profiles have been recorded in a database. Biolog GP plates with wells are inoculated with a predetermined aliquots (150 µl) of target bacterial species. The plates are incubated at 25 °C for 24 h and they are evaluated on a microplate autoreader at 590 nm and outputs are compared by the Microlog Software (Biolog release 3.5). The Biolog System provides a printout of identification choices that include the identification of the target bacterial species followed by the next ten closest species.

The Biolog system GN and the MIDI (Microbial ID Inc, Newark, DE) identification systems were employed for identification of *Pantoea agglomerans* antagonistic to *Pseudomonas syringae* pv. *syringae* causing barley basal kernel blight disease (Braun-Kiewnick et al. 2000). *Serratia plymuthica* isolates obtained from the rhizosphere of oilseed rape were identified using API and BIOLOG systems. The identification rate achieved using API system was 57.5 %, whereas BIOLOG system provided an identification rate of 86.3 %, indicating the greater effectiveness of the latter system (Kurze et al. 2001). Seventy four isolates of bacteria obtained from rice were subjected to analysis by Biolog System, using 96-well MicroPlate designed to test the ability of the bacterial isolates to utilize a panel of 95 different carbon sources and nutrients in the plate. The Gram-negative (GN) MicroPlate characterized and differentiated GN aerobic bacteria. Gram-positive (GP) Microplate was used to characterize GP aerobic bacteria. The Biology, MicroLog release 4.01software allowed identification of the bacteria from the metabolic patterns based on the similarity in carbon sources utilization. Isolates of *Bacillus subtilis* and *B. megaterium* were identified by the Biolog system (Jin-yan et al. 2005). *Burkholderia cepacia* occurs as complex. The biotype causing human cystic fibrosis (in respiratory organ) is differentiated from the biotype used as biocontrol agent against plant pathogen based on the production of bacteriocin and proteolytic enzymes by the latter (Gonzalez and Vidaver 1979). The fresh cultures of *B. cepacia* on nutrient agar medium was streaked on Biolog Universal Growth(BUG) medium. Bacterial isolates were initially tested for the Gram reaction and oxidase tests to categorize them into enteric bacteria or non-enteric bacteria. Enteric bacteria are Gram-negative and oxidase negative. In contrast, non-enteric bacteria are Gram-negative and oxidase positive. Bacterial suspension was inoculated into GN microplate using the 8-channel repeating pipette. After covering the microplate with the lid, it was incubated at 28–30 °C for 24 h to allow utilization of carbon sources. The results were obtained by inserting the microplate into the Biology reader installed with the software of Biolog. Identification system for the identification of the target bacterial isolate up to the species level (Sijam and Dikin 2005).

The rhizobacteria isolated from tomato grown in replant soil and tomato roots were multiplied in nutrient broth at 28 °C for 36 h. Carbon source utilization of the rhizobacteria and reference organism *Pseudomonas putida* strain 17 was analyzed using Biolog microplates. Cultural characterization and carbon sources utilization patterns were very similar to that of *P. putida*. This result was confirmed by the fatty acid methyl esterase (FAME) analysis. Furthermore, the 16S rDNA sequence showed the highest homology of 98.5 % to *P. putida*. The biocontrol agent isolated from tomato was identified as *P. putida* (Lee et al. 2005). Bacterial isolates possessing biocontrol activity have to be identified simultaneously. Identification of bacterial isolates that suppressed development of *Leptosphaeria maculans* causing canola (*Brassica napus*) black leg disease was taken up, using the Microlog® systems. After performing Gram staining, the isolates were streaked onto Biolog universal growth (BUG) agar medium (Biolog). The bacterial population was approximately quantified with a turbidimeter and the bacterial suspensions (150 µl) were pipetted into each of the 96-well Biolog microplates. The plates were incubated at 32 °C for 16–24 h. Observations were made with an automated plate reader (Biolog) and assessed visually. The isolates were identified as *Pseudomonas chlororaphis* and *P. aurantiaca* according to carbon source utilization patterns as determined by the Biolog identification system (Ramarathnam and Fernando 2006). *Lysobacter capsici* strain PG4, a health-promoting rhizobacterium, reduced tomato seedling infection by the *Fusarium oxysporum* f.sp. *lycopersici, F. oxysporum* f.sp. *radici-lycopersici* and *Rhizoctonia solani*. The Biolog Identification System was applied to determine the physiological profile of the strain PG4. After incubation for 24 h, the utilization patterns were recorded. A high similarity was observed between the type strain and the PG 4 strain used in this study. The identification procedure allowed the PG 4 strain to be identified as a new *L. capsici* strain (Puopolo et al. 2010).

Pseudomonas fluorescens isolates producing the antibiotic 2-4 diacetyl phloroglucinol (2,4-DAPG) effective against soilborne diseases were grouped into superior or average root colonizers. Strain Q 8r 1-96 (genotype D) utilized trehalose, benzoate and valerate as sole carbon sources, while the average colonizers Q2-87 (genotype B) and IMI-96 (genotype L) did not utilize them. This study was enlarged, including 55 strains from 17 genotypes of DAPG producers to determine their ability to utilize the three carbon sources. No correlation between the strain's ability to utilize the carbon sources and the level of rhizosphere competence on wheat and pea. The genotype D strains utilized all three carbon sources. The results indicated that the rhizosphere competence of 2,4-DPAG–producers was not dependent on their ability to utilize the three carbon sources tested (De La Feunte et al. 2007). The strain HX2 effective against grape crown gall pathogen *Agrobacterium vitis* was small, rod-shaped, Gram-negative bacterial strain. This strain was identified as *Rahnella aquatilis* using Biolog identification system. Further, the biochemical test results showed that it was negative for cytochrome oxidase and positive for catalase. These characteristics indicated that it was a typical member of the family Enterobacteriaceae and closely related to *Pantoea agglomerans*, an important biocontrol agent (Chen et al. 2007).

Fluorescent bacteria isolated from the roots of healthy tomato plants were tested by Gram staining and for their ability for phosphate solubilization and production of siderophores, hydrolytic enzymes, indole-3-acetic acid and hydrogen cyanide. Further, they were analyzed using the API ZONE system with computer software (Bio Me'rieux, France). This system facilitated the identification of the isolates within 48 h of nonfastidious Gram-negative rods belonging to Enterobacteriaceae. The API ZONE strip had microtubes containing dehydrated media and substrates. The microtubes containing material for tests were inoculated with a bacterial suspension. After incubation, the metabolic end products were detected by indicator systems or addition of reagents. The isolates capable of utilizing the substrates available in the tubes developed well. All isolates were Gram–negative rods, oxidase-positive and capable of metabolizing glucose in an oxidative form. API ZONE analyses showed that nine isolates belonged to the genus *Pseudomonas*. The API biochemical test for *Pseudomonas* sp. TEI 1 and *Pseudomonas* sp. TR1 indicated that these two isolates belonged to *P. fluorescens*. The results of the biochemical tests were corroborated by those of ERIC-PCR analysis (Pastor et al. 2010).

4.1.2.3 Fatty Acid Methyl Esters Analysis

The fatty acid composition of the bacteria has been used as a basis of their identification. Gas chromatography (GC)-FAME analysis was used as a basis for the identification of *Pseudomonas fluorescens* strain SS 101 isolated from wheat rhizosphere (de Souza et al. 2003a, b). Two endorhizosphere bacterial isolates K-165 and 5-127 were identified as *Paenibacillus alvei* and *Bacillus amyloliquefaciens* based on the results of fatty acid analyses. These two isolates effectively inhibited the mycelial growth of *Verticillium dahliae* and reduced the disease severity significantly (Tjamos et al. 2004). Pure cultures of bacterial isolates from rice were multiplied in nutrient agar at 28 °C for 24 h and transferred to trypticase soy agar (TSA) plates containing 3 % trypticase soy broth (TSB) and 1.5 % Bacto agar (Difco) for 24 h at 28 °C. Loopfuls of bacterial cells were dispensed to test tubes covered with Teflon-lined screw cap. Gas-liquid chromatography equipped with a 5 % phenylmethyl silicone capillary column, an automated sampler, a flame ionization detection system and an integrator was employed to analyze the fatty acid composition of each bacterial isolate. FAME fingerprints were identified using a microbial identification system software package and a calibration mixture of known standards. The Gram-positive bacteria *Bacillus subtilis, B. cereus, B. megaterium* and *B. licheniformis* were identified based on FAME analysis. Similar results were obtained using Biolog System, confirming the identity of the bacterial species associated with rice (Jin-Yan et al. 2005). Isolates of *Bacillus megaterium* were effective against *Phytophthora capsici* causing, Phytophthora blight or crown blight of pepper (chilli). The species level identification of the isolates was achieved by applying FAME analysis. The similarity indices of the isolates ranged from 52 to 55 % (Akgül and Mirik 2008).

4.1.2.4 Green Fluorescent Protein Assay

The green fluorescent protein was isolated from the jelly fish *Aequorea victoria* and used as a reporter in a wide range of areas in biological and biotechnological investigations (Chalfie et al. 1994). Green fluorescent protein (GFP) assay has been shown to be useful for detecting the presence and distribution of GPF-tagged organisms in their substrates. Advantages of the use of GFP are that GFP is present within the cell as a product of gene expression and the visualization does not require any fixation or preparation methods that are time-consuming. Bacterial plasmids have been used to construct set of marker plasmids carrying *egfp* (green), *ecfp* (cyan), *eyfp* (yellow), *ebfp* (blue) and *rfp* (red). These marker plasmids have been useful to visualize plant growth-promoting rhizobacteria (PGPR) like *Pseudomonas fluorescens* (Bloemberg et al. 2000). If necessary plant material may be stored by fixing it before visualization with paraformaldehyde which keeps GFP intact for fluorescent studies (Bloemberg 2007).

It is possible to distinguish three different populations of *Pseudomonas fluorescens* WCS365 cells tagged with *ecfp, egfp* and *rfp* simultaneously in the rhizosphere (Bloemberg et al. 2000). The presence of mixed populations of *P. fluorescens* WCS 365 and *P. chlororaphis* PCL 1391 was detected by tagging a combination of GFP and Ds Red. Mixed colonies were mostly present on the upper root portions and *P. chlororaphis* preferentially colonized the root hairs (Dekkers et al. 2000). The GFP tagging approach was applied for monitoring of antifungal metabolite production by *P. fluorescens* CHA0 (Baehler et al. 2005). *Pseudomonas fluorescens* strain 1100-6 effectively protected apple against blue mold disease. The population levels of strain 1100-6 on wounded apples in the presence or absence of the blue mold pathogens *Penicillium expansum* or *P. solitum* were determined using the GFP as a marker. The GFP-transformed BCA cells were monitored by fluorescence microscopy or direct fluorescence scanning (Storm® Scanning System) with a digital imaging software for quantifying fluorescence produced by both techniques. A strong linear relationship between BCA cell number and fluorescence intensity of transformants was observed in 'Gala' apples inoculated with 1100-6 GFP in the presence or absence of challenge inoculation with pathogen species. The concentration of GFP-transformed cells increased over time at 20 and 5 °C in the presence or absence of pathogens. The presence of GFP-transformed BCA cells could not be detected in tissues taken one cm away from the site of inoculation, suggesting that the activity of the BCA was confined to the inoculation sites (Etebarian and Sholberg 2006).

Detection of bacteria in environmental samples using genetic markers has provided valuable information on microbial ecology. *Pseudomonas fluorescens* cells tagged with a red-shifted *gfp* reporter gene were assayed under nutrient starvation conditions at 40, 23 and 30 °C. The *gfp*-tagged *P. fluorescens* strain R2fG1 cells were introduced into bulk soil microcosms and soil microcosms with wheat seedlings. GFP-marked cells were enumerated immediately after inoculation into the soil and again in soil and root samples after 10 days by using epifluorescent microscope. Microcolonies were observed on root surfaces after immobilizing in agar and

incubation for 24 h (Cassidy et al. 2000). *P. putida* KT 2442, a derivative, was genetically tagged with *gfp* gene to study the development of biofilm. The biofilm formation by the BCA was studied using flow cells and confocal laser scanning microscopy (CLSM). Intrinsic labeling of this model biofilm using constitutively expressed proteins like GFP may be used for real-time biofilm, observation and generation of reliable quantitative data, comparable to the data obtained using conventional methods like nucleic acid staining (Nancharaiah et al. 2005).

4.1.2.5 Direct Colony Thin Layer Chromatography

Direct colony thin layer chromatography (TLC) was developed for rapid identification of lipids present in bacteria, making it possible for identification of bacterial species. One loopful of some bacterial colony (cells) suspension was applied directly on the origin line of a silica gel TLC plate followed by drying. The silica gel plate was developed with chloroform-methanol (2: 1, v/v) in well–moistured glass tank at 25 °C for 10 min, until the solvent front reached 6 cm line from the origin spots. The first run extracted the lipids from bacterial cells. The bacterial cells were scraped out from the glass plate, which was developed again at the same direction using chloroform-methanol-water (60: 25; 4 v/v/v) for 90 min at 25 °C in an incubator. Ninhydrin was sprayed after drying the plate and kept at 100 °C for the detection of aminolipids. Chromium containing sulfuric acid (0.6 % K_2CrO_7 in 55 % H_2SO_4) was sprayed and heated at 11 °C for visualizing the bench mark spots of *Pseudomonas* spp. The lipid profiles of different bacterial species showed significant variations. The effectiveness of direct colony TLC procedure for rapid identification of different *Pseudomonas* spp. in rRNA homology group II was demonstrated by Matsuyama (1995). High performance liquid chromatography (HPLC) in conjunction with direct colony TLC was effective in detecting the isolates of *Burkholderia* spp. (Matsuyama et al. 1998).

4.1.3 Immunoassays

BCAs have various spore forms containing different surface proteins. These proteins are antigenically distinct and useful for differentiating fungal species. The bacteria, on the other hand, have various immunodeterminants of capsular polysaccharide antigens, lipopolysaccharide (LPS) O and K antigens and murein lipoproteins that have been characterized in medically important genera of bacteria. Polyclonal antibodies (PAbs) and monoclonal antibodies (MAbs) specific to different species of bacteria have been raised. The procedures for the production of antisera containing PAbs and MAbs have been described in earlier publication (Narayanasamy 2001, 2005).

After application of bacterial BCAs, in the field, their populations have to be monitored at several intervals. This is necessary to improve biocontrol efficacy

through better formulation, minimizing wasteful applications and optimizing spray scheduling (Knudsen and Spurr 1987). Monoclonal antibodies against *Bacillus subtilis* were raised and MAb secreted by the clone 6B7E5 (IgG2b) was selected for optimizing in enzyme-linked immunosorbent assay (ELISA). The protocol developed in this study could be effectively applied to monitor *B. subtilis* throughout the season. Further optimization of ELISA was necessary to rule out variation between batches of commercial production (Towsen and Korsten 1995). Different formats of ELISA and other immunoassays have been employed for the detection, identification, differentiation and quantification of bacterial plant pathogens. However, only very few studies have been carried out for the detection and identification of bacterial biocontrol agents, compared to fungal biocontrol agents. It will be advantageous to adapt the immunoassays with necessary modifications of protocols for sensitive detection and precise identification of closely related bacterial species/strains with biocontrol potential. Since conventional isolation-based methods need long time, in addition to being less sensitive and reliable (Table 4.1).

Pseudomonas fluorescens (*Pf*) reduced the severity of banana Fusarium wilt disease caused by *Fusarium oxysporum* f. sp. *cubense* (FOC), when banana plants were pretreated with the BCA at the time of planting. Polyclonal antibodies against *Pf* and *FOC* were raised in rabbits. After purification the antibodies were conjugated with fluorescein isothiocyanate (FITC) for studying immunolocalization of *Pf-FOC*-and specific antibodies in banana root tissues. Colonies of *Pf* and *FOC* could be located in banana plants pretreated with *Pf* and challenged with *FOC*. The bacterial population was relatively greater towards the cortex region of the root, as compared to the stele region. A reduction (72 %) in the number of FOC colonies in treated plant tissues was noted. Once the bacteria gained entry into the root system followed by colonization of tissues, production of antifungal metabolites and growth-promoting compounds may be stimulated in treated plant tissues (Fig. 4.1; Mohandas et al. 2004) (Appendix 4.2).

4.1.4 Nucleic Acid-Based Techniques

In general, nucleic acid (NA)-based techniques have been found to be more sensitive, specific and reliable for detection, identification and quantification of bacterial species, than techniques based on biological, biochemical and immunological properties. Closely related organisms share greater nucleotide sequence similarity than those that are distantly related. A DNA fragment containing highly specific sequence in the bacterial genome can be identified. Probes or primers based on the sequences of nucleotides present in the DNA fragment may be employed as probes or primers for specific hybridization or amplification by polymerase enzyme. Among the nucleic acid (NA)-based techniques, polymerase chain reaction (PCR) assay or its variants have been extensively employed for detection and identification of bacterial biocontrol agents.

Table 4.1 Identification of bacterial biocontrol agents based on cultural and physiological characteristics

Bacterial biocontrol agent	Cultural characteristics (Medium)		Reference
A cultural characteristics *Pseudomonas* spp.	Yeast dextrose agar		Mavrodi et al. (2001), Yoshida et al. (2001)
Myxobacteria	CY agar		Bull et al. (2002)
Pseudomonas putida	MGY agar Trypticase soy agar (TSA)		Steddom and Menge (2001) Anith et al. (2004)
P. fluorescens strain SS 101	Kings's B medium with rifampicin		Mazzola et al. (2007)
P. fluorescens strain EPS 62 e	Luria-Bertani agar		Pujol et al. (2006)
Bacillus subtilus	Nutrient yeast dextrose agar (NYDA)		Kim et al. (2003)
Physiological characteristics *B. subtilis, B. Pumilus, B. amyloliquefaciens* *B. stearothermophilus*		API 50 CH	Gacitúa et al. (2009)
Pantoea agglomerans *B, subtilis*		Biolog System GN Physiological characteristics	Braun-Kiewnick et al. (2000), Cavaglieri et al. (2005)
B. subtilis		Biolog System	Jin-yan et al. (2005)
Burkholderca cepacia		Biolog Identification System	Sijam and Dikin (2005)
Lysobacter capsici		Biolog Identification System	Puopolo et al. (2010)
Bacterial biocontrol agent	Metabolic finger printing		References
B. Physiological characteristics *Pseudomonas chlororaphis* *P. aurantica*	Biolog Identification System		Ramarathnam and Fernando (2006)
P. fluorescens strains Q8r 1-96, Q2-87, IMI-96	Biolog Identification System		De La Feunte et al. (2007)
Pseudomonas sp. TEI 1	API ZONE		Pastor et al. (2010)
Pseudomonas sp. LSW25R	Biolog GN Microplate		Lee et al. (2010)
Streptomyces spp.	Bennett medium		Errakhi et al. (2007)
Serratia plymuthica HRO-C48	API and BIOLOG Systems		Kurze et al. (2001)
Pseudomonas fluorescens (A 506)	BIOLOG System		Fessehaie and Walcott (2005)
Pseudomonas aeruginosa	API 20 NE System		Al-Hinai et al. (2010)

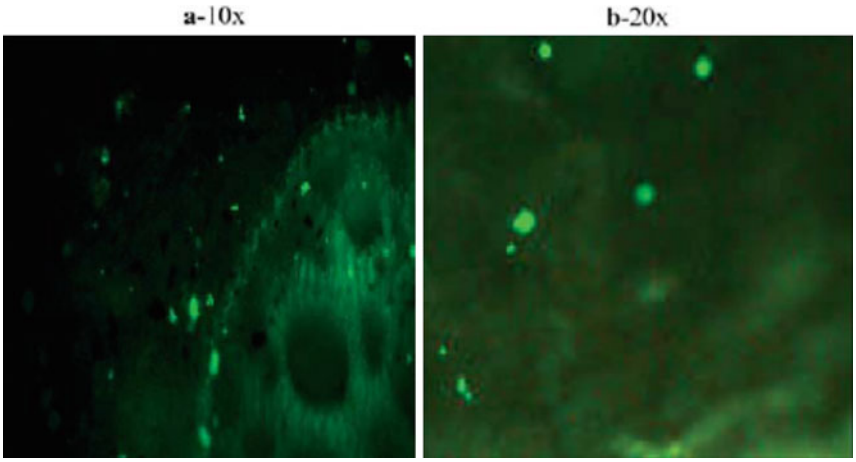

Fig. 4.1 Immunolocalization of *Pseudomonas fluorescens* and *Fusarium oxysporum* f.sp. *cubense* in the vascular tissues of banana plants as observed using fluorescence microscope (**a**) inoculated with pathogen alone; (**b**) plants treated with the bacterial biocontrol agent at 90 days before challenge inoculation with the pathogen. Note the reduction in pathogen colonies in plants pretreated with *P. fluorescens* (Courtesy of Mohandas et al. 2004 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

4.1.4.1 Polyacrylamide Gel Electrophoresis

In molecular biological investigations, analysis and manipulation of DNA of organisms are the fundamental features. Separating complex mixtures of DNA into different sized fragments is achieved more frequently by electrophoresis technique. The isolated DNA from target organism is treated with restriction enzymes which cleave the DNA at specific sites generating pieces small enough, to be resolved by electrophoresis in agarose or acrylamide gel. In conventional electrophoresis involving constant electrical field, DNA fragments migrate to different distances that are inversely proportional to the logarithm of their length. The sensitivity of the technique reduces with increasing size of the DNA. Polyacrylamide gel electrophoresis (PAGE) is useful especially to resolve the DNA fragments amplified by polymerase chain reaction (PCR). Two-dimensional (2-D) PAGE technique was employed to detect and differentiate *Erwinia* spp. and *Pseudomonas* spp. Electrophoretic profiles of acid ribosome enriched proteins of different strains of the bacterial species exhibit distinct patterns that were consistent (Moline 1985). Pulsed-field gel electrophoresis (PFGE) is useful for overcoming the limitations of PAGE procedure. It allows the electrophoretic separation of larger molecules in agarose gels. The PFGE patterns are obtained by assaying the digests of bacterial genomic DNA, using the specified restriction enzyme like Xba1 (Zhang et al. 1998).

Isolates of *Pseudomonas* sp. producing 2,4-diacetyl phloroglucinol (2,4-DAPG) have been found to be predominant constituents of rhizospheres of wheat and tobacco plants grown in soils that exhibited suppressiveness respectively to take-all

and black root diseases (de Souza et al. 2003a, b; Ramette et al. 2003). A simple and rapid method was developed to detect and determine the presence and genotypic diversity of 2,4-DAPG-producing strains in rhizosphere samples. The *phlD* gene is conserved among DAPG–producing *Pseudomonas* strains found world-wide. Denaturing gradient gel electrophoresis (DGGE) of the 350-bp fragments of *phlD*, a key gene involved in DAPG biosynthesis allowed discrimination of the genotypically different *phlD*$^+$ reference strains and indigenous isolates. The PCR-DGGE analysis provided a higher level of discrimination between *phlD*$^+$ genotypes than the *phlD* RFLP analysis. The detection limit of the PCR-DGGE analysis was approximately 5×10^3 CFU/g of root. Further, DGGE also allowed simultaneous detection of multiple *phlD*$^+$ genotypes present in mixtures of rhizosphere samples. The indigenous *phlD*$^+$ isolates (184) obtained from the rhizospheres of wheat, sugar beet and potato plants resulted in the identification of seven *phlD*$^+$ genotypes. The results showed that DGGE analysis of the *phlD* gene might be useful for the identification of new genotypic groups of specific antibiotic-producing *Pseudomonas* with different rhizosphere competence in soils cropped to sugar beet (Bergsma-Vlami et al. 2005).

Pseudomonas spp. producing the antifungal compound 2,4-diacetylphloroglucinol (Phl) have been detected in soils suppressive to the fungal pathogen *Thielaviopsis basicola* causing black root rot of tobacco as well as in conducive soils. However, these experiments were conducted only with a limited number of *Pseudomonas* isolates. Hence, an approach based on denaturing gradient gel electrophoresis (DGGE) of dominant *phlD* (gene encoding Phl) alleles from tobacco rhizosphere provided different *phlD* migration patterns. Sequencing of *phlD*- DGGE bands revealed a novel phylogenetic cluster of *phlD* sequences found in both suppressive and conducive soils. The population structure of *phlD*$^+$ pseudomonads depended more on the individual soil analyzed and its suppressiveness status than on inoculation of tobacco with *T. basicola*. The results indicated that *phlD*-DGGE might reveal additional *phlD* diversity compared with analyses of individual *Pseudomonas* isolates by other methods. This technique showed existence of differences in the *phlD*$^+$ *Pseudomonas* population structure in relation to disease suppressiveness (Frapoli et al. 2010).

4.1.4.2 Hybridization Techniques

Plant growth-promoting rhizobacteria (PGPR) have the ability to suppress development of diseases caused by a range of phytopathogens. Strains of *Pseudomonas fluorescens* (Pf) producing the polyketide antibiotic 2,4-diacetylphloroglucinol (DAPG) are the most effective in controlling root and seedling diseases such as black root rot of tobacco, crown and root of tomato, Pythium damping-off of cucumber and take-all of wheat (by *P. fluorescens* CHA0). Pythium damping off of sugar beet and soft rot of potato (by *P. fluorescens* F113) and wheat take-all of wheat (by *P. fluorescens* Q2–87 and Q8r-96). The DAPG-producing *Pseudomonas* spp. have major role in suppressing take–all disease during extended monoculture of wheat or barley (Cook and Weller 1987). The 2,4-DAPG biosynthetic locus contains six

genes *phlA, phlB, phlC, phlD, phlE*, and *phlF*, coding for the regulation, synthesis and export of 2,4–DAPG (Bangera and Thomashow 1999). Two distinct groups of 2,4-DPAG producers have been identified (Mavrodi et al. 2001). Strain Q 8r 1-96 is a representative of D-genotype 2,4-DAPG producers which account for the *phlD*$^+$ isolates effective against *Gaeumannomyces graminis* var. *tritici* (take-all disease) and Fusarium wilt of pea (*F. oxysporum* f. sp. *pisi*). This strain has exceptional root colonizing ability, distinguishing it from other pseudomonads in the rhizosphere (Landa et al. 2002b).

Studies on the process of root colonization by PGPR and other BCAs are important to understand how PGPRs establish and maintain a minimum threshold population size in the rhizosphere to be effective. Detecting the PGPRs introduced into the soil is challenging especially, when they are at low densities. Colony hybridization technique was employed to isolate and determine the frequency and diversity of indigenous 2,4-DAPG-producing fluorescent *Pseudomonas* spp. in the natural environment. This procedure was considered to be preferable for isolating the broadest range of genetically diverse 2,4-DAPG producers from soil and the rhizosphere, especially, when information on about genomic diversity is not available. On the other hand, the phlD-specific PCR-based assay was found to be less efficient for isolation of the full spectrum of genotypes present in a sample. The probe developed to detect 2,4-DAPG producers by colony hybridization was derived from a PCR fragment amplified from strain Q2-87 by primers Phl2a and Phl2b which were designed, based on the sequence of *phlD* from Q2-87. Regression analysis of population densities of ten 2,4-DAPG producing *Pseudomonas fluorescens* determined by traditional dilution plating on selective media, colony hybridization followed by PCR and phlD-specific PCR-based dilution end point assay showed that the relationship was linear ($P < 0.001$) and results of the techniques were similar. Colony hybridization technique revealed that the *phlD* probe derived from strain *P. fluorescens* Q8r1-96 hybridized more strongly to colonies of D genotypes than with colonies of A genotypes (Fig. 4.2). Colony hybridization alone overestimated the actual densities of some strains, thus requiring an additional PCR step to obtain realistic estimates of bacterial population (Landa et al. 2002a).

Isolates of 2,4–DAPG producers (over 300) from Europe and United States were differentiated into 17 distinct genotypes using fine-structure genetic analyses. The D-genotype isolates predominated in the roots of wheat and pea grown in the USA. The structural differences between the genomes of closely related bacteria may be resolved more precisely by genomic subtraction. The genomic suppressive subtractive hybridization (SSH) procedure was applied for identifying genes that contributed to the exceptional rhizosphere competence of D-genotype strains. The specific DNA sequences present in the superior root colonizer *P. fluorescens* Q 8r1-96, but not in the less rhizosphere-competent strain Q2-87 were cloned and their sequences were determined and analyzed. Their expression in the rhizosphere and distribution among 29 other 2,4-DPAG-producing strains, representative of 17 different genotypes, were assessed. Randomly selected clones containing subtracted fragments were amplified by PCR with nested primers 1 and 2 R and screened by hybridization to identify those containing tester-specific sequences. Over 80 % of

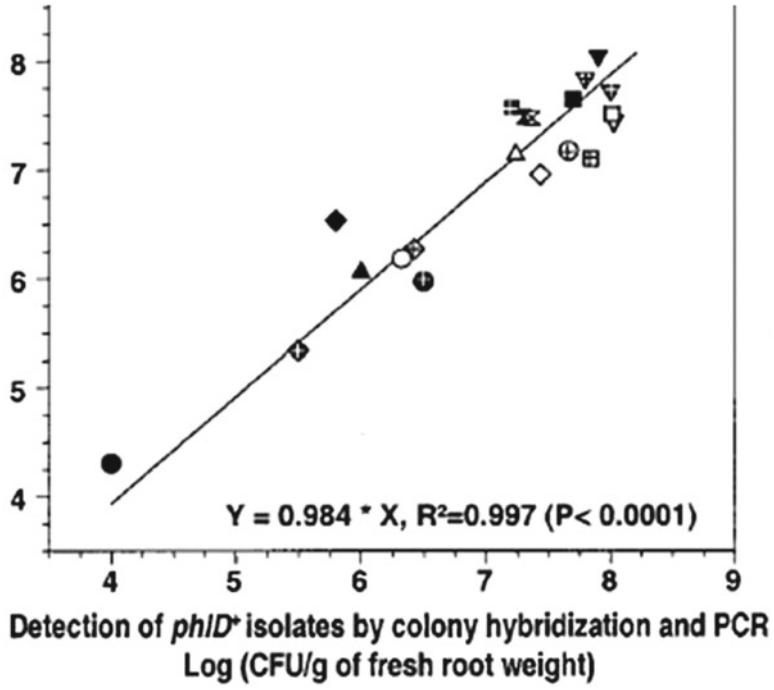

Fig. 4.2 Detection of *phlD*⁺ colonies of *Pseudomonas fluorescens*, using colony hybridization assay and enumeration of population densities of 2,4-diacetylphloroglucinol (DAPG) producers in the wheat rhizosphere (Courtesy of Landa et al. (2002a) and with kind permission of The American Phytopathological Society, MN, USA)

the 180 screened clones contained tester-specific DNA fragments ranging from 0.3 to 1.5 kb. Only 13 of these were present at least twice, among the 180 clones screened, indicating that the subtracted library represented a subset of the unique sequences present in the strain Q8r1-96. Of the 17 different genotypes were assessed, seven clones hybridized preferentially to DNA from strains with superior rhizosphere competence and sequences in two others were highly expressed in vitro and in the rhizosphere (Mavrodi et al. 2002).

Pseudomonas fluorescens Q8r1-96 strain produces 2,4-DPAG capable of suppressing several soilborne fungal pathogens including *Gaeumannomyces graminis* var. *tritici,* causing wheat take-all disease. This strain showed exceptional capacity to colonize aggressively and maintain large populations on the roots of crop plants like wheat, sugar beet and pea. Three genes, an *sss* recombinase gene, *ptsP* and *orfT* which are important in the interaction of *Pseudomonas* spp. with various host plant species were studied to assess their contributions to the highly efficient colonization properties of the strain Q8r1-96. Clones containing these genes were identified in a Q8r1-96 genomic library and sequenced. Mutants of Q8r1-96 were characterized to determine their ability to produce 2,4-DAPG, motility, fluorescence, colony morphology, exoprotease and hydrogen cyanide (HCN) production,

carbon and nitrogen utilization and ability to colonize the rhizosphere of wheat grown in natural soil. The *ptsp* mutant was impaired in wheat root colonization, whereas mutants with mutations in the *sss* recombinase and *orfT* genes were not affected. All three mutants were less competitive than the wild-type Q8r1-96 strain in the wheat rhizosphere. This investigation indicated that it would be possible to detect and identify strains of *Pseudomonas* spp. containing the genes involved in colonization of rhizosphere of different hosts by employing appropriate probes for hybridization with complementary DNA fragments of the test isolates of rhizobacteria (Mavrodi et al. 2006).

Strains of *Bacillus subtilis* have been shown to suppress fungal pathogens like *Botrytis cinerea* and *Rhizoctonia solani* which cause a range of crop diseases. The genomes of *B. subtilis* strains GB03 and QTS713 present in two commercial products Kodiak and Serenade respectively were compared with that of the strain 168 which has no defined biocontrol capacities to obtain a pool of DNA fragments unique to the two biocontrol strains. Suppressive subtractive hybridization (SSH) technique was employed to identify genetic markers that may be useful to select strains with biocontrol potential. The sequences of 149 subtracted fragments were compared with those available in GenBank. Sixteen subtracted fragments shared a high degree of similarity to sequences found in multiple *B.subtilis* strains with proven biocontrol potential. By using oligonucleotide primers specific to nine of genes involved in antibiotic synthesis and four additional genes not previously associated with biocontrol activities were identified. All nine markers were amplified from the commercialized strains GB03, OST713 and MB1600 with the exception of *ituC* which was absent in GB03. Sequencing of amplified markers revealed that all strains that scored positive for multiple markers were genotypically distinct strains. The strains containing amplifiable markers were more effective in inhibiting the growth of *R. solani* and *Pythium ultimum* than other *Bacillus* isolates that lacked the markers. This study showed that defined genetic markers might be very useful in studying the genetic diversity, ecology and biocontrol efficiency of bacterial species (Joshi and McSpadden Gardener 2006).

4.1.4.3 Polymerase Chain Reaction

Bacterial biocontrol agents (BCAs) are present in different substrates such as soil, water and plants. The traditional isolation-based methods have been found to be inadequate, because of the unculturability of many microorganisms and lack of sensitivity to detect them, when they are in low population densities. Molecular methods based on the polymerase chain reaction (PCR) have been demonstrated to be preferable alternatives to classical culture-dependent methods. Hybridization technique may not be useful, if the target bacterial species is contaminated with saprophytic microorganisms. The PCR assay may be applied by employing specific primers that can amplify the unique sequences of nucleotides present in the bacterial species even in the presence of contaminants. Furthermore, the sensitivity of detection is also at a higher level, in addition to the possibility of characterizing the

genotypic diversity of the plant pathogens, their antagonists and other plant associated microorganisms. Molecular analysis of microbial communities relies primarily on the availability of high quality DNA of the target organism. Hence, the method(s) of extraction of DNA especially from soil assumes great importance, since several compounds present in the substrates may inhibit PCR to varying extent. Several studies have been carried out to improve the efficiency of extraction and purification of soil DNA.

The effect of DNA extracts on the bacterial diversity detected within DNA extracted from three soils exhibiting contrasting physico-chemical properties was assessed. Two commercial DNA purification kits and a laboratory-devised method based on mechanical lysis were employed to extract DNA directly from soils. Amplified ribosomal DNA restriction analysis (ARDRA) and ribosomal intergenic spacer analysis (RISA) were employed to estimate the effect of the DNA extraction methods. The polymerase chain reaction (PCR)-based 16S ribosomal DNA analysis was performed to detect and quantity the bacteria. The results showed that soil DNA extraction methods could affect both phylotype abundance and composition of the indigenous bacterial community. The PCR efficiency of 16S or 16S-23S rDNA was affected by the extraction method or the soil matrix respectively. The RISA data indicated that direct molecular methods allowed the differentiation of soils according to their bacterial communities (Martin-Laurent et al. 2001). Four rhizobacterial strains effective against radish bacterial leaf spot disease caused by *Xanthomonas campestris* pv.*armoraciae* were identified as *Bacillus* sp. based on partial 16S rRNA gene sequencing, after amplifying the 16S rRNA genes using the oligonucleotide primers 8F and 519R (Krause et al. 2003).

The effectiveness of polymerase chain reaction (PCR)-based quantification of DNA in soil and other natural environments is influenced by variability in cell lysis efficiency and yield of DNA extraction. A technique was developed to minimize the effects of these constraints, providing at the same time a reliable internal control to distinguish between PCR-inhibition and negative results. *Pseudomonas fluorescens* Pf153 effective against tobacco and cucumber black root rot disease was used as the target organism for PCR quantification. The genetically engineered reference strain *P. fluorescens* CHA0/c2 was inoculated in a reference soil. CHA0/c2 and Pf 153 were lysed in parallel in respective soil samples and the lysates were mixed in known proportions. CHA0/c2 contained the plasmid pME6031-cmp2 that had an allelic variant (competitor) of the Pf 153 specific sequence. In the quantitative competitive PCR (QC-PCR) assay the competitor allowed the quantification of the target strain up to 0.66 Pf 153 CFU/mg of soil. Processing the reference strain in the same way as Pf 153 allowed exact quantification of the target strain in biocontrol assays performed in natural soil eliminating differences in DNA extraction efficiency and PCR amplification from different soil environments (Gobbin et al. 2007).

A bacterial species isolated from the soil suppressive to tomato Fusarium wilt disease caused by *F. oxysporum* f.sp. *lycopersici* was evaluated for its potential to control the disease. The genomic DNA was isolated using a DNeasy tissue kit (Qiagen). A fragment of the 16S rDNA was amplified by PCR assay. The PCR assay was performed by using universal eubacterial primers 27f and 1492r.

The PCR amplicon was cloned with a TOPO TA vector kit (Invitrogen) and sequenced using the M13 reverse and forward primers. Based on the sequence similarity to bacterial species available online from the National Center for Biotechnology Information (NCBI), the bacteria isolated, was identified as *Achromobacter xylosoxydans.* This bacterial species significantly reduced Fusarium wilt disease in tomato. The pathogen could not be isolated from the stem sections of asymptomatic plants protected by treatment with *A. xylosoxydans* (Moretti et al. 2008). Precise identification of the bacterial species/strain found to be effective against microbial plant pathogens is essential for registration of the bacterial BCA for commercial production as well as for monitoring the fate of the BCA strain introduced into the crop environment. Strain-specific sequence-characterized amplified region (SCAR) markers were developed to monitor the strain MA250 of *Pseudomonas brassicacearum* which effectively suppressed the wheat snow mold disease caused by *Microdochium nivale*. The SCAR marker OPA2-73 was employed for quantitative real-time PCR (Q-PCR) assay for estimating the population of the strain MA 250 in wheat seeds samples treated with the bacterial BCA. The strain MA 250 persisted for up to 3 weeks after sowing on kernel residues and the bacterial BCA colonized the roots of treated seedlings. The population of MA250 on bacterized seedling after 3 weeks was approximately 10^6 cells, compared with original inoculum of 10^6–10^7 cells/seed. The results demonstrated that the SCAR marker OPA2-73 could be used as a specific and sensitive tool for monitoring the bacterial strain employed for the management of wheat snow mold disease in environmental samples (Holmberg et al. 2009).

The bacterial biocontrol agent *Pseudomonas brassicacearum* strain MA250 was applied as seed treatment in a field trial with winter wheat. The fate and behavior of the seed-applied BCA was monitored, using sequence-characterized amplified region (SCAR) markers. Samples of below ground plant parts from healthy and snow mold-affected seedlings were collected at about 1 and 7 months after sowing, which was performed in early autumn. DNA was extracted from the root tissues and remaining parts of seeds with adhering soil. The population of the bacterial BCA was determined, using quantitative real-time PCR (q-PCR) assays. The BCA introduced through seeds persisted over the whole trial duration of 7 months. The below-ground plant tissues of each plant contained 10^6–10^7 cells, substantially less than the concentration of bacterial cells inoculated into the seed. In healthy seedlings, a shift in cell numbers from seeds to roots between the samplings was observed, suggesting colonization of roots during this time. The results indicated that the SCAR markers in combination with qPCR could provide information regarding the population of the bacterial BCA introduced through seeds that persists in the soil under field conditions (Holmberg et al. 2012).

Pseudomonas spp. have been shown to produce biosurfactants. Isolates of *Pseudomonas* obtained from wheat grown in agricultural soils produced a halo on SW medium, indicating their ability to produce a biosurfactant. These isolates were able to lyse zoospores of *Pythium ultimum* var. *sporangiferum.* The cell contents of the zoospores changed to a granular appearance and lysis of entire zoospore population was noticed within 60 s. Similar effects on zoospores of *Phytophthora*

infestans, *P. intermedium* and *Albugo candida* were observed. The cell culture supernatants of each of the six isolates induced lytic effect on zoospores. Random amplified polymorphic DNA (RAPD) analysis employing the 10-mer primers M12 and D7 revealed that the six isolates of *Pseudomonas* spp. were genotypically identical. The representative strain SS101 was identified as *P. fluorescens* biovar II based on the biochemical characterization by API20NE testing and gas chromatography (GC)-fatty acid methyl ester (FAME) analysis. The strain SS101, showed multiple characteristics indicative of surfactant production (de Souza et al. 2003a, b). Pyrrolnitrin (PRN) is an effective antibiotic involved in the suppression of a range of phytopathogenic fungi. An operon consisting of four genes *prnA, prnB, prnC* and *prnD* encoding the biosynthesis of pyrrolnitrin have been identified in *Pseudomonas fluorescens* (Kirner et al. 1998). A direct PCR assay was applied on soil DNA from agricultural soils for assessing the prevalence of *prnD* genes. The conventional PCR performed with the PRND1/PRND2 primer set yielded semi-quantitative data. The real-time quantitative (TaqMan™) PCR with specific primers located in the same *prnD* gene was employed to obtain more precise quantitative assessment. A range of soil-derived clones containing *prnD* gene fragments were analyzed by RFLP technique. Based on the restriction patterns generated by restriction enzymes *Hae*II, *Hinf*I and *Mnl*I, polymorphism among the *prnD* clones were observed and they were grouped in five different clusters. The TaqMan PCR system based on the *prnD* gene was found to be specific for *prnD* sequences from *Pseudomonas, Serratia* and *Burkholderia* species. The *prnD* gene was detectable up to a level of 60 fg or approximately 10 gene copies for amplification reaction. The real-time PCR assay of field soil samples indicated an enhanced presence of *prnD* genes in grassland or grassland-derived plots. The prevalence of PRN producers appeared to correlate with the extent of suppression of *Rhizoctonia solani* AG3, suggesting that PRN producing organisms might contribute to the natural suppressiveness of soils for *R. solani* AG3 (Garbeva et al. 2004; Appendix 4.3).

Strains of *Bacillus cereus* (4) and *B. substilis* (2) were found to inhibit the mycelial growth of *Fusarium oxysporum* f.sp. *sesame* causing sesame wilt disease. The genetic relationship of the six strains was investigated by employing RAPD-PCR technique using seven random primers. These primers generated multiple band profiles with a number of amplified DNA fragments ranging from 1 to 11. The size and number of amplified fragments also varied with different primers which indicated random pattern of amplification. These patterns of amplification revealed a genetic heterogeneity between the bacterial strains investigated. The primer (B3) generated a maximum of 53 fragments, while another primer (A3) produced the minimum number (20) of fragments. The markers employed were found to be very useful for differentiating the strains of *Bacillus*. The mean of genetic similarity among the six strains was 0.32 with a range of 0.15–0.49. Identification of the fragments specific to each strain may assist in the rapid identification of the strains of the bacterial species (El-Hamshary et al. 2008). Detection of bacterial BCAs unambiguously is a prerequisite for the valid large scale application in agricultural crops. Traditional methods based on phenotypic characteristics lack specificity and may introduce bias due to over or under estimation of target bacterial population. Converting random

amplified polymorphic DNA (RAPD) markers into sequence characterized amplified region (SCAR) markers that are specific for target DNA has been shown to be a diagnostic tool for detection and identification of bacterial isolates up to genus, species or even strain level (Felici et al. 2008). *Bacillus cereus* strain TS02 was found to be effective against the strawberry powdery mildew disease caused by *Sphaerotheca macularis*. The SCAR marker TSS_1, was used to monitor the dynamic changes in the population of the strain TS02 by amplification of the DNA collections from the strawberry leaves after the application of TS02 in the fields. The expected 400-bp SCAR marker of TSS, was amplified only in the DNA samples containing the strain TS02, but not in the DNA samples from 6 other strains of *B. cereus,* indicating the specificity of the assay. The detection limit of 78.13 pg/μl of DNA corresponded to a density of about 8×10^5 CFU/ml cells of strain TS02. The results demonstrated that the SCAR marker TSS_1, was highly effective in providing sensitive detection and identification of the strain TS02 in the samples from strawberry fields (Chen et al. 2010).

Pantoea agglomerans strains have been found to be effective in suppressing the growth of fungal and bacterial pathogens and the development of diseases caused by them. However, commercial registration of the *P. agglomerans* biocontrol products was hampered, because this species is listed as a biosafety level 2 (BL2) organism, due to clinical reports as an opportunistic human pathogen. Hence, precise identification and differentiation of the strains of *P. agglomerans* is essential to overcome the hurdles for registration. The taxonomy of a collection of clinical and plant-associated isolates was assessed using fluorescent amplified fragment length polymorphism (fAFLP) analysis of total genomic DNA and sequence analysis of specific genes such as 16S rDNA gene *crrs*, *gyrB* encoding DNA gyrase subunit B and the *P. agglomerans* quorum-sensing regulatory genes *pagRI* encoding homoserine lactone receptor and synthase. A single discriminatory marker was identified for biocontrol strains using fAFLP which could be used in biosafety decisions for registration of beneficial isolates. Only the isolates with biocontrol potential had this unique fAFLP band, even though all strains of *P. agglomerans* sensu stricto have indication of the gene found within the bank. For the differentiation purposes, this marker might be useful, because the purpose is to identify a genomic marker, but not a specific gene (Rezzonico et al. 2009).

Fluorescent *Pseudomonas* spp. are present abundantly in the rhizosphere and their ability to protect the plants against microbial plant pathogens and to promote plant growth has been demonstrated (Cook et al. 1995). The population of *Pseudomonas* in New Zealand soils was evaluated using a culture-dependent (Gould's S1) population estimate and a culture-independent real-time PCR combined with fluorescent TaqMan technology. As it was not possible to isolate all fluorescent pseudomonads, the cultivated fluorescent pseudomonads were not numerically dominant in comparison to the population determined by real-time PCR assay. Culturable *Pseudomonas* represented only a small proportion of <1 % of total *Pseudomonas* population. Further, the total *Pseudomonas* population itself accounted for only a small proportion of <1 % of the total bacterial population (Lloyd-Jones et al. 2005). The biocontrol activity of *Pseudomonas* spp. is

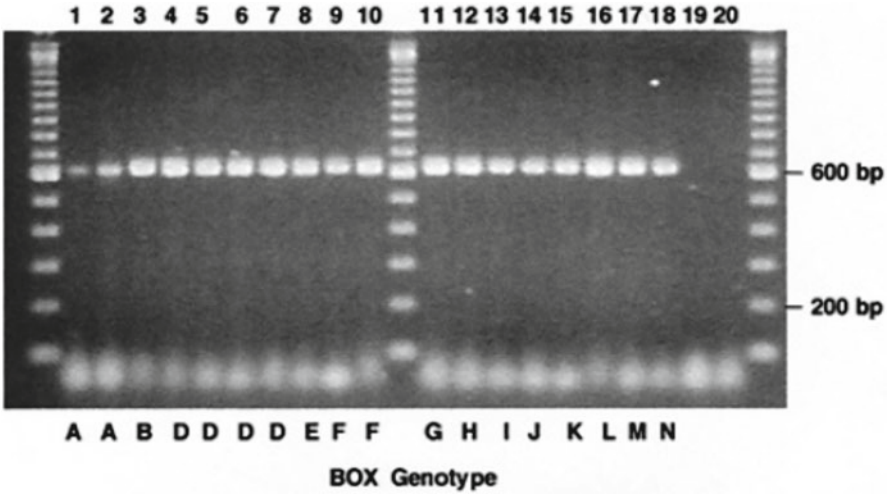

Fig. 4.3 Detection of genotypically distinct 2,4-DAPG-producing *Pseudomonas* spp. with primers B2BF and BPR4. Note the presence of a 629-bp PCR product amplified from whole-cell templates prepared from a diverse set of *phlD*$^+$ *Pseudomonas* spp. (*lanes 1* to *18*), but not from a *phlD*$^-$ strain (*lane 19*) or from cell-free reaction mix (*lane 20*). *Lane M*: 100-bp DNA size standard (Courtesy of McSpadden Gardener et al. 2001 and with kind permission of The American Phytopathological Society, MN, USA)

attributed to the presence of the *phlD* gene involved in the biosynthesis of 2,4-diacetylphloroglucinol (2,4-DAPG), capable of suppressing the development of several phytopathogens in the rhizosphere of crop plants. A rapid polymerase chain reaction (PCR)-based assay targeting the *phlD* gene which is predicted to encode a polyketide synthase that synthesizes monoacetyl phloroglucinol, the immediate precursor of 2,4-DPAG. Several new *phlD*-specific primers were designed based on the sequences of this gene. Only two primer sets BF2/BR4 and B_2BF/BPR4 were useful for amplifying *phlD* sequences from all the six strains tested. The latter set was chosen for further testing, since it amplified a greater portion of the *phlD* gene (629-bp as against 535-bp by the former set) (Fig. 4.3). The restriction patterns indicative of the distinct genotypes of *Pseudomonas* spp. were obtained by employing suitable restriction enzymes (Fig. 4.4). The procedure developed in this study could be used to quantify the abundance of *phlD*$^+$ pseudomonad populations and to directly characterize the genotype of the most abundant *phlD*$^+$ populations inhabiting the rhizosphere of wheat. This PCR assay, being a significant improvement over colony hybridization and less expensive, required less labor, because of using multiwell plates and an eight-channel multipipettor. The sensitivity of detection of the *Pseudomonas* spp. could be improved by performing an additional step of incubating the aliquots of the rhizosphere washes in the selective medium for pseudomonads (MCSpadden Gardener et al. 2001).

The genus *Pseudomonas* has been subjected to several taxonomic revisions. The DNA-RNA hybridization techniques recognized 5 rRNA similarity groups. The group 1 is considered as the "true" *Pseudomonas* or *Pseudomonas* "sensu stricto"

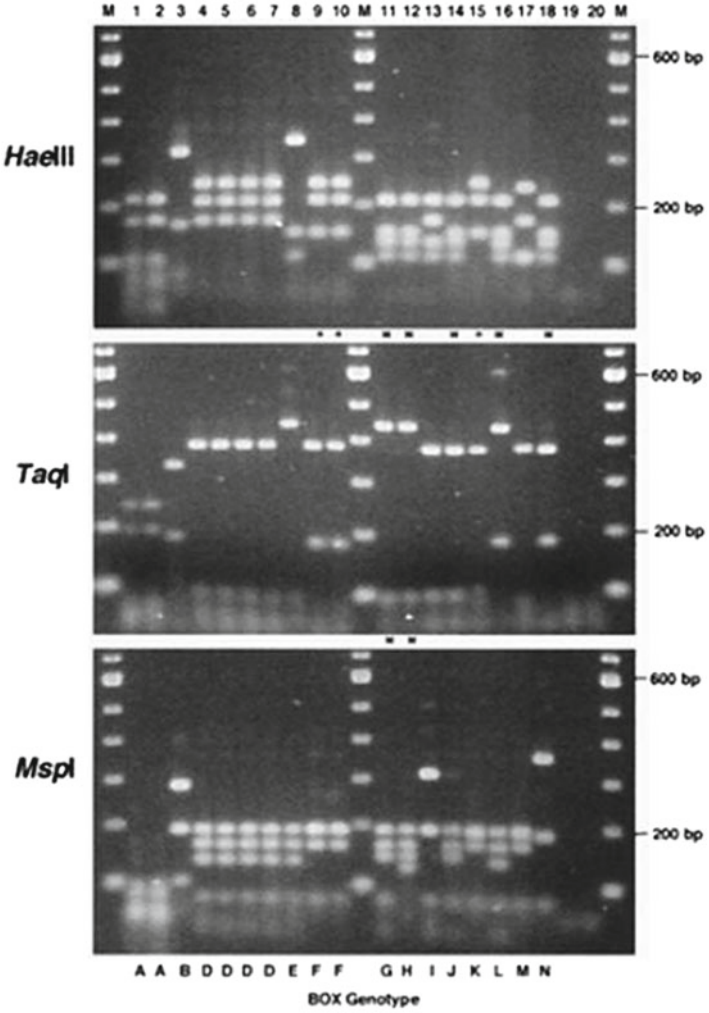

Fig. 4.4 Restriction fragment length polymorphism (RFLP) analysis of *phlD* sequences amplified with B2BF and BPR4 and digested sequentially with restriction enzymes *Hae*III, *Taq*I or *Msp*I RFLPs detected after treatment with these enzymes could differentiate all 13 genotypes of *Pseudomonas* spp. *Lane 1*: CHA0; *Lane 2*: Pf-5; *Lane 3*: Q2-87; *Lane 4*: Q8r1-96; *Lane 5*: W2-6; *Lane 6*: OC4-1; *Lane 7*: FEL1R9; *Lane 8*: Q2-2; *Lane 9*: JMP6; *Lane 10*: JMP7; *Lane 11*: FEL1R18; *Lane 12*: CV1-1; *Lane 13*: FTAD1R36; *Lane 14*: FEL1R22; *Lane 15*: F113; *Lane 16*: W4-4; *Lane 17*: D27B1; *Lane 18*: HT5-1; *Lane 19*: 2-79 and *Lane 20*: none; and *Lane M*: 100-bp DNA size standard (Courtesy of McSpadden Gardener et al. 2001 and with kind permission of The American Phytopathological Society, MN, USA)

(Kozo 1995). *P. fluorescens* included in group 1 is a heterogeneous species that can be subdivided into several biotypes/strains. A rapid PCR-based species-specific procedure was developed to detect, identify and distinguish different biotypes of this species by combining molecular and biochemical methods. The target regions

for amplification by PCR primers 16SPSE flu F and 16SPSER were identified at locations 482–521-bp and 1311–1351-bp for all the biotypes of *P. fluorescens* tested. A single DNA fragment of 850-bp for 16S rRNA was amplified only from the DNA of *P. fluorescens*. Restriction fragment length polymorphism (RFLP) analysis was performed by digesting the 16S rRNA region with the restriction enzymes *Vsp*1, *Hae* III and *Taq*I. The comparison of the RFLP profile from the combination of three enzymes along with a biochemical test (levan production) provided a reliable, rapid and less expensive method for routine identification and differentiation of *P. fluorescens* and its biotypes A, B, C and 3 (Scarpellini et al. 2004; Appendix 4.4).

Grapevine crown gall disease is one of the serious diseases, causing substantial production losses. *Agrobacterium vitis* strain E26 has been found to be effective in suppressing the development of the crown gall disease in grapevine. Risk assessment of BCAs for the control of plant diseases under field conditions has become a necessary requirement, before commercialization of the potential biocontrol agents. The information on the safety or environmental risks of the strain E26 was lacking. Hence, the PCR assay and Southern blot analysis were employed to determine the presence of five essential virulence genes viz., *virA*, *virG, iaaH, iaaM* and *ipt* in the strain E26. Primers and probes were designed based on the conserved regions of each gene. The results indicated that the essential virulence genes *virA* and *virG* determinants were absent in the strain E26, suggesting that this strain would be unlikely to elicit crown gall symptoms in either host or nonhost plant species. It appeared that the *iaaH*, *iaaM* or *ipt* genes were also not present in the strain E26. A new procedure by combining PCR and Southern blot analysis was developed facilitating the examination of the pathogenicity of potential BCAs belonging to the genera enclosing the pathogen(s) (Wei et al. 2009).

Production of antibiotics by *Pseudomonas fluorescens* for the suppression of pathogen and the disease development is important. *P. fluorescens* Pf-5 is reported to produce at least three antibiotics including 2,4-diacetylphloroglucinol (2,4-DAPG). Among the three genes involved in the biosynthesis of 2,4-DAPG, *phlD* has been used as a genetic marker for detecting and identifying DAPG-producing pseudomonads. A rapid and inexpensive method was developed for assessing the abundance and diversity of the most prolific *phlD*$^+$ *Pseudomonas* spp. present in the rhizosphere (McSpadden Gardener et al. 2001). Likewise, the abundance and diversity of phlD$^+$ *Pseudomonas* spp. colonizing the rhizospheres of young field grown corn and soybean plants were investigated over a period of 3 years by employing a PCR based-technique to characterize *phlD*$^+$ populations. The D genotype was detected on all soybean plants collected from all 15 countries examined. The most frequently observed genotypes of the seven genotypes (A, C, D, F, I, R and S) were A and S genotypes, both of which were found on corn and soybean roots obtained from many locations. All isolates showed ability to inhibit the growth of *Pythium irregulare* SF2 in vitro. However, the A genotype exhibited was significantly more inhibitory, when tested against multiple oomycete pathogens isolated from soybean. Furthermore, the A genotype was able to supplement the activities of native population resulting in increased soybean stand and yields (McSpadden Gardener et al. 2005). Two strains of *P. fluorescens* effective against. Septoria tritici blotch (STB) disease of

wheat caused by *Mycosphaerella graminicola* were identified based on their 16S rRNA, ITS1, *gyrB* and *RpoD* sequence homologies (Kildea et al. 2008).

Pseudomonas fluorescens (*Pf*) strains F113 and CHA0 have been used as model strains in biocontrol investigations. It is essential to monitor the persistence of the bacterial BCA strains applied for the suppression of the plant pathogens and the diseases induced by them. A strain-specific real-time PCR quantification assay was developed based on the sequence characterized amplified regions (SCAR) for strains F113, CHA0 and Pf 153. By using the plasmid APA9 as the internal standard to normalize CT values, the differences in DNA extraction efficiencies from rhizosphere sample could be circumvented. The detection limits of the real-time PCR assays for all three strains were about 10 cells for genomic DNA and 10^4 cells/g rhizosphere for maize samples grown in different natural soils. Population sizes of these strains in the rhizosphere were similar and they persisted differently over a period of 5 weeks. The procedure developed in this study was found to be rapid and resource-efficient for monitoring target biocontrol strain(s) in natural soil, making it a suitable technique for large scale field application (Von Felten et al. 2010).

Pseudomonas spp. are known to be present abundantly in the soil as saprophytes. But the abundance and population structure of pseudomonads may vary at different depths in the soil. Soil samples were collected from long- (>1,000 years) and short- (>50 years) term grapevine monocultures in Switzerland. Soil samples were baited with grapevine and rhizosphere pseudomonads containing the biocontrol genes *phlD* (2,4-diacetylphloroglucinol synthesis) and/or *hcnAB* (hydrogen cyanide synthesis) were analyzed by the most probable number (MPN)-PCR format. The numbers of total, *phlD*$^+$ and *hcnAB*$^+$ pseudomonads decreased with increase in soil depth. Further, the percentages of *phlD*$^+$ (except in short-term monoculture) and *hcnAB*$^+$ pseudomonads were also lower in deep soil layers. Restriction fragment length polymorphism (RFLP)-profiling of *phlD*$^+$ and *hcnAB*$^+$ pseudomonads revealed the presence of 3 *phlD*$^+$ alleles and 12 *hcnAB*$^+$ alleles, which decreased in relation to soil depth. The *phlD* allele was found both in top soil and deeper layers, while one *hcnAB* allele (*K*) was observed only in deeper horizons in long-term monoculture. The results suggested that certain *Pseudomonas* ecotypes might have adapted to specific soil depths and the *phlD* and *hcnAB* genotypes could be selected based on soil depth (Svercel et al. 2010).

Two distinct culture-independent approaches have been made to rapidly recognize functionally important microbes such as biocontrol agents (BCAs). The first approach depends on the use of genetic markers for a functionally important activity such as antibiosis. This approach was applied to identify and recover novel genotypes of 2,4-diacetylphloroglucinol (2,4-DAPG)-producers from rhizosphere of field-grown crop plants (McSpadden Gardener et al. 2005). The second approach referred to as microbial community profiling is based on molecular profiling of microbial population structure. The ribosomal gene sequences are targeted, amplified from the rhizosphere environment and analyzed. The terminal restriction fragment length polymorphism (T-RFLP) which is a low-cost, low-resolution technique has been shown to be very useful to recognize generalist populations that have the potential to contribute to the suppression across environments. The T-RFLP

analyses were employed to compare the bacterial community structure in soils differing in their disease-suppressive potential. Multiple bacterial populations differentiated by different TRFs showed a positive association with disease suppression in tomatoes and soybean (Benítez et al. 2007). The effectiveness of T-RFLP technique in differentiating two microbial communities present in soils suppressive (SS) and conducive (CS) to potato common scab disease caused by *Streptomyces scabies* was demonstrated by Meng et al. (2012). The biological basis of naturally occurring soil suppressive to potato common scab disease caused by *Streptomyces scabies* in the field was studied. This field with suppressive soil and an adjacent nursery conducive to scab were investigated using pyrosequencing to compare the two microbial communities. Total DNA was extracted from both the disease-suppressive and conducive soils. A phylogenetically taxon-informative region of the 16S rRNA gene was used to establish operational taxonomic units (OTUs) to characterize bacterial community richness and diversity. In total, 1,124 OTUs were detected and 565 OTUs (10 % dissimilarity) were identified in disease-conducive soil and 859 in disease-suppressive soil, including 300 shared both between sites. Based on relative sequence abundance, Acidobacteria, Proteobacteria and Firmicutes were identified. Sequences of *Lysobacter* were found in significantly higher numbers in disease suppressive soil, as were sequences of group 4 and group 6 Acidobacteria. The relative abundance of sequences identified as the genus *Bacillus* was significantly higher by an order of magnitude in the disease conducive soil (Rosenzweig et al. 2012).

A molecular-profile data of bacterial community structure was applied to recover two novel groups of rhizosphere bacteria by using sequence-based terminal restriction fragment length polymorphism (T-RFLP)-derived molecular markers for detection, identification and isolation of the bacterial species *Mitsuaria* and *Burkholderia* involved in the suppression of damping-off disease. Multiple sequences matching TRF M139 and M141 were cloned. Sequences matching TRF M148 displayed greater sequence diversity. *Mitsuaria* spp. and *Bukholderia* spp. with high levels of sequence similarity to the targeted M139 and M141 TRF respectively could be isolated. As predicted, these isolates of the two bacterial species displayed the expected function of reducing the growth of oomycete plant pathogen (*Pythium aphanidermatum*) growth in vitro and reducing disease severity in infected tomato and soybean seedlings. The protocol developed in this study established the direct connection of TRF-derived molecular markers to isolates capable of expressing an ecologically important microbial function like biocontrol potential. Further, the procedure was shown to be less expensive, providing the possibility for wider applications (Benítez and Mc Spadden Gardener 2009). Fluorescent pseudomonads have a world-wide distribution providing protection to crop plants against biotic stresses. A multilocus sequence analysis of a world-wide collection of *Pseudomonas* strains producing 2,4-diacetylphloroglucinol (2,4-DAPG) indicated the existence of six main groups (genotypes) (A-F). In order to achieve precise identification of these groups in environmental samples, a T-RFLP method was developed based on the *phlD* gene encoding the 2,4-DAPG. A combination of six restriction enzymes was useful to identify group-specific terminal fragments (T-RF). The detection limits of

the *phlD*-T-RFLP procedure for the two *P. fluorescens* strains F113 (group B) and CHA0 (group F) obtained from the rhizosphere samples were found to be 5×10^3 CFU/g and 5×10^4 CFU/g respectively. The *phlD*-T-RFLP and *phlD*-DGGE analysis of wheat and maize root samples from greenhouse and field revealed the existence of the multilocus groups A, B and D. The frequency of detection by *phlD*-TRFLP of these groups was greater, in addition to the detection of pseudomonads belonging to groups C and F which were not detected by *phlD*-DGGE technique. The results showed that the *phlD*-T-RFLP method was rapid and reliable for detecting the strains of all groups of DAPG-producers in environmental samples more efficiently (Von Felten et al. 2011).

Pseudomonas spp. producing 2,4-DAPG have been shown to have a key role in the natural suppression of *Gaeumannomyces graminis* var. *tritici* causing take-all disease of cereals. Two major phenotypic groups have been distinguished among 2,4-DAPG-producers, based on the production of antifungal compounds. One group synthesizes 2,4-DAPG, hydrogen cyanide and pyoluteorin, while the second group can synthesize only the first two compounds. Diversity within *phlD*, an essential gene in the biosynthesis of 2,4-DAPG was assessed, using the restriction fragment length polymorphism (RFLP) analysis of 123 isolates, producing DPAG from various locations in the US and other countries. Clusters defined by RFLP analysis of *phlD* correlated closely with clusters defined earlier by BOX-PCR genomic fingerprinting, indicating the usefulness of *phlD* as a marker of genetic diversity and population structure among 2,4-DAPG producers. Random amplified polymorphic DNA (RAPD) analysis revealed the highest degree of polymorphism among strains studied. Five of the groups (B, D, E, F, and J) defined by *phlD*$^+$ RFLP and BOX-PCR techniques were divided into 17 groups by RAPD analysis. The RAPD analysis also revealed more genotype diversity than rep-PCR assay. Both methods could provide a high degree of discrimination in analysis of population structure. The results indicated that the gene *phlD* could be used to study the genetic diversity and population structure of *Pseudomonas* strains (Mavrodi et al. 2001). Biocontrol agents introduced into the soil or other substrates, must be able to establish and maintain a certain threshold population density to preempt or limit infection by the pathogen or induce host defenses. Selecting suitable rhizosphere competent strain may considerably contribute to enhance the effectiveness of biocontrol. Study was taken up to determine the diversity of indigenous 2,4-DAPG-producing *Pseudomonas* spp. occurring in the roots of wheat grown in soil naturally suppressive to take-all disease. Random amplified polymorphic DNA (RAPD) analysis resulted in the formation of 16 different groups. One RAPD group accounted for 50 % of the total population of DAPG-producing *Pseudomonas* strains. This dominant group represented by *P. fluorescens* Q8r1-96 was found to be highly adapted to wheat rhizosphere. The Q8r1-96 strain was able to maintain a rhizosphere population density of approximately 10^5 CFU per g of root after eight successive growth cycles of wheat in three different raw virgin soils. In contrast, population of two other strains dropped rapidly and they could not be detected after seven cycles. However, the biochemical analysis showed that the superior rhizosphere competence of Q8r1-96 strain was not dependent on in situ DAPG production levels. The results indicated

that by exploiting diversity of antagonistic bacterial species, the efficiency of biocontrol might be improved (Raaijmakers and Weller 2001).

Colonization of *Pseudomonas fluorescens* strain Pf 29A was assessed on the roots of healthy wheat plants and of plants with take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (*Ggt*). The effect of this bacterial species on indigenous populations of fluorescent pseudomonads was also investigated. The efficacy of the strain Pf29A in suppressing wheat take-all disease in 5-week old wheat seedlings was assessed in non-sterile conducive soil in a growth chamber. Random amplified polymorphic DNA (RAPD) fingerprinting with a decamer primer was used to monitor the strain Pf29A and culturable indigenous rhizoplane populations of fluorescent pseudomonads. The strain Pf29A reduced the disease severity and accounted for 44.6 % of the culturable fluorescent pseudomonads on healthy plant rhizoplane and 75.8 % on diseased plant rhizoplane. Fewer RAPD patterns were obtained, when Pf29A was introduced into the soil with *Ggt*. In the presence of *Ggt* and necrotic roots, the strain Pf 29A became the dominant root colonizer and drastically changed the diversity and the structure of indigenous fluorescent pseudomonad populations. The results revealed that *Ggt* and reduction of lesion on roots might trigger a specific increase in antagonist populations and introduction of the bacterial BCA into the soil could influence the structure of native bacterial populations (Chapon et al. 2002). The root-colonizing fluorescent pseudomonads including *Pseudomonas fluorescens* produce different kinds of metabolites extracellularly with antimicrobial properties. Strains of *Pseudomonas* spp. with biocontrol potential have been identified functionally, based on amplified ribosomal DNA restriction analysis (ARDRA) fingerprints (Tran et al. 2008). The genetic diversity of *P. fluorescens* strains isolated from the rhizosphere was assessed by two PCR-based molecular techniques, random amplified polymorphic DNA (RAPD) and rep-PCR assay. A total of 40 oligonucleotide primers were used in RAPDs and 26 were found to be polymorphic. The number of DNA bands amplified varied from 2 to 10 with each primer and the level of polymorphism was 100 % in all assays. *P. fluorescens* isolates were divided into 5 major classes. A high level of polymorphism was seen in PCR with REP, ERIC and BOX set of primers. The total number of polymorphic DNA bands obtained in this method was almost similar to that observed with RAPD analysis. The rep-PCR profiles of *P. fluorescens* strains revealed that with this technique each set of subspecies could be easily distinguished, as they generated unique profiles with each primer set. The rep-PCR assay could be used to identify the subspecies to which each of the isolates belonged, which was not the case with RAPDs. Rep-PCR format may be preferable, because it targeted a specific region of the genome of the test organism and the results were found to be reproducible (Charan et al. 2011).

The interactions between two bacterial BCAs *Pseudomonas corrugata* IDV1 and *P. fluorescens* UA5-40 with the bacterial pathogen *Ralstonia solanacearum* biovar 2, causative agent of potato brown rot disease were investigated by a polyphasic approach. Molecular techniques, fluorescent in situ hybridization (FISH) and PCR-DGGE were applied on plant DNA extracts. Concomitant with the presence of *P. corrugata* IDV1, plant invasion by *R. solanacearum* was hampered, but not

abolished. The PCR-DGGE analyses of the tomato rhizoplane supported the evidence for the antagonistic activity against the pathogen. Using the FISH technique, differences in root colonization between the pathogen and *P. corrugata* IDV1 could be observed. *R. solanacearum* was detected in the vascular cylinder of tomato plants, while the presence of strain IDV1 was not detected by this technique. Potato plants were protected against *R. solanacearum* inoculated in soil as revealed by FISH technique, since the pathogen cells were not found in stems of several plants tested. This investigation indicated that the polyphasic analysis might be a valuable tool for testing antagonistic strains for approval as biocontrol agents in agricultural applications (van Overbeek et al. 2002).

The microorganisms present in used rockwool slabs employed in hydroponic systems for cucumber were found to be responsible for suppression of root rot disease caused by *Pythium aphanidermatum.* The composition of the bacterial and actinomycete population in the rock wool responsible for disease suppression was studied by applying PCR-DGGE technique. The diversity of the bacterial and actinomycete PCR-DGGE patterns had a lesser effect on disease suppressiveness, but showed a positive association in all treatments. Three bacterial PCR-DGGE bands that occurred only in the two suppressive treatments and not in sterilized treatments, were identified as *Serratia marcescens* (100 % similarity), *Pseudomonas* spp. (98 %) and *Pseudomonas putida* (100 % similarity) which are known for the antagonistic and root colonizing potential (Paulitz et al. 1992). In a later study, the bands from actinomycete-specific PCR-DGGE patterns which were sequenced, exhibited high similarity (96–100 %) with sequences of *Streptomyces* spp. Disease suppressiveness showed high correlation with the culturable number of filamentous actinomycetes. The filamentous actinomycetes enumerated with the plate counts were mainly *Streptomyces* spp. of which 10 % were antagonistic towards *P. aphanidermatum* in dual culture (Postma et al. 2005). The role of microorganisms present in soilless crop systems has been studied. The effectiveness of antagonistic strains against *Phytophthora cryptogea* infecting gerbera in closed soilless systems was assessed by Garibaldi et al. (2003). A key role was reported by Minuto et al. (2007) for resident microflora in used rockwool, in suppressing tomato root rot disease caused by *Fusarium oxysporum* f.sp. *radicis-lycopersici.* The bacterial strains isolated from the recycled soilless substrates and suppressive soils were evaluated for their efficacy in suppressing the Fusarium wilt disease of tomato. Five bacterial strains were identified as *Pseudomonas* spp. by PCR amplification of 16S rDNA sequences, using universal bacterial primers 704F and 1495R. The PCR products were resolved on 2 % agarose gels. The bacterial strains belonged to *Pseudomonas putida* FC-6B; *Pseudomonas* sp. FC-7B; *P. putida* FC-8B; *Pseudomonas* sp. FC-9B and *Pseudomonas* sp. FC-24B. All bacterial species increased plant growth, in addition to the protection against Fusarium wilt disease. The strains *P. putida* FC-6B and FC-8B and *Pseudomonas* sp. FC-9B were more effective in suppressing the pathogen and the disease induced (Srinivasan et al. 2009).

Pseudomonas putida strain P9, a novel competent endophyte causes cultivar-dependent suppression of potato late blight pathogen *Phytophthora infestans*. The fate of P9 strain and its rifampicin-resistant derivative P9R was monitored by

examining the colony growth and by performing PCR-denaturing gradient gel electrophoresis (DGGE) procedure. The PCR-DGGE with bacterial and *Pseudomonas-specific* primers was performed with rhizoplane and endosphere DNA extracts during growth in different potato cultivars. A clear and conspicuous band comigrating with the P9 band was found in the bacterial and *Pseudomonas* rhizoplane endosphere fingerprints of all P9-treated plants. The band representing P9 was detected in nontreated plants. The strain P9R band comigrated with the P9 band in PCR-DGGE performed with bacterial and *Pseudomonas*-specific primers. The numbers of P9R CFU and the P9R-specific band intensities were positively correlated. The results indicated that the strain P9 could be an efficient colonizer of potato plants capable of competing with microbial population indigenous to the potato phytosphere (Andreote et al. 2009). A Gram-negative *Pseudomonas* sp. isolate LSW25 was antagonistic to *Pseudomonas corrugata* causative agent of tomato vein necrosis disease. A spontaneous rifampicin-resistant mutant LSW25R was selected to monitor the pattern of colonization on tomato radicals using scanning electron microscope (SEM). For the identification of LSW25R, sequences of 16S rRNA and 16S/23S rRNA intergenic spacer (ITS) region were compared. In addition to fatty acid composition and carbon sources utilization profiles, the spontaneous mutant LSW25R retained its resistance to the antibiotic even after successive transfers on King's medium B without antibiotics. Sequences of 16S rRNA from LSW25R showed 99 % sequence identity to the corresponding region in different *Pseudomonas* spp. and the ITS sequence had 98 % sequence similarity with that of *P. fluorescens* strains. However, the fatty acid analysis and profiles of Biolog Identification System did not corroborate the results of sequence analysis. Hence, the strain LSW25 and its mutant LSW25R were tentatively identified as strain of *Pseudomonas* sp. (Lee et al. 2010).

The biocontrol efficacy of three bacterial antagonistic species *Pseudomonas fluorescens* L13-6-12, *P. trivialis* 3 Re2-7 and *Serratia plymuthica* 3Re4-18 was assessed against *Rhizoctonia solani,* causing bottom rot disease of lettuce. The BCAs were inoculated onto lettuce seedlings at 7 days before and 5 days after planting in naturally pathogen-infested field soil. The bacterial BCA provided significant protection to lettuce seedlings against *R. solani*. Single-strand conformation polymorphism (SSCP) analysis of 16S rRNA gene or ITS1 fragments revealed a highly diverse rhizosphere and less diverse endophytic microbial community for lettuce. The complexity of the rhizosphere *Pseudomonas* communities increased with lapse of time. SSCP profiles from endophyllosphere communities formed a unique group at 50 % similarity, apart from the root-associated communities. Negligible short-term effects on bacterial communities, after the introduction of endophytic and rhizospheric BCAs into *Rhizoctonia*-infested lettuce fields, were observed. Comparison of the number of SSCP bands of total bacterial profiles suggested that pseudomonads represented a large proportion of the total bacterial communities of field-grown lettuce. This investigation clearly revealed the presence of potentially human pathogens such as *Staphylococcus* sp., *Burkholderia* sp., *Cladosporium* sp. and *Aspergillus* sp. on or inside lettuce that is eaten as salad, indicating the need for assessing the pathogenic potential of plant-associated bacteria (Scherwiniski et al. 2008).

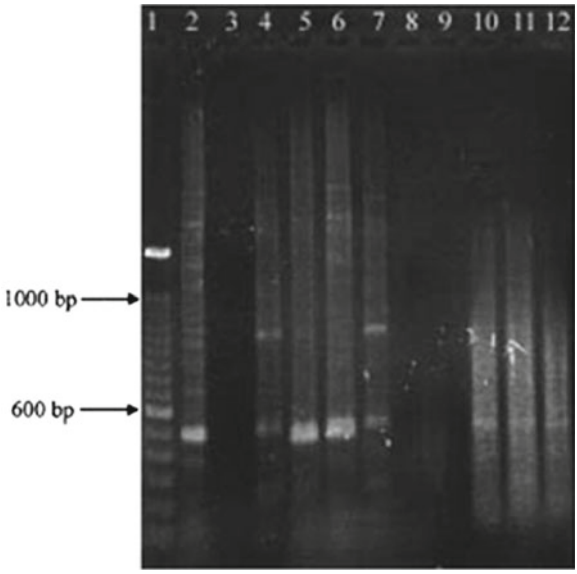

Fig. 4.5 Detection of strains of *Bacillus subtilis* from rhizosphere samples by agarose gel electrophoresis of the PCR products using TGD and LGG primers *Lane 1*: 100-bp DNA size marker; *Lane 2*: *B. subtilis* strain ATCC33677; *Lane 3*: sterile water; *Lane 4*: strain UBA30-35; *Lane 5*: strain UBA30-31; *Lane 6*: strain UBA30-32; *Lane 7*: strain UBA30-33; *Lane 8*: strain UBA30-34; *Lane 9*: UBA30-35; *Lane 10*: rhizosphere sample from field 1; *Lane 11*: rhizosphere sample from field 2; *Lane 12*: rhizosphere sample from field 3 (Courtesy of Giacomodonato et al. 2001 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

Rhizosphere supports the development of various kinds of bacteria, fungi and other organisms. Isolation of bacteria directly from suppressive soybean soils might be an effective method for selection of putative biocontrol agents, since they may be well adapted to survive in the soil in contrast to laboratory-modified strains. Selection and evaluation of bacterial strains for antifungal activity in natural environments is time-and energy-consuming. The genus *Bacillus* includes *B. subtilis*, *B. cereus*, *B. megaterium, B. pumilus*, *B. licheniformis* and *B. brevis* capable of producing antifungal antibiotics which have been effective against crop pathogens (Edwards et al. 1994). *Bacillus* spp. also produce spores that are dormant structures resistant to desiccation, heat, UV-irradiation and organic solvents. The ability to produce antibiotics and spores makes *Bacillus* spp. attractive candidates for formulation and commercialization for use against phytopathogens. Polymerase chain reaction (PCR)-based methods provide greater level of specificity, sensitivity and rapidity, compared with isolation-based tests. Screening of *Bacillus* strains from soybean field soils was performed by PCR amplification, using degenerate primers to target sequences of peptide synthetase genes. The DNA obtained from six *Bacillus* strains isolated from soybean rhizosphere and *B. subtilis* ATCC 33677 was amplified by primers TGD and LGG. The PCR amplification of the product (500-bp) occurred in four *Bacillus* strains and *B. subtilis* (Fig. 4.5). These four *Bacillius*

strains, giving positive PCR amplification, were tested for their antifungal activity in dual cultures. Three of them showed significant inhibitory effect on the growth of *Sclerotinia sclerotiorum* infecting soybean and other crops. The present study showed that by employing PCR assay, preliminary screening of bacterial population in the rhizosphere could be performed for selecting the effective strains rapidly (Giacomodonato et al. 2001).

The genetic basis of biocontrol activity of *Bacillus subtilis* strains remains unclear. By employing suppressive subtractive hybridization (SSH), the genomes of GB03 and QST713 strains for *B.subtilis* were screened for strain-specific genetic elements that could be related to biocontrol activities. Cyclic lipopeptides (CLPs), antibiotic compounds synthesized nonribosomally by large peptide synthetases, contribute to the biocontrol activities of many *B. subtilis* strains. Genetic disruption of the CLP iturin biosynthesis reduced the suppression of *Rhizoctonia solani* by the strain RB14 (Tsuge et al. 2001). Analyses of the mutants showed that fengycin and bacillomycin D acted synergistically to inhibit the growth of *Fusarium oxysporum* in vitro (Koumoutsi et al. 2004). Isolates of *Bacillus* sp. were obtained from soil samples from soybean-grown fields. Strain AP-3 with biocontrol potential was used for establishing its identity by amplifying the 16S rRNA region by PCR. Partial sequences of the 16S rRNA ribosomal gene of AP-3 showed a high identity (98 %) of nucleotide bases with *B. subtilis* strain BS-2 from China. Another strain PRBS-1 also exhibited high sequence similarity with BS-2 and strain TKSP21 (available in GenBank). Hence, these two strains were identified as *Bacillus subtilis* strains (Aroújo et al. 2005). In another study, bacterial isolates from the rhizosphere of healthy red pepper plants were multiplied and the DNA was extracted from bacterial cells. The 16S rDNA was amplified using PCR assay employing the universal primers 27f and 1492r. PCR amplification of the target 16S rDNA resulted in the formation of the predicted 1.55-kbp amplicons in R33 and R13 isolates which were the most effective in suppressing the pathogen *Phytophthora capsici,* causing the devastating *Phytophthora* blight disease of red pepper. The amplified products were sequenced and compared with the 16S rDNA sequences in National Center for Biotechnology Information (NCBI) database. The two isolates were identified as *Bacillus subtilis* based on the partial sequences comparison by BLAST search (Lee et al. 2008). *B. subtilis* strains GB03 and F2B24 effectively suppressed *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL), causing tomato wilt disease. Combined application of GB03 and *B. amyloliquifaciens* strain IN937 provided higher level of protection, than when they were applied separately (Myresiotis et al. 2012).

Bacillus spp. with high level of biocontrol potential have special characteristics that make them desirable candidates as biological control agents. In order to identify and group 51 isolates, the universal primer (UP)-PCR format was developed. The bacterial genome was isolated using the Promega Wizard genomic DNA purification kit for Gram-positive bacteria. Amplification of the unique DNA fragments was performed, using the UP primers (15-20-bp) that targeted intergenic areas of the bacterial DNA which were more variable. The primer AS 15inv had the ability to distinguish different *Bacillus* sp. according to the intense polymorphic band patterns. The isolates of *Bacillus* were clustered into three groups.

Isolates of group 1 mainly identified as *B. amyloliquefaciens* exhibited an ability to produce secondary metabolites, characteristic of this group. The group 2 isolates showed similar UP-PCR profiles and had ability to produce surfactin and arthobactin in culture. They belonged to *B. subtilis*. Isolates included in group 3 were heterogeneous based on UP-PCR profiles. Eight different UP-PCR profiles for the 14 isolates were generated and the profiles were found to be different from those of two reference isolates. But the fatty acid methyl ester (FAME) analysis identified them as *B. pumilus*. The results showed that FAME analysis may be useful, when applied as a complementary test for identification of bacterial biocontrol agents (Wulff et al. 2002). A bacterial strain KJ2C12 with high potential for suppressing the development of Phytophthora blight disease infecting pepper (chilli) was identified as *Bacillus luciferensis* based on the similarity of 16S rRNA sequences. The 16S rDNA gene sequence (1,545-bp) of strain KJ2C12 was compared with published sequences of species within the genus and other members of the family Bacillaceae. The BCA strain showed 98.2 % similarity to that of *B. luciferensis*. Morphological, physiological, biochemical characteristics and fatty acid methyl ester (FAME) analysis of the strain KJ2C12 were considered for its identification (Kim et al. 2009).

In order to understand the underlying mechanisms of production of antibiotics characteristic of different species of *Bacillus*, a PCR-based assay was developed. *Sclerotinia sclerotiorum* was used to determine the antifungal activity of the compounds produced by *Bacillus* spp. Primers specific for bacillomycin D, iturin A, surfactin, mycosubtilin, fengycin and zwittermicin A were employed to amplify biosynthetic genes. Three strains, *B. subtilis* 3057, *B. amyloliquefaciens* BS6, and *B. mycoides* 4079 were positive for bacillomycin D, whereas 4 strains *B. subtilis* H-08-02, *B. subtilis* 3057, *B. amyloliquefaciens* BS6, and *B. mycoides* 4079 possessed the fengycin biosynthetic gene. The presence of zwittermicin A gene was detected in *B. mycoides* S, *B. thuringiensis* BS8, and *B. amyloliquefaciens* BS6. Sequence analysis of purified PCR products revealed homology with corresponding genes from other *Bacillus* sp. in the GenBank database. This study showed that identification of the *Bacillus* spp. may be aided in detecting the presence of genes governing the synthesis of antifungal antibiotics by employing primers specific for these genes in PCR assays (Athukorala et al. 2009). Isolates of *Bacillus subtilis* produce surfactin and other antibiotics. The isolates possessing biocontrol potential against the aflatoxin-producing fungus *Aspergillus flavus* and citrus anthracnosis pathogen *Colletotrichum gloeosporioides* were characterized using blood agar, drop collapse, reverse-phase high performance liquid chromatography and PCR assay. Isolates containing the *sfp* gene, governing the biosynthesis of surfactin were detected by PCR assay. The presence of *sfp* gene was detected in 14 isolates of *B. subtilis*. The isolates produced different quantities of surfactin ranging from 55 to 1,610 mg/l of broth medium, as determined by HPLC analysis. The biocontrol potential of the surfactin-producing isolates was demonstrated by dual culture and chloroform vapor methods. The isolate BS119 produced maximum quantity of surfactin and it showed high inhibitory activity against *A. flavus* (100 %) and *C. gloeosporioides* (88 %) (Mohammadipour et al. 2009).

The bacterial BCA *Bacillus subtilis* strain BD170, the principal active ingredient of Biopro was applied for the control of fire blight disease affecting apple and pear. The fate of the applied BD170 on plants was monitored. A specific molecular marker was developed to monitor the spread of this strain on blossoms after Biopro spray-application in a Swiss apple orchard throughout the bloom period for 2 years. Direct spraying facilitated efficient colonization of pistils in flowers that were open at the time of treatment. Subsequent dissemination (secondary colonization) of flowers that remained closed or at bud stage at the time of BCA application was also observed. The secondary colonization was dependent on the timing of the treatments, relative to bloom stage in the orchard. Foraging honey bees were found to be responsible for secondary spread of the BCA strain. The presence of the strain BD170 was detected in honey collected from hives where bees were exposed by placing the commercial product Biopro at the entrance or in the hatching nest and from hives that were placed in orchards receiving the BCA sprays (Broggini et al. 2005).

Paenibacillus spp. associated maize rhizosphere could be detected by employing a specific PCR assay based on the specific primer PAEN515F in combination with bacterial primer R1401. The primers amplified specific fragments of the 16S rRNA gene from the rhizosphere DNA. The PCR products were used in a second (semi-nested) PCR for DGGE in which bacterial primers F968GC and R1401 were employed. The amplification products, thus derived, were separated into community fingerprints by DGGE. The diversity of *Paenibacillus* sp. was evaluated to assess the reliability of the protocol, using the DNA extracted directly from the rhizospheres of four maize cultivars grown in natural soils. The results indicated that considerable diversity among *Paenibacillus* spp. could be detected by the *Paenibacillus* specific PCR-DGGE technique. The *Paenibacillus* fingerprints generated via semi-nested PCR followed by DGGE showed a clear distinction between the maize plants grown in different soils (da Silva et al. 2003). The HX2 strain isolated from vineyard soils was able to suppress the grapevine crown gall disease caused *Agrobacterium vitis*. The 16S rDNA was subjected to PCR amplification by employing the primers 63F and 1494R. The PCR product was sequenced and the sequence was 99 % identical to that of the genus *Rahnella*. Biolog identification system depending on the carbon source utilization pattern identified the HX2 strain as *R. aquatilis* (Chen et al. 2007).

Pantoea agglomerans CPA-2 has been shown to be an effective biocontrol agent suppressing the development of postharvest disease in citrus and pome fruits. A monitoring technique, consisting of a semi-selective medium and molecular markers, was developed. Malonate Broth Agar supplemented with tetracycline hydro-chloride and incubation at about 40 °C was efficient in selective recovery of *P. agglomerans* CPA-2 colonies. Strains (13) of *P. agglomerans* including the CPA-2 strain were tested using the RAPD technique. The primer OPL-11 amplified a fragment (about 720-bp) specific to CPA-2 strain. Based on the sequences of this fragment, two SCAR markers were amplified using a primer pair derived from OPL-11 elongation. The SCAR marker of 720-bp was specifically amplified for the identification of CPA-2 strain. The marker of 270-bp amplified the sequences in all other strains including CPA-2 strain was tested. Population

dynamics of CPA-2 in commercial trials were monitored on fruit surfaces and in the environment by employing both plating technique and PCR assay with SCAR primers. In general, both methods provided similar trend in the population levels of CPA-2 strain. The results indicated that CPA-2 strain had limited persistence and capacity for dispersion from the site of application (Nunes et al. 2008). *Agrobacterium vitis* strain E26 has been shown to be a promising biocontrol agent for the control of grapevine crown gall disease. A plating-PCR technique was developed for specific detection and quantification of the strain E26 by combining classical microbiological techniques with molecular tools. Random amplified polymorphic DNA fingerprints were employed to differentiate E26 from other strains. A differentially amplified fragment from E26 was sequenced and characterized as a sequence characterized amplified region (SCAR) marker. Two primer pairs were then designed and evaluated for their specificity against E26. One of the two SCAR primer pairs 740F/R was further selected for specific detection of strain E26. A plating assay coupled to PCR with the SCAR primers 740F/R allowed assessment of population dynamics of E26 in non-sterile grape rhizosphere soil under controlled conditions (Bi et al. 2012).

Endophytic bacterial species including *Burkholderia* have been shown to be efficient biocontrol agents of several plant pathogens. Endophytic actinomycetes isolated from the interior of leaves and roots of healthy and wilting banana plants were found to be effective against *Fusarium oxysporum* f.sp. *cubense* causing Panama wilt disease. These isolates were identified as *Streptomyces griseorubiginosus* based on sequence similarity of the 16S rRNA genes. The similarity was up to the maximum of 99 % (Cao et al. 2004). *B. cepacia* was originally reported to be pathogenic to onion by Burkholder (1950). This bacterial species was later found to be an ubiquitous soil bacteria with biocontrol potential for suppressing many fungal diseases infecting different crops. As *B. cepacia* could cause respiratory ailments in human beings, it is important to identify *B. cepacia* complex (Bcc) at genomovar level for risk assessment. *B. cepacia* complex consists of nine genomovars. The Biolog Identification System could not differentiate between the pathogenic and nonpathogenic human forms. Hence, molecular techniques were applied to differentiate the genomovars based on the sequences of the gene *recA*. The gene *recA* has been shown to be useful for the identification of *B. cepacia* complex, with phylogenetic analysis of sequence variation with the gene enabling the discrimination of all the nine current species within the genus *Burkholderia* (Mahendralingam et al. 2000). *Burkholderia cepacia* strain UPMB3 was able to effectively suppress *Ganoderma boniense* causing oil palm basal stem rot (BSR) disease. The strain UPMB3 was isolated from the oil palm roots. Biolog Identification System and DNA fingerprinting procedures were performed to differentiate UPMB3 strain from other strains. The strain UPMB3 was identified as genomovar I of *B. cepacia* complex. The primers BCR 1 and BCR 2 amplified the expected product size (1 kb amplicon) from the bacterial DNA. This product was amplified from all strains representative of *B. cepacia* complex. The identification of the PCR product was subsequently confirmed to be *recA* by direct nucleotide sequence analysis of PCR products. The primers BCR 1 and BCR 2 were specific

to members of *B. cepacia* complex and did not amplify this gene from other *Burkholderia* spp. Genomovars II and III of *B. cepacia* complex had the highest isolation frequency from clinics of America, Canada and Europe. Members of genomovars I, III, VII and IX included strains that showed good biocontrol potential especially effective against *Pythium* and *Rhizoctonia*. The UPMB3 showed strong antagonistic effect on *G. boninense* and suppressive effect on development of BSR in oil palm seedlings (Azadeh et al. 2010).

4.2 Assessment of Biocontrol Potential of Bacteria

Bacterial species isolated from plants, soil and environment have to be evaluated for their capacity to suppress the development of the pathogens and the disease(s) caused by them in parallel to their identification by employing various methods. The bacterial species, isolates are screened in vitro to eliminate less effective ones, so that more efficient ones are advanced to further testing in the greenhouse and under field conditions. However, caution has to be exercised in not eliminating the species/isolates that are not inhibitory directly to the target pathogen(s). Such species/isolates may act indirectly by stimulating host defense systems. The effectiveness of the bacterial species in reducing the disease incidence and/ or intensity has to be demonstrated under natural field conditions or normal storage conditions in the case of postharvest diseases. The in vitro tests are useful to demonstrate the ability of the bacterial species to secrete the enzymes or toxic metabolites involved in the biocontrol activity of the species isolates concerned. Various kinds of tests applied to demonstrate the biocontrol potential of the test bacterial species/isolates are discussed below:

4.2.1 Laboratory Tests

Different laboratory tests are performed to demonstrate antifungal and/or antibacterial property of the bacterial species/strains/isolates under in vitro conditions. In the case of fungal pathogens, the inhibitory effects of the bacteria on spore germination and mycelial growth are determined, using appropriate nutrient media.

4.2.1.1 Dual Culture or Confrontation Assay

Potato dextrose agar (PDA) medium is placed in petridishes. Bacterial isolates are streaked on the medium at 2 cm from the periphery of the plate. Agar plugs containing the mycelium of the fungal pathogen are placed at the rate of one plug/plate at the centre of the plate and incubated at room temperature for 5–7 day, depending on the rate of growth of the fungus. An inhibition zone between the bacterium and

fungus may be observed, if the bacterial isolate is antagonistic to the fungal pathogen. Percentage of inhibition is calculated in comparison to the control plate which does not contain any bacterial isolate as detailed below.

$$\text{Percentage of growth In hibition} = C - T/C \times 100$$

where C = mean diameter of the fungal colony in control; T = mean diameter of the fungal colony in plates with test bacterial isolates.

The growth inhibition of mycelial growth may be expressed using a scale from 0 to assess the relative effectiveness of different treatments as follows: 0 = no growth inhibition; 1 = 1 to 25 %; 2 = 26–50 %; 3 = 51–75 % and 4 = >75 %.

Alternatively the effect of the putative bacterial biocontrol agent may be assessed by placing three µl drops from bacterial suspension (10^8 CFU/ml) at predetermined positions at equidistance on the margins of PDA plates and incubate plates for 24 h at 28 °C. Agar discs (6 mm) from cultures of the fungal pathogen are placed at the center of each plate, containing test bacterial isolates. The plates are incubated for 7 days at room temperature (28 °C). The radii of the fungal pathogen colony towards and away from the bacterial isolate colonies are measured. The growth inhibition is determined using the formula:

Percentage of inhibition = $(R - r/R) \times 100$, where r = he radius of the fungal colony towards the bacteria and R = the radius of the fungal colony away from the bacteria. The mean inhibition in calculated for each plate.

Myxobacteria are soilborne Gram-negative gliding bacteria that form fruiting bodies containing myxospores. They produce a wide range of antibiotics and lytic enzymes which assist in their ability to prey on other microorganisms. Myxobacteria were inoculated in a 60 mm ring on diluted casitone agar medium and incubated for 8 days at room temperature. The fungal pathogens were grown in PDA for 7–14 days and plugs of mycelium and agar (3 mm diameter) were removed from the plates and transferred to the center of the myxobacterial rings. Effect on growth inhibition was determined at different periods from 1 to 30 days after inoculation. The myxobacteria *Myxococcus coralloides* (*Mc*), *M. flavescens* (*Mfl*), *M. flavus* (*Mf*), *M. stipitatus* (*Ms*), *M. virens* (*Mv*), *M. xanthus* strain K (MxK) and *M. xanthus* strain L (MxL) were evaluated for their biocontrol potential against soilborne fungal pathogens. The myxobacteria inhibited the mycelial growth of *Sclerotinia sclerotiorum*, *Pythium ultimum*, *Rhizoctonia* spp. and *Phytophthora capsici*. *Pythium ultimum* was completely inhibited by all myxobacteria. *Verticillium dahliae*, *V. albo-atrum*, *Cylindrocarpon* spp. and *Fusarium oxysporum* f.sp. *apii* were less sensitive to inhibition by the myxobacteria (Fig. 4.6; Bull et al. 2002).

Another variant of the dual culture method was applied to assess the in vitro antagonism of *Rhizobium* isolates on *Fusarium oxysporum* f.sp. *ciceris* race O infecting chickpea. The bacterial isolates were streaked across the petriplate at the center and a second streak was made at right angles to the first streak. Discs (5 mm diameter) of the mycelium of the fungal pathogen cut from the edge of a 7-days old culture were placed at each side of the bacterial streak maintaining a distance of 2.5 cm between the reactants. After an incubation period of 7 days at room temperature, the percent inhibition of mycelial growth was calculated, based on the colony

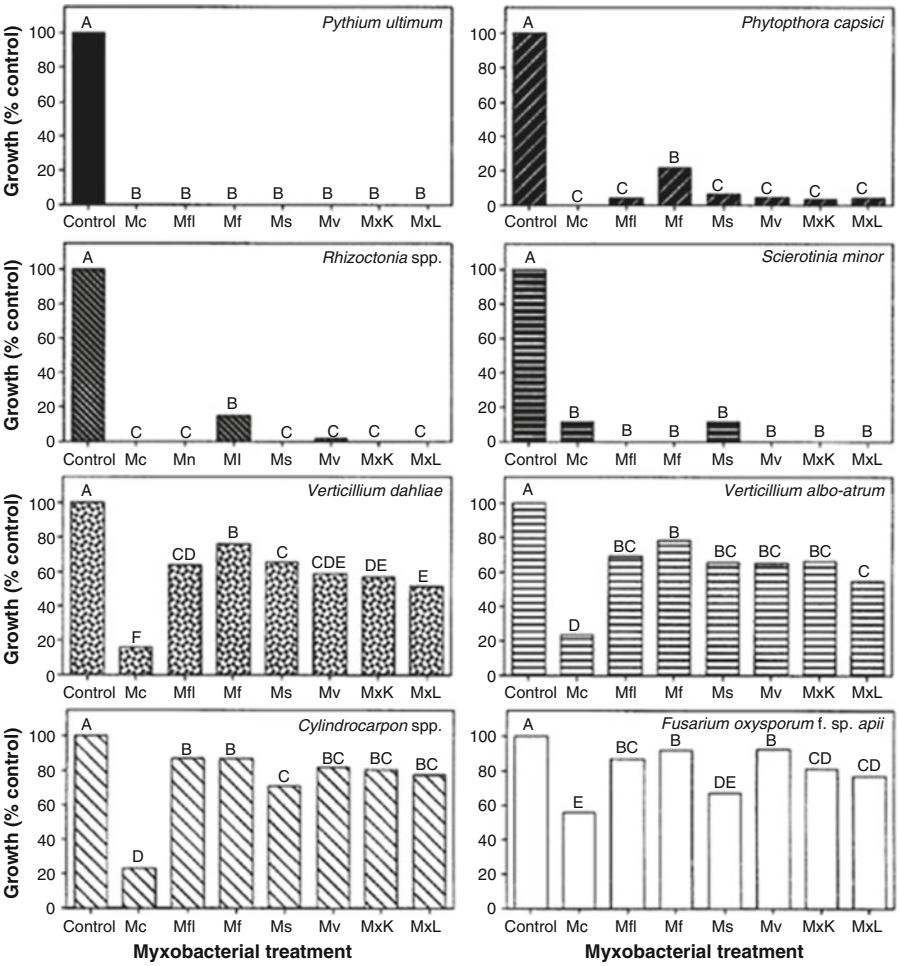

Fig. 4.6 Inhibition of mycelial growth of fungal pathogens *Pythium ultimum, Phytophthora capsici, Rhizoctonia* spp. and *Sclerotinia minor* by myxobacteria. *Bars labeled with the same letter* are not significantly different (P>0.05) as per Turkey's HSD test. Myxobacteria evaluated were as follows: MC *Myxococcus corralloides,* Mfl *M. flavescens,* Mf *M. flavus,* Ms *M. stipitatus,* Mv *M. virens,* MxK *M. xanthus* strain K, MxL *M. xanthus* strain L (Courtesy of Bull et al. 2002 and with kind permission of The American Phytopathological Society, MN, USA)

diameter in the treatments and control without the antagonistic bacterial isolates (Arfaoui et al. 2006; Appendix 4.5). Inhibition of pathogen growth by isolates of *Mitsuaria* sp. and *Burkholderia* sp. associated with soybean and tomato was assessed against soilborne pathogens. *Mitsuaria* isolates were grown on trypticase soy agar, while King's medium B was used to multiply *Burkholderia* isolates. Bacteria from 48 h-old cultures were scrapped and suspended in sterile distilled water. A 10-µl drop of bacterial cells was placed in the center of the plate containing

suitable medium seeded with the pathogen. Plates were incubated at room temperature. Extent of growth inhibition of the pathogens *Pythium aphanidermatum*, *Pythium sylvaticum Phytophthora capsici*, *Phytophtora sojae*, *Rhizoctonia solani, Alternaria solani*, *Fusarium graminearum* and *F. oxysporum* was determined. Mycelial growth inhibition was scored as positive or negative for each isolate. Positive scores were based on the formation of clear inhibition zone between the pathogen and bacteria, diminished total growth of the pathogen in comparison to the control. Melanization or morphology change in pathogen, colony growth and/or bacterial swarming over pathogen culture. *Mitsuaria* isolates inhibited the growth of all pathogens tested exhibiting greatest level of inhibition against *P. aphanidermatum P. sojae, R. solani* and *A. solani* with least inhibition against *P. sylvaticum*. On the other hand, *Burkholderia* isolates showed more variable and less frequent inhibition against the pathogens tested (Benítez and Mc Spadden Gardener 2009).

The biocontrol potential of the entomopathogenic bacterial species *Xenorhabdus bovienii* YL002 for suppressing the development of the fungal pathogens, *Botrytis cinerea* causing tomato gray mold rot and *Phytophthora capsici* causing pepper leaf scorch diseases was assessed in vitro. The cell-free filtrate of *X. bovienii* YL002 strongly inhibited the mycelial growth of *P. capsici* and *B. cinerea* to the extent of >98 %. The 50 % inhibition concentrations (EC_{50}) of the methanol-extract, containing bioactive compounds of the cell-free extract were determined to be 164.83 and 42.16 μg/ml for *P. capsici* and *B. cinerea* respectively. The methanol extract at 1,000 μg/ml exhibited curative effect of 70.8 % and protective effect of 77.4 % against *B. cinerea* on tomato plants, compared to controls. The methanol extract was less effective in suppressing the development of *P. capsici* on pepper both as protective and curative treatment compared with the effect on *B. cinerea* (Fang et al. 2011).

The antibacterial activity of the bacterial biocontrol agent *Rahnella aquatilis* HX2 against grape crown gall bacterium *Agrobacterium vitis* was assessed in vitro. Drops of 10 μl HX2 strain were dispensed at the center of PDA medium plates and incubated for 48 h at 28 °C. The BCA cells were killed with chloroform. After evaporation of chloroform, 50-μl aliquots of *A. vitis* cells (2×10^8 CFU/ml) mixed with 5 ml of 0.7 % yeast extract broth (YEB) soft agar was poured over the surfaces of PDA plates inoculated with BCA isolates. The plates were then incubated for 24 h at 28 °C. Observations were made for the development of inhibition zones around the killed BCA cells. Diameter of the inhibition zone was measured. Five replicates were maintained for each isolate of *R. aquatilis* and the experiment was repeated again (Chen et al. 2007). Antibacterial activity of *Lysobacter capsici* on five Gram-positive bacteria *Clavibacter michiganensis* subsp. *michiganenesis (Cmm)* and Gram-negative bacteria *Agrobacterium tumefaciens, Ralstonia solanacearum, Erwinia carotovora* subsp. *carotovora* and *Xanthomonas euvesicatoria* was assessed by dual culture technique. No antagonistic activity of *L. capsici* could be observed on Gram-negative bacterial species. Inhibition zone produced by *L. capsici* against C*mm* was increased with iron supply up to certain level, indicating the influence of iron supplied as $FeCl_2$ to King's medium B (Puopolo et al. 2010).

The antagonistic activity of *Paenibacillus polymyxa* strains against *Phytophthora palmivora* and *Pythium aphanidermatum* was demonstrated using agar plates, liquid media and soil. *P. polymyxa* strains significantly reduced colonization of pathogens in liquid assays. Most plants treated with *P. polymyxa* survived the infection by *P. aphanidermatum* in soil assays. The antagonistic abilities of the BCA strains correlated well with mycoidal substance production, but not with the production of antagonistic substances from the bacterial strains (Timmusk et al. 2009). Based on the antifungal activity of 25 strains of *P. polymyxa* against *Phytophthora capsici* in vitro, they were differentiated into strongly antimicrobial, weakly antimicrobial and without antimicrobial activity. The strain GBR-462 showed strong antifungal activity against *P. capsici* and it could form biofilm, only when they were applied at higher concentration (10^8 CFU/ml) (Kim et al. 2009). *P. polymyxa* strains WI-14-3 and C1-8-b inhibited the mycelial growth of *Fusarium graminearum,* causative agent of wheat Fusarium head blight disease and reduced the production of deoxynivalenol (DON). A novel concurrent screening procedure was developed for selecting promising biocontrol agents effective against FHB disease (He et al. 2009). The bacterial strain SG-6 isolated from the root tissue of *Sophora tonkinensis* was identified as *Paenibacillus polymyxa* based on morphological characteristics, 16S rDNA gene analysis and Biolog tests. The strain SG-6 effectively suppressed *Penicillium digitatum*, causing postharvest green mold disease of citrus. In the in vitro dual culture assay, the strain SG-6 significantly inhibited the growth of *P. digitatum* on potato dextrose agar plates. Conidial germination of *P. digitatum* was drastically inhibited in the presence of live cells of *P. polymyxa*. Unwashed cell suspension of SG-6 was more effective than the washed cell suspension and culture filtrate. The effectiveness of suppression of green mold disease could be improved by increasing the period between treatment with strain SG-6 and challenge inoculation with the pathogen. This report appears to be the first indicating the effectiveness of *P. polymyxa* against the postharvest green mold of citrus fruit (Lai et al. 2012).

The inhibitory effect of cell-free filtrates of *Brevibacillus brevis* culture was affected by the culture medium used for growing the bacterial BCA (Chandel et al. 2010). The dual culture method has been employed to assess the antagonistic capacity of the bacterial species/isolates obtained from different sources/substrates. Some of the bacterial biocontrol agents that have been shown to be effective include the bacteria in the genera *Bacillus, Pseudomonas, Rahnella* and *Serratia* against *Phytophthora infestans* (Daayf et al. 2003). *Burkholderia cepacia* against *Fusarium oxysporum* f.sp. *lycopersici* (Sijam and Dikin 2005), *Bacillus subtilis* against *Phytophthora capsici* (Lee et al. 2008), *Bacillus* spp. against *Phytophthora capsici* (Melnick et al. 2008), *Bacillus megaterium* against *Phytophthora capsici* (Akgül and Mirik 2008), *Lysobacter enzymogenes* strain C3 against *Bipolaris sorokiniana* (Li et al. 2008), *Bacillus subtilis* against *Phytophthora ramorum* (Elliott et al. 2009) and against *Monilinia vaccinii-corymbosi* (Thornton et al. 2009), *Pseudomonas fluorescens* strain CV6 against *Phytophthora drechsleri* (Maleki et al. 2010) and *Lysobacter capsici* strain PG4 against *F. oxysporum* f.sp. *lycopersici, F. oxysporum* f.sp. *lycopersici-radicis* and *Rhizoctonia solani* (Puopolo et al. 2010).

The comparative efficacy of four biocontrol agents *Bacillus subtilis* QST 713, *Streptomyces lydicus* WYEC 108, *Coniothyrium minitans* CON / M /91-08 and *Trichoderma harzianum* T-22 in reducing the survival of sclerotia and production of apothecia of the pathogen *Sclerotinia sclerotiorum* was assessed under controlled conditions in the growth chamber. For each BCA treatment, 25 sclerotia were buried in pots containing potting soil and BCA suspensions were drenched into the soil at various concentrations and five soybean seeds were planted in each pot. Sclerotia of the pathogen were retrieved at 6 weeks after seeding and their viability was assessed on agar plates. All the four BCAs, were effective in reducing *S. sclerotiorum* inoculum to different levels. In general, the efficacy was positively correlated with the rate of application of BCAs. *B. subtilis* reduced apothecia and sclerotia by 91.2 and 26.2 % respectively, whereas *C. minitams* reduced apothecia and sclerotia by 81.2 and 50 % respectively. *S. lydicus* could arrest production of apothecia completely (100 %), but sclerotial production by 29.6 % only. *T. harzianum* was less efficient than other three BCAs in reducing apothecia production but equally effective in reducing sclerotial formation as the other BCAs. The results indicated the potential diversities of biological control strains and the need to employ appropriate methods to select the most effective strains for large scale use (Zeng et al. 2012).

Bacillus strains (400) isolated from roots of cucumber plants grown in the greenhouses and fields were evaluated for the antagonistic potential against *Fusarium oxysporum* f.sp. *cucumerinum* causing cucumber wilt disease. A strain BO68150 was the most effective in suppressing the development of Fusarium wilt disease of cucumber in in vitro assays and under greenhouse conditions. The biocontrol efficiency of this strain was up to 50.68 % in the seedling stage. The strain BO68150 showed no obvious antagonistic activity against *F. oxysporum* f.sp. *cucumerinum* on PDA plates. The strain BO68150 was identified as *Bacillus subtilis* by using morphological, physiological and biochemical tests, cellular fatty acid analysis and Biolog based substrate utilization test. In addition, 16S rRNA gene- and *gyrA* gene-based phylogenetic analysis revealed high levels of similarity between BO68150 and known *Bacillus* spp. The strain was designated *B. subtilis* BO68150. The results indicated that this BCA could be effective in reducing the incidence of cucumber wilt disease caused by *F. oxysporum* f.sp. *cucumerinum* and consequently the yield loss considerably (Li et al. 2012).

4.2.1.2 Detached Leaf Assay

The inhibitory effects of live bacterial cells and bacterial broth supernatant on the development of potato late blight pathogen *Phytophthora infestans* were determined by detached leaf assay. The bacterial inoculum (10^8 CFU/ml) was applied into Russet Burbank potato leaflets by dipping and incubated for 5 h at room temperature. The leaflets were challenged by applying the pathogen (20 µl droplets of 10^4 zoospores/ml suspension) into the lower side of the leaflet. The inoculated and control leaflets were placed on moist sterile filter paper kept in a covered sterile petridish and then transferred to a moist chamber to provide high humidity (>95 %) for the

development of the pathogen. The bacterial-broth supernatant was prepared by inoculating the nutrient both in flasks and keeping them on a shaker continuously for 3 days at room temperature. After centrifuging at 4,500 rpm for 10 min, the supernatant was decanted and frozen. The effect of supernatant of bacterial nutrient broth was assessed, as in the case of live bacterial cells. The inoculated leaves treated with live bacteria or water were scanned, using the image analysis soft ware Assess® to determine the area of leaflets infected by *P. infestans*. The percent inhibition of disease development was calculated using the formula:

$$\text{Percent inhibition} = 100 - \left[\left(\text{BP} / \text{WP} \right) \times 100 \right]$$

where BP is infected area in leaflets treated with bacteria/supernatant and WP is infected area in leaves treated with water (control). The disease intensity is expressed using a rating scale of 1–5 as follows: 1 = <10 %; 2 = 10–20 %; 3 = 21–40 %; 4 = 41–75> % and 5 = >75 % (Daayf et al. 2003).

Healthy leaves of pistachio (three leaves/bacterial isolate) were placed on plastic mesh platforms resting in plastic containers. They were inoculated with drops (7.5 µl) of cell suspension (10^8 CFU/ml) of bacterial isolates at a distance of 10 mm from the midrib. A drop of spore suspension (7.5 µl) of the fungal pathogen *Botryosphaeria dothidea* was also placed at the same site immediately. Control leaves were inoculated with the fungal spore suspension. High relative humidity (>95 %) was maintained by pouring 200 ml of water in each plastic container to favor disease development. The containers were covered and incubated at room temperature (23 ± 2 °C) for 10 days. Diameter of lesions developed on leaves for each treatment (bacterial isolate) was measured. The isolate *Paenibacillus lentimorbus* CBCA-2 was found to be the most effective in suppressing panicle and shoot blight disease in pistachio caused by *B. dothidea*. The inhibitory effects of washed bacterial cells and culture filtrate of the CBCA-2 strain were also determined, using the detached leaves of pistachio and measuring the diameter of the lesions developed in different treatments (Chen et al. 2003). The detached leaf assay was performed to determine the relative antagonistic activity of the isolates of *Bacillus subtilis* against Phytophthora blight of red pepper caused by *Phytophthora capsici*. The suspensions of bacterial isolates (10^8 cells/ml) were sprayed individually on the detached leaves and they were placed in the petriplates lined with moistened filter paper. Spore suspension (10^5 spores/ml) of the pathogen was sprayed on the leaves already treated with different bacterial isolates. The leaves were incubated in a growth chamber at 20 °C with 12 h light, dark periods for 10 days. Disease severity in different treatments was determined by using 0–4 disease rating scale as follows: 0 = no visible; symptoms1 = 1–12 %; 2 = 13–25 %; 3 = 26–50 % and 4 = 51–100 % of leaf area covered by the disease. Each treatment consisted of three detached leaves in three replications (Lee et al. 2008).

A detached leaf disk assay was adopted to assess the ability of *Bacillus* spp. to suppress the development of cocoa black pod rot caused by *Phytophthora capsici*, leaf disks (9 cm diameter) were punched out from leaves in such a manner that the midrib was in the centre of the leaf disk. The disks were placed adaxial side up on a moist sterile 9 cm Whatman filter paper in an inverted petridish. Each leaf disk was

inoculated with six isolated drops (10 µl) of zoospores suspension (5×10^3 zoospores/ml or 50 zoospores per 10 µl drop). Then the dishes were sealed with parafilm and incubated at 28 °C with a 12-h light/dark cycle. The diameter of lesions developed in each leaf disk was measured at 8–12-h interval for about 52 h after inoculation. Disease severity was calculated based on a 0–8 scale. The area under disease progress curve (AUDPC) was worked out based on the measurements of lesion size at different time intervals of the two strains BP24 and BT8 of *Bacillus cereus* tested. Only BT8 suppressed lesion expansion following infection by *P. capsici* (Melnick et al. 2008).

Colletotrichum gloeosporioides causes the anthracnose disease in strawberry and grapevine. *Bacillus amyloliquefaciens* S13-3 isolated from soi, suppressed the mycelial growth of *C. gloesporioides* in plate tests. The strain S13-3 reduced the severity of strawberry anthracnose on detached strawberry leaves. The biocontrol activity of the strain S13-3 against grape ripe rot was also observed. This BCA strain exhibited tolerance to some chemical pesticides and copper, suggesting that S13-3 could be applied before and/or after treatment with pesticide or Bordeaux mixture in the field. The strain S13-3 had *itu D* and *lpa-14* genes, both of which may play a role in iturin A production. Production of iturin A by S13-3 was detected in the BCA culture medium. The inhibitory effects of *B. amyloliquefaciens* S13-3 against *C. gloesporioides* could indicate its usefulness for application against the anthracnose diseases of grapes and strawberry (Mochizuki et al. 2012).

The antagonistic activity of the endophytic strains of *Streptomyces* sp. recovered from cucumber and pumpkin plants was assessed for the control of anthracnose pathogen *Colletotrichum orbiculare*. Eleven strains that strongly inhibited the mycelial growth of *C. orbiculare* in plate tests were further tested using detached cotyledons of cucumber. Spore suspensions of test strains were used to soak the cucumber cotyledons 1 day prior to challenge inoculation with the pathogen. Six strains of the BCA significantly reduced the number and size of the lesions on the cotyledons, compared to the untreated control. These strains significantly inhibited lesion development on intact leaves of 3-week old cucumber plants. Strain MBCu-56 was the most effective in suppressing the disease development and its suppressive efficiency increased with increasing concentration of the BCA. Pretreatment of cucumber leaves with concentrations of 10^7, 10^8 and 10^9 CFU/ml suppressed the disease by 72, 79 and 93 % respectively. The results showed that the *Streptomyces* sp. strain MBCu-56 had the potential for using it as an alternative for the conventional fungicide treatment (Shimizu et al. 2009).

4.2.1.3 Excised Dormant Stem Assay

Dormant 1-year old pistachio stem pieces (20 cm × 20 mm) were surface-sterilized with sodium hypochlorite (5.25 %) solution for 10 min and the cut ends were sealed with parafilm. A cavity was made by removing the bark with cork borer and an aliquot of bacterial BCA (*Paenibacillus lentimorbus*) suspension or the medium (for control) was placed in each cavity. Agar plugs containing mycelium of *B. dothidea* was placed in each cavity at 3 days after application of the BCA cell suspension.

Inoculated stem pieces were incubated in plastic screens raised 2 cm above the bottom of the plastic containers at 24° ± 1 °C. Water (200 ml) was placed in the plastic container to maintain high relative humidity (>95 %) to favor development of lesions. The length of lesions was measured at 9 days after incubation. The antagonistic activity of the bacterial BCA *Paenibacillus lentimorbus* strain CBCA-2 was determined, based on the extent of suppression of development of lesions on the stem pieces (Chen et al. 2003).

4.2.1.4 Seed Assays

The efficacy of *Pseudomonas fluorescens* (*Pf*) (A506) was assessed in suppressing the watermelon seed infection by *Acidovorax avenae* subsp. *citrulli (Aac,)* causing bacterial fruit blotch (BFB) disease. The extent of seed infestation by *Aac* was determined by immunomagnetic separation (IMS)-PCR technique and by seedling grow-out assay. Naturally infested seed samples (25 seeds/sample) were vacuum-infiltrated with different isolates of *Pf* by exposing seeds suspended in suspension of BCA isolates (10^8 CFU/ml) under continuous vacuum for 20 min. The treated seeds were air-dried overnight at 25 °C and assayed for BFB seedling transmission by the seedling growing-out assay. Seeds were incubated in transparent plastic boxes on two layers of blotter paper saturated with sterile water under high humidity of >90 % at 30 °C and continuous fluorescent light. Seedling germination and BFB incidence in different treatments were recorded daily for 3–15 days after placing the seeds in plastic boxes. Area under disease progress curve (AUDPC) for each treatment was calculated and compared to select the most efficient BCA isolate (Fessehaie and Walcott 2005). Infection of wheat seeds by *Fusarium graminearum* and related species cause wheat scab and maize ear rot disease as well as accumulation of the mycotoxins such as deoxynivalenol and zearolenone that cause ailments in human beings and animals consuming contaminated grains. The biocontrol potential of a patented endophytic bacterial strain *Bacillus mojavensis* RRC 101 was assessed in growth rooms. All strains of *B. mojavensis* were antagonistic to the strain of *F. graminearum, F. pseudograminearum* and *F. verticillioides*. Seed germination of the highly susceptible cv. 2552 was increased from 77 to 97 %, following treatment with the BCA and planting in *Fusarium* spp.-infested soil. Likewise, seedling emergence was also greatly increased from 20 to 82 % due to treatment with the BCA. The results indicated that the seed treatment with the bacterial antagonist might provide effective protection against the mycotoxin producing fungal pathogens (Bacon and Hinton 2007).

The biocontrol efficacy of *Pantoea agglomerans* isolate LRC 8311 against *Curtobacterium flaccumfaciens* pv. *flaccumfaciens* (*Cff*) causing bean bacterial wilt disease was assessed under greenhouse conditions. Seed soaking in a suspension of 3×10^8 CFU/ml of *P. agglomerans* resulted in a thorough endophytic colonization of the entire bean seedling from root to apical stem after 7 days and the treatment did not have negative effect on seedling emergence. High rate of colonization of seedlings by the BCA reduced the frequency of infection by *Cff* and disease severity, compared with control treatment with pathogen inoculation alone. Seed treatment with

Table 4.2 Efficacy of isolates of *Mitsuaria* spp. and *Burkholderia* spp. in suppressing development lesions induced by *Pythium aphanidermatum* and *Rhizoctonia solani* in soybean and tomatoes (Benítez and Mc Spadden Gardener 2009)

Biocontrol agent/crop	Treatments	Severity of lesions[a] induced by	
		P. aphanidermatum	*R. solani*
A. *Mitsuaria* isolates			
Soybean	H23L1	44.2**	22.9
	H24L5A	62.5*	26.7
	H29L1B	64.3	33.3
	Pathogen alone	91.0	32.3
	No pathogen	27.0	11.7
Tomatoes	H23L1	57.8**	45.0**
	H24L5A	41.7***	33.3***
	H29L1B	60.4**	33.3***
	Pathogen alone	917	55.6
	No pathogen	477	34.8
B. *Burkholderia* isolates soybean	R2C2	–	36.1***
	R2G3	–	34.5***
	R4F2	–	34.2**
	Pathogen alone	–	56.1
	No pathogen	–	29.1
Tomatoes	R2C2	–	46.2***
	R2G3	–	42.3***
	R4F2	–	48.3***
	Pathogen alone	–	63.6
	No pathogen	–	45.2

[a] Lesion severity expressed as percentage of root length marked by a lesion
Statistical significance as per Wilcoxon two-sample test:
***P<0.01; **P<0.05; *P<0.1

P. agglomerans provided additional benefit of improved seedling emergence and greater plant height. Application of *P. agglomerans*, as a soil drench at 24 h after planting also suppressed development of bacterial wilt disease. But drenching was generally less effective and cumbersome, compared with seed treatment which is a more practical method of BCA application (Hsieh et al. 2005).

Seedling bioassays were performed to assess the biocontrol potential of *Mitsuaria* and *Burkholderia* isolates obtained from the rhizosphere of soybean and tomato against soilborne pathogens. Development of lesions on developing seedlings was suppressed by inoculation with isolates of *Mitsuaria*. Lesion severity in soybean challenged with *Pythium aphanidermatum* and in tomatoes challenged with *P. aphanidermatum* and *Rhizoctonia solani* was significantly reduced by all isolates. *Mitsuaria* isolates could also reduce lesion severity in soybean seedlings, when challenged with inoculation of *Phytophthora sojae*. Overall, lesion severity reduced by *Mitsuaria* isolates ranged from 5 to 20 %. *Burkholderia* isolates reduced the lesion severity induced by *R. solani*. Overall, these isolates reduced the lesion severity by at least 15 % in soybean and 20 % on tomato seedlings. The results suggested that multiple isolates of *Mitsuaria* spp. and *Burkholderia* spp. might contribute to the general suppression of soilborne diseases (Table 4.2; Benítez and Mc Spadden

Gardener 2009; Appendix 4.6). The antagonistic potential of bacterial species isolated from the rhizosphere soil of tobacco against *Phytophthora parasitica* var. *nicotianae* causing tobacco black shank disease was assessed by employing a rapid microbioassay. Tobacco seedlings grown in petridishes were used in the bioassay for standardization for quantitation of initial zoospore inoculum. The petridishes were inoculated with zoospore suspension containing 10^2 to 10^5 spores/petridish on 14-day old tobacco seedlings for the susceptibility test. The optimum inoculum was found to be 10,000 zoospores. The bacterial species were screened for protective ability against the pathogen. Fifteen bacterial isolates showing high level of antifungal activity against *P. parasitica* var. *nicotianae* were identified by Biolog GEN III Microplate. These effective isolates were distributed in *Bacillus amyloliquefaciens, B. licheniformis, Paenibacillus pabuli, B. atrophaeus, B. subtilis, B. pumilus* and *B. endophyticus*. Four isolates showed 100 % protective activity in planta also, as in petridish assay. This microassay was found to be a rapid, reproducible and efficient method that could be employed for screening a large number of isolates of microorganisms to determine their biocontrol potential against microbial plant pathogens (Wang et al. 2012).

A vermiculite test tube assay was applied to assess the biocontrol efficacy of bacterial isolates in suppressing the pathogen *Gaeumannomyces graminis* var. *tritici* (*Ggt*) causing wheat take-all disease. Plugs containing PDA medium alone or mycelium of *Ggt* were mixed with sterilized vermiculite and homogenized for 30 s using a blender. This mixture was packed in test tubes (18×95 mm). Then a layer of vermiculite (1 cm) was overlaid. Wheat seeds were surface-sterilized with 0.6 % sodium hypochlorite and soaked in different suspensions containing test bacterial isolates (10^8 cells/ml) and 1 % carboxymethyl cellulose. The seeds were placed over vermiculite layer in the test tubes. One millilitre of sterile deionized water was added to each tube and tubes were capped and incubated at room temperature under fluorescent light to maintain 12-h photoperiod. After 17 days of incubation, the severity of root infection was determined, using a 0–4 disease rating scale. Three bacterial isolates belonging to *Pseudomonas aureofaciens*, *Bacillus subtilis* and *Burkholderia glathei* were found to be more effective than other isolates in suppressing the development of take-all disease in wheat (Nasraoui et al. 2007). The biocontrol potential of *Lysobacter capsici* strain PG4 was assessed for protecting the emerging seedlings from tomato seeds against three fungal pathogens *Fusarium oxysporum* f.sp. *lycopersici, F. oxysporum* f.sp. *radicis-lycopersici* and *Rhizoctonia solani*. Glass tubes filled with sterile perlite and Hoagland solution (18 ml) were used. Tomato seeds were disinfected by soaking in 0.5 % $NaClO_3$ for 5 min and then washed with sterile distilled water three times in a laminar flow cabinet. Seeds dipped only in PG4 suspension, seeds treated with PG4 and challenged with the fungal pathogen (individually), seeds inoculated with the pathogen alone, and seed with no treatment (control) formed three kinds of treatments. The seeds were placed on the top of the perlite at the rate of three seeds per tube. In the case of the first and second treatments, a second bacterization of BCA suspension (2 ml of 10^8 CFU/ml) was provided. Suspension of pathogen conidia (10^4/ml) were added to the second and third treatments. Observations on the percentage of dead plantlets were recorded at 2 weeks after inoculation of pathogen into the tubes (Puopolo et al. 2010).

4.2.1.5 Visualization by Microscopes

Interactions between bacterial BCAs and microbial pathogens follow certain sequence of events such as initial attraction, attachment, proliferation and lysis of pathogen cells. A fluorescent *Pseudomonas* sp. strain GRC_2 isolated from potato rhizosphere exhibited necrotrophic antibiosis against *Macrophomina phaseolina* and *Sclerotinia sclerotiorum* infecting groundnut (peanut). The interaction between the BCA and fungal pathogens was examined under scanning electron microscope (SEM) by taking mycelial growth from inhibition zones in dual cultures. Loss of sclerotial integrity, hyphal shriveling, mycelial and sclerotial deformities were observed in *M. phaseolina,* due to the antagonistic activity of *Pseudomonas* sp. GRC_2.

Observation on the interaction of the BCA with *S. sclerotiorum* revealed hyphal perforations, lysis and fragmentation of cells. The adverse effects were attributed to the secondary metabolites secreted by the BCA (Gupta et al. 2001). *Sclerotium rolfsii*, a soilborne pathogen infects peanut (groundnut) and other leguminous plants, causing the death of infected plants. Observations under scanning electron microscope revealed that *Pseudomonas fluorescens* Pf1 and *Streptomyces exfoliatus* effectively suppressed the development of *S. rolfsii* by parasitizing the sclerotia. *P. fluorescens* entirely covered sclerotia, resulting in their malformation (Fig. 4.7a). *S. exfoliatus* suppressed mycelial growth and parasitized the sclerotia, leading to failure of sclerotial germination (Fig. 4.7b) (Jamuna Rani and Dr. T. Ganapathy, Tamil Nadu Agricultural University, Coimbatore, India; personal communication). The interaction between the bacterial antagonists *Bacillus cereus* X16 and *B. thuringiensis* 55T and the fungal pathogen *Fusarium roseum* var. *sambucinum,* causing potato dry rot disease, was investigated, using light microscope. Inhibition of the pathogen by *B. cereus* was due to fungistasis, rather than fungicidal activity. In contrast, *B. thuringiensis* caused marked damage to pathogen cells with partial to complete cell wall disintegration and disorganization and generally complete loss of protoplasm of fungal cells, indicating the fungicidal activity of *B. thuringiensis*. Similar effects of *B. thuringiensis* on fungal cells, like disintegration of cytoplasm were observed in ultra-thin sections under transmission electron microscope (Chérif et al. 2002). The antagonistic activity of *Pseudomonas* sp. strain EC-S101 against *Aphanomyces cochlioides* causing spinach root rot disease was investigated. Confocal laser scanning microscopic observations using fluorescent strains indicated an increased quantity of nuclei and lipid bodies in the affected hyphae during early stage at 3 days of interaction between the bacterial pathogen and fungal pathogen. Later on, the nuclei became smaller and round-shaped, compared with oval shaped nuclei on control samples. At 7 days, nuclei disintegrated and the nuclear materials were released into the disorganized cytoplasm. Observations under transmission electron microscope (TEM) revealed that the cell walls of pathogen hyphae were considerably thickened in the presence of the bacterial BCA. Enlarged vacuoles, lipid bodies sunk into vacuoles and vacuoles filled with electron-dense material, followed by an invagination of the hyphal cell wall were frequently observed. Non-membranous electron-transparent inclusion bodies irregular in size were also seen often distributed in the affected hyphae. The results indicated that direct killing

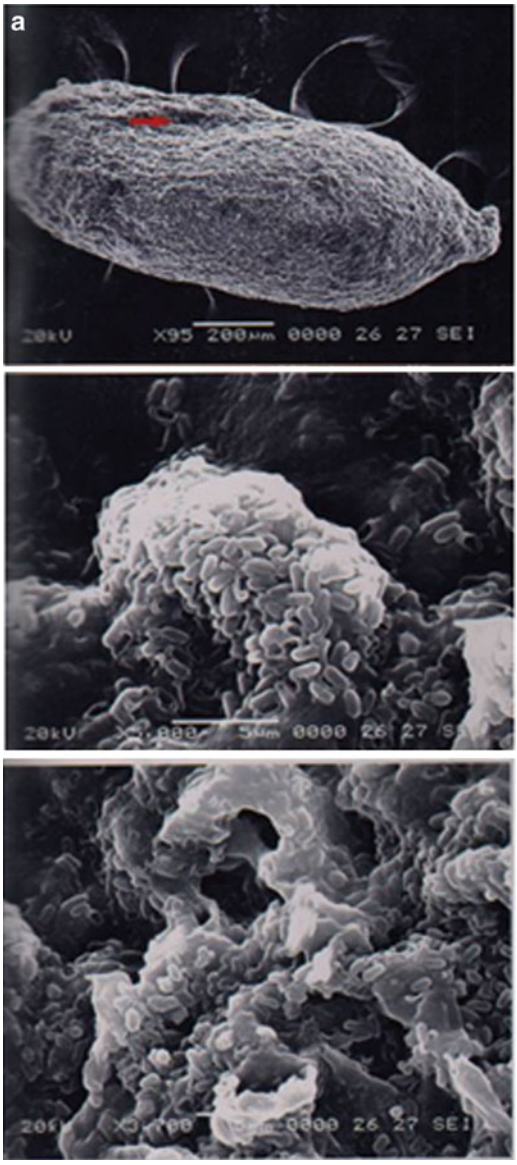

Fig. 4.7 (**a**) Visualization of parasitization of sclerotium of *Sclerotium rolfsii* by *Pseudomonas fluorescens* using scanning electron microscope. *Top*: parasitized sclerotium; *Middle*: proliferation of *P. fluorescens* on parasitized sclerotium; *Bottom*: degeneration of parasitized sclerotium (With kind permission of Ms. Jamuna Rani and Dr. T. Ganapathy, Tamil Nadu Agricultural University, Coimbatore, India). (**b**) Scanning electron microscopic observation on parasitization of sclerotium of *Sclerotium rolfsii* by *Streptomyces exfoliatus* (*top*) and magnified view of a portion of the parasitized sclerotium (With kind permission of Ms. Jamuna Rani and Dr. T. Ganapathy, Tamil Nadu Agricultural University, Coimbatore, India)

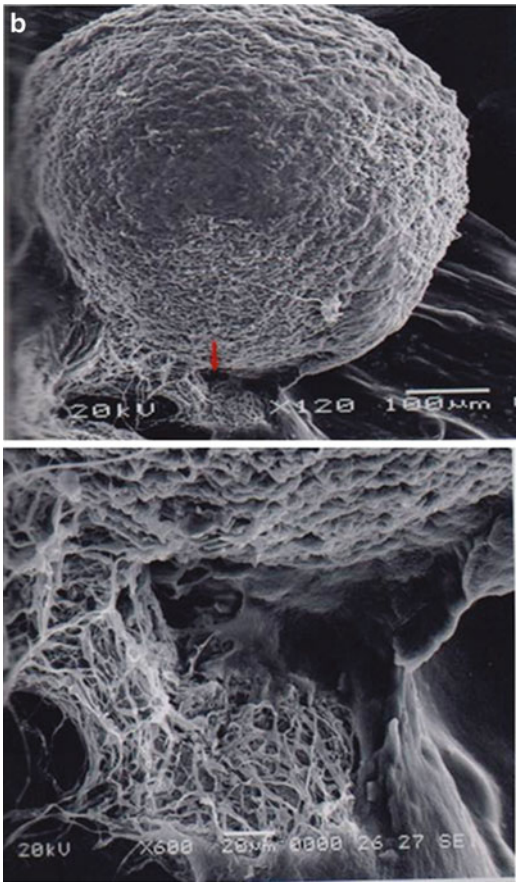

Fig. 4.7 (continued)

of the pathogen hyphae by the antagonistic bacteria resulted in the suppression of the development of the spinach root rot disease caused by *A. cochlioides* (Deora et al. 2006). *Pseudomonas jessenii* antagonistic to *Aphanomyces cochlioides* and *Pythium aphanidermatum* produced two metabolites viz., (i) (1) 3-[(1R)-hydroxyoctyl]-5-methylene-2(5H)-furanone and (ii) 3-[(1R)-hydroxyl-]-5-methylene-2(5H)-furanone. These metabolites inhibited radial growth and induced morphological abnormalities characterized by hyperbranching and periodic swelling in *A. cochlioides* and *P. aphanidermatum* respectively (Deora et al. 2010).

The influence of flower age on the development of *Erwinia amylovora* (*Ea*), causative agent of fire blight disease of apple and pear and the biocontrol agents *Pseudomonas fluorescens* Pf A506nr and *Pantoea agglomerans* (*Erwinia herbicola*) Eh 318nr was investigated. The stigmas on 1-to 3-day old flowers often supported

growth or only limited growth of *Ea*. Scanning electron microscope (SEM) observation showed that rapid and significant changes in stigma morphology of young flowers, when compared with stigmas on flowers older than 4 days, correspond with the marked difference in the ability of *Ea* to grow on these sites. The inability of *Ea* to multiply on stigmas older than 4 days appeared to be associated with collapse of the majority of papillae. Upon inoculation, both Pf A506nr and Eh318nr behaved similarly to the pathogen, as other saprophytic bacteria. They were unable to multiply exponentially, when inoculated on pistils older than 4 days. Hence, the timing of application of BCAs was considered as important to ensure successful colonization and growth (Thomson and Gouk 2003). The antibiotic activity of particularly purified metabolites of *Pseudomonas* sp. LV strain against *Xanthomonas citri* pv. *citri (Xcc*) causing citrus canker disease was studied using scanning electron microscopy (SEM) and transmission electron microscopy (TEM). The antibiotic activity of the dichloromethane phase (DP) and all fractions from vacuum liquid chromatography (VLC) were tested against *Xcc*. The minimum inhibitory concentration (MIC) of the DP was 78.12 µg/ml and the fractions of VLC were less effective in inhibiting the pathogen growth as determined by the halos formed around the wells containing VLC fractions. The SEM analysis showed the biofilm formed by exopolysaccharide (EPS) was present in the untreated control bacterial cells. After 1 h of treatment, F3 fraction destroyed the EPS entirely, but did not disrupt the *Xcc* cells. After 3–6 h, the cells appeared to be shrunken and rough, compared with untreated control cells of *Xcc.* Observations under TEM showed that cell morphology was altered within 1 h of F3 treatment. By 3–6 h after treatment with F3, the cell wall and the cytoplasmic membrane of *Xcc* became indistinct, followed by apparent lysis of pathogen cells. The EPS produced by the *Xcc* cells facilitated their dispersal on plant surface and survival in the environment. The F3 fraction stopped the EPS production and biofilm formation in treated *Xcc* cells, probably resulting in the reduction in lesion formation observed in plants treated with F3 fraction in the greenhouse experiment (de Oliveira et al. 2011).

The effects of the putative biocontrol agents on germination of spores of the fungal pathogens are assessed to determine the possible multifunctional capacity of the BCA being investigated. To assess the effect of *Paenibacillus lentimorbus* on the hyphal elongation and spore germination of *Botryosphaeria dothidea,* causing pistachio panicle and shoot blight disease, a mycelial plug from PDA culture of the pathogen was transferred to the center of the petriplate containing potato dextrose broth (PDB) and incubated at 25 °C in the dark. When the mycelial growth on the surface of the broth reached 10 mm diameter, a loopful of the BCA suspension from a 2-day old PDB culture was placed over the pathogen colony and incubated at 25 °C for 3 more days. Hyphal strands taken from the edge of the pathogen colony were examined under the microscope for abnormalities and hyphal lysis. Hyphal tips of *B. dothidea* showed malformations, thickening, vacuolation and swelling of hyphal cells and tips. No lysis of hyphal cells could be seen (Chen et al. 2003). Effect on the spore germination was assessed by transferring pycnidia from pistachio leaves inoculated with *Botryosphaeria dothidea* to test tubes containing 500 µl of sterile distilled water and shaken vigorously to dislodge the pycnidiospores from

pycnidia and to produce a suspension containing 1×10^6 spores/ml. The bacterial suspension (20 µl/well) was dispensed to wells in double-depression slide, followed by transfer of pycnidiospore suspension to each well (20 µl/well). The slides were incubated at 25 °C in a sterile petriplate containing moistened filter paper to maintain high relative humidity. Percentages of germination of pycnidiospores were determined by counting 100 spores per well per treatment after incubation for 8 h. The experiment was repeated three times. The pycnidiospore germination was entirely inhibited by the bacterial BCA, while the mean germination in the control was 100 % after 8-h incubation. Further, vacuolation, lysis and irregular texture of cytoplasm of the spores were the adverse effects of the BCA observed under the microscope (Chen et al. 2003).

Interaction between the bacterial BCA *Paenibacillus brasilensis* strain PB177 and two phytopathogenic fungi *Fusarium moniliforme* and *Diplodia macrospora* infecting maize was investigated using microscopic techniques. The pathogens were pre-cultured in trypticase soy broth (TSB) and one set was challenged with strain PB177, while the other set was left as such (control). At different intervals the paired cultures were sampled, stained with trypan blue (0.8 %) and observed under the microscope. In the cultures challenged with the bacterial BCA, the bacterial-fungal interaction led to rapid death of hyphae of both pathogens. The bacterial cells were attached to fungal hyphae and this was followed by hyphal deformation enlargement of cytoplasmic vacuoles and loss of cellular impermeability to trypan blue which could be recognized by the incorporation of the stain commencing after 24 h of interaction. The strain PB177 appeared to be more inhibitory to *D. macrospora*. Furthermore, formation of conidial structures could not be seen in these cultures, indicating that cellular differentiation of both pathogens was inhibited by the BCA. In contrast, in cultures not challenged with bacteria, abundant conidia were formed by the pathogens (von der Weid et al. 2005). The biocontrol efficacy of *P. fluorescens* BAM-4, *Burkholderia cepacia*BAM-6 and *B. cepacia* BAM-12 isolated from the rhizosphere of *Vigna radiata* was assessed. Light and scanning electron microscope (SEM) observations showed that the BCA strain induced morphological abnormalities, such as fragmentation, swelling, perforation and lysis of hyphae of *Macrophomina phaseolina* causing root rot disease (Minaxi and Saxena 2010).

The ability of *Paenibacillus brasilensis* PBI77 to protect seeds and roots of maize against *Fusarium moniliforme* and *Diplodia macrospora* was assessed using green fluorescent protein (GFP), tagged bacterial cells of strain PB177. Maize seeds were sterilized with ethanol (70 %) for 2 min followed by sterilization with NaClO (2 %) for 10 min and washing four times with sterile water. The sterilized seeds were pre-germinated on filter paper kept in petridishes for 4 days in the dark. Then these seeds were immersed in a GFP-tagged PB177 suspension (10^9 cells/ml) and placed in water agar plates containing 10 µg/ml erythromycin that were pre-inoculated with the fungal pathogens separately. The plates were incubated at 25 °C in the dark for 10 days. The colonization pattern of the GFP-tagged PB177 along with the fungal pathogens was monitored using a stereo-and epifluorescence-microscope. Parts of the seeds and roots were examined using confocal microscope also. The presence of

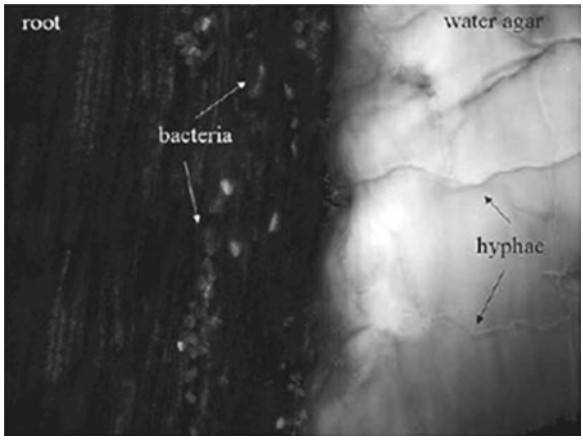

Fig. 4.8 Visualization of GFP-tagged *Paenibacillus brasilensis* in pretreated maize seeds and challenged with *Fusarium moniliforme* using confocal scanning laser microscope (CSLM) Presence of the bacterial biocontrol agent and the fungal pathogen can be observed (Courtesy of von der Weid et al. 2005 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

GFP-tagged strain PB177 and hyphae of both pathogens could be detected in most of the samples. In the absence of fungal pathogens, bacterial colonization was restricted to the seed surface (Fig. 4.8; von der Weid et al. 2005). Competitive tomato root tip colonization assay was performed to select isolates of bacteria with enhanced competitive root colonizing capacity from total rhizosphere soil samples. Strains of bacteria grown overnight in King's medium B were tested. Sterile tomato seedlings were dipped in suspension of test isolates (10^8 cells/ml) and incubated for 10 min. Inoculated seedlings were placed in sterile glass tubes containing quartz sand and plant nutrient solution. Groups of 3–4 strains were allowed to compete, using three plantlets for each mixture of bacterial isolates. After incubation for 7 days, samples of root tips (1 cm) were removed and the bacterial isolates present were isolated on King's medium B. The average number of different bacterial isolates as well as the standard deviation was calculated. Of the 216 bacterial strains tested, 24 strains performed equally well or better than the well known good root colonizer *Pseudomonas fluorescens* WCS365, as revealed by competitive tomato root tip colonization assay. Finally seven new strains were selected, based on their ability to suppress the tomato foot and root rot (TFRR) disease caused by *Fusarium oxysporum* f.sp. *radicis-lycopersici*. Six Gram-negative and one Gram-positive bacteria were identified as three *Pseudomonas putida* strains and one strain each of *Delftia tsuruhatensis*, *Pseudomonas chlororaphis*, *P. rhodesiae* and *Paenibacillus amylolyticus*. The procedure developed in this study minimizes the laborious screens that are a common feature in the isolation of biocontrol strains (Validov et al. 2007).

4.2.2 Greenhouse Bioassays

The biocontrol potential of a large number of bacterial species/strains/isolates is assessed by different in vitro tests conducted in the laboratory. The most effective ones are advanced to the next stage of assessing their efficacy in suppressing the

disease development in whole plants under greenhouse conditions. These bioassays are useful in identifying the putative biocontrol agents which may act either directly by inhibiting the development of pathogens or indirectly on the pathogens by activating the host plant defense systems leading to enhancement of disease resistance. The in vitro tests may not be useful to recognize the isolates that reduce disease incidence and/or severity by activating the host responses to pathogen infection. Assessment of the biocontrol activity of the bacterial isolates under greenhouse conditions will be required to confirm the results obtained from in vitro tests, paving the way for field evaluation as the ultimate proof for the suitability of the bacterial isolates for commercialization. Bacterial biocontrol agents have been applied against soilborne, seedborne and airborne fungal and bacterial pathogens and against a few viral pathogens. Methods of assessing the biocontrol potential of bacterial BCAs under greenhouse conditions are discussed hereunder.

4.2.2.1 Soil Application

Strains of *Pseudomonas* spp. isolated from agricultural soils, river silt and rhizosphere soils were evaluated for their antagonistic potential against *Rhizoctonia* sp. and *Pythium* sp., causing root rot of wheat. They were tested for their ability to suppress *Rhizoctonia* and *Pythium* damage in greenhouse assays to produce antifungal metabolites, to promote plant growth and to colonize wheat rhizosphere. Strains 14B2r, 15G2R, 29G9R, 39A2R, 48G9R and wood 3R reduced the severity of symptoms induced by *R. solani* AG-8 and *P. ultimum*. The latter two strains suppressed the development of *R. oryzae* and *P. irregulare* also. Four strains were able to promote growth of wheat plants which correlated with disease suppression. Based on 16S rDNA typing, the strains were identified as *P. borealis, P. chlororaphis, P. fluorescens, P. marginalis, P. poae, P. putida, P. syringae* and *P. vranovensis* (Mavrodi et al. 2012). Of the 420 bacterial strains tested, *Bacillus subtilis* strain JN2, *Myroides odoratimimus* 3YW8, *Bacillus amyloliquefaciens* 5YN8 and *Stenotrophomonas maltophila* 2JW6 showed biocontrol efficacies greater than 50 % against wilt disease of ginger caused by *Ralstonia solanacearum* under greenhouse conditions (Yang et al. 2012).

Soilborne *Streptomyces* spp. have been shown to produce antibiotics inhibitory to soilborne fungal pathogens. *Sclerotium rolfsii* causes the damping-off disease of sugar beet. The biocontrol potential of four isolates of *Streptomyces* spp., J-2, B-11, B-5 and B-40 was assessed. They inhibited the germination of sclerotia of *S. rolfsii* in infested soil significantly, compared to control. The inhibition of sclerotial germination by mycelial inoculum of BCA isolates was greater than the culture filtrate. The isolate J-2 was the most effective in inhibiting the sclerotial germination to the extent of 93 and 88 % by mycelial inoculum and culture filtrate respectively (Fig. 4.9). The disease severity was significantly reduced in seedlings growing from the BCA-treated seeds. The isolate J-2 reduced the severity of damping-off disease more effectively than the other isolates of *Streptomyces* spp. (Errakhi et al. 2007). The disease suppressive effect of putative BCA isolates in reducing the severity of

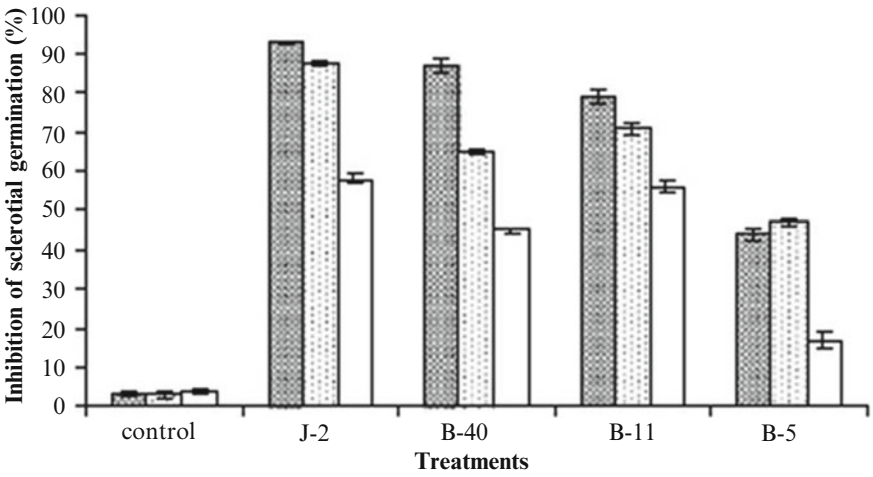

Fig. 4.9 Effects of different inocula of *Streptomyces* spp. on sclerotial germination of *Sclerotium rolfsii*, causing damping-off disease of ginger. *Cross-check column*: biomass inoculum; *dotted column*: culture filtrate; *Open column*: spore suspension (Courtesy of Errakhi et al. 2007 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

potato scab disease caused by *Streptomyces turgidiscabies* was assessed. The isolates with antagonistic potential were selected in the pot trials. Of the 26 isolates, five actinomycetes isolated from either the rhizosphere soil of wild oat grown earlier in potato fields or the soil adhering to potato stolons and tubers were selected as antagonists. A comparison of partial sequences of 16S rRNA genes of the five isolates indicated that they belonged to the genus *Streptomyces*. The isolate WoRs-501 exhibited stronger inhibition of mycelial growth of *S. turgidiscabies* compared to other isolates. In addition, this isolate was the most effective in suppressing potato scab disease in further pot trials. A 10 % (v/v) mix of WoRs-501 (6.2×10^8 CFU/g dry mass) reduced the disease severity by 78–94 % in comparison with untreated control at pathogen concentration of 5×10^4 to 5×10^6 CFU/g dry soil. The isolate WoRs-501 could tolerate a wide range of pH levels and temperatures of the soil, indicating that this isolate could be a promising candidate for biological control of potato scab disease (Kobayashi et al. 2012).

The underlying mechanism of soil suppressiveness to potato common scab disease caused by *Streptomyces scabies* was investigated. The putative common scab-suppressive soil (SS) was either treated with various temperatures or mixed with autoclaved SS at various ratios under greenhouse conditions. The soils were inoculated with *S. scabies* at 10^6 CFU/cm^3 of soil, followed by planting of either potato or radish. Disease severity was negatively correlated with the percentage of SS in the mixture and positively correlated with temperature above 60 °C. The presence of antagonistic organisms among general bacteria, streptomycetes, fluorescent pseudomonas and bacilli was examined by employing dual culture technique using the bacterial pathogen. The frequency of antagonistic bacteria in SS was higher

than in conducive soil (CS) in all the four groups. However, pseudomonads and streptomycetes populations were significantly higher among antagonistic isolates tested. The population of pathogenic *Streptomyces* spp. was significantly higher in CS than in SS sample. The terminal restriction fragment polymorphism (T-RFLP) technique differentiated two distinct microbial communities in SS and CS samples, whereas dilution plating method could not provide any clear trends or differences in population of total fungi, total bacteria, streptomycetes, fluorescent pseudomonads and bacilli (Meng et al. 2012). Gummy stem blight disease of melon (*Cucumis melo*) caused by *Didymella bryoniae* was a major limiting factor in melon production. Two *Streptomyces* spp., *S. pactum* A12 and *S. globisporus* subsp. g*lobisporus* C28 exhibited significant inhibitory effects on the development of gummy stem blight pathogen in vitro. Both strains A12 and C28 significantly reduced disease severity and area under disease progressive curve (AUDPC) of the disease in vivo ($P < 0.05$). Ten-fold dilution of C28 culture filtrate was more effective in controlling the disease compared with other treatments, showing disease reduction to the extent of 41–64.2 %. In addition, treatment with the BCA strain A12 increased the mean fresh weights of plants, roots and aerial plant parts, when applied in both nursery and transplanted soil. Both A12 and C28 strains exhibited substantial colonization abilities in the rhizosphere and on the rhizoplane of melon plants. The results indicated the ability of *Streptomyces* strains to protect the melon plants against gummy stem blight disease and also to promote the growth of melon plants (Zhao et al. 2012).

4.2.2.2 Seed/Seed Material Treatments

Of the several methods of treatment with bacterial BCA, seed treatment is the method of choice, since it is less expensive and easier to be adopted. Seeds of cabbage after disinfestation with ethanol were treated with sodium hypochlorite for 3 min. After washing with sterile distilled water, the seeds were dried in the flow cabinet in petriplates containing sterile filter paper. The cultures of bacterial BCA *Bacillus* spp. and pathogen *Xanthomonas campestris* pv. *campestris* (*Xcc*) were grown on trypticase soy agar (TSA, Difco Laboratories) and the suspensions of bacterial cells were prepared with sterile saline water. The concentration of *Xcc* was adjusted to $OD_{600} = 0.01$ (10^7 CFU/ml) and that of *Bacillus* spp. was adjusted to $OD_{600} = 1.0$ (10^9 CFU/ml). The disinfested seeds were inoculated with *Xcc* for 30 min and dried in the flow cabinet. After 24 h, the pathogen-inoculated seeds were soaked for 18 h under agitation (150 rpm) in the BCA suspension (10 ml). Suitable controls were maintained. The treated and control seeds were sown in trays containing pine bark that were watered twice a day and placed in glasshouse without controlled conditions. Effect on seed germination and symptom development were monitored. Seed treatment with *Bacillus* spp. generally reduced germination of seeds and the incidence of black rot disease caused by *Xcc* (Wulff et al. 2002). Plant growth-promoting rhizobacterial strains *Bacillus pumilus* SE 34 and *Pseudomonas putida* SE 89B61 were evaluated by treating the seeds of tomato cv. Solar Set for

Table 4.3 Population of *Fusarium verticillioides* in the rhizosphere and endorhizosphere in maize plants growing from the seeds treated with *Bacillus subtilis* (Cavaglieri et al. 2005)

Bacterial seed treatments (cells/ml)	$\log_{10}$ *F. verticillioides* CFU/g of root tissue*			
	Rhizoplane	Inhibition (%)	Endorhizosphere	Inhibition (%)
Untreated control	5.064 ± 3.061a	0	5.756 ± 3.238a	0
10^6	4.889 ± 3.397ab	3.45	4.948 ± 3.238b	14.03
10^7	4.497 ± 4.0616	11.20	3.695 ± 3.364c	35.79
10^8	0.649 ± 2.447c	98.55	0.008 ± 0.384d	99.86

*Means followed by the same letter are not significantly different in the columns as per the LSD test ($P < 0.001$)

their biocontrol efficacy against the bacterial wilt pathogen *Ralstonia solanacearum*. Tomato seeds were sown in separate flats containing 32 cells each. Seeds were cleaned with deionized water, followed by treatment with PGPR suspensions (5 ml/treatment) at a concentration of 5×10^8 CFU/ml. Second application of BCA was carried out for some treatments at 7 days before inoculation with *R. solanacearum*. The pathogen suspension (5 ml at 6×10^7 CFU/ml) was drenched into each transplant flat cell. Tomato plants were transplanted at 3 days after challenge inoculation in pots containing moist soil. Treatment of tomato plants with *B. pumilus* and *P. putida* reduced the wilt disease incidence compared to the controls. Two applications of the BCA strains were more effective than single application (Anith et al. 2004). The ability of *Bacillus* strains to inhibit colonization of maize roots by *Fusarium verticillioides* that produces the fumonisin, a mycotoxin, in maize grains was assessed. Seed bacterization with *B. subtilis* strain CE1 at 10^8, 10^7 and 10^6 cells/ml inoculum concentrations inhibited *F. verticillioides* counts at rhizosphere and endorhizosphere levels. Maximum inhibition of colonization by the pathogen was observed in plants growing from seeds treated with 10^8 CFU/ml concentration of bacterial cells (Table 4.3; Cavaglieri et al. 2005).

The antagonistic potential of *Rhizobium* isolates on the development of chickpea wilt disease caused by *Fusarium oxysporum* f.sp. *ciceris* was assessed under greenhouse conditions. The bacterial inoculum was multiplied in yeast extract medium. The pathogen was grown in PDA and mixed with the mixture of soil, sand and peat (1:1:1). Pregerminated seeds of chickpea were inoculated with liquid suspension of *Rhizobium* isolates and planted in pathogen-infested soil and maintained in the greenhouse at 25 ± 5 °C and 60-90 RH for a period of 12 weeks. Five isolates of *Rhizobium* were able to reduce percentage of wilted plants in both susceptible and moderately susceptible chickpea cultivars (Arfaoui et al. 2006). The biocontrol potential of *Rhizobium leguminosarum* bv. *viceae* (*Rlv*), applied as seed treatment against damping-off disease caused by *Pythium* spp. affecting pea and lentil was evaluated. Treatment of pea seeds with *Rlv* strains R12, R20 or R21 significantly ($P < 0.05$) reduced incidence of damping-off disease, promoted seedling growth and increased root nodule mass, root biomass and shoot biomass. Increase in seed yield also was seen, when treated with strain R12 or R21. Further, seed treatment with strain 21 was the most effective among four strains of *Rlv* tested in providing

Table 4.4 Effect of seed bacterization of tomato with *Lysobacter capsici* strain PG4 on incidence of Fusarium wilt disease caused by *F. oxysporum* f.sp. *radicis-lycopersici* (FORL) and cell density of the BCA recovered from the roots of tomato (Puopolo et al. 2010)

Treatments	Plants infected (%)	PG4 density[a]	Plant fresh weight
Tomato (no inoculation)	0c	0	168.31 ± 28.85b
Tomato + PG4	0c	2.3×10^6	240.98 ± 32.24a
Tomato + FORL	24 b	3.6×10^6	243.34 ± 50.74a
Tomato + FORL	86 a	0	172.84 ± 35.31b

[a]Recoverable cell density of *L. capsici* PG4 on tomato plants expressed as CFU/g of roots

disease suppression to a level equal to that offered by the fungicide Thiram™. In the case of lentil, the strain R12 was the most effective in protecting the plants against damping-off and in enhancing plant growth, root nodule mass and shoot biomass. Treatment of lentil seeds with strain R12 was as effective as Thiram in reducing disease incidence, in addition of to enhancing plant growth and seed yield. The efficacy of disease suppression was strain-specific, the strain R21 being more effective for the damping-off affecting pea, while the strain R12 was more effective for treatment of lentil seeds to obtain more effective disease control (Huang and Erickson 2007).

The effectiveness of seed treatment with *Pseudomonas fluorescens* (A506) and *Acidovorax avenae* subsp. *avenae* (AAA99-2) was assessed for the suppression of bacterial fruit blotch disease (BFB) of watermelon caused by *Acidovorax avenae* subsp. *citrulli*. Seeds treated with AAA99-2 showed greater level of disease suppression reducing BFB transmission to plants by 96.5 % AAA99-2 treated watermelon seeds developed symptoms after a delay of 2 days, compared with control and they showed lowest levels of BFB incidence. Seedling emergence was affected by seed treatments (Fessehaie and Walcott 2005). Assessment of the efficacy of *Lysobacter capsici* strain PG4 in suppressing the tomato Fusarium wilt disease caused *F. oxysporum* f.sp. *radicis-lycopersici* (FORL) was carried out under greenhouse conditions. The results of rapid seed assay in which PG4 was found to be effective, were confirmed by the greenhouse tests. PG4 proved to be a good root colonizer. High population of PG4 (about 10^6 CFU/g of roots) could be recovered from the roots of PG4-coated seeds. Seed treatment with PG4 reduced the wilt disease incidence to 24 % as against 86 % in untreated plants inoculated with FORL (Table 4.4). In addition to the reduced disease incidence, PG4-treatment enhanced plant fresh weight, indicating growth-promoting effect of the treatment with *L. capsici* PG4 (Puopolo et al. 2010; Appendix 4.7).

Potato soft rot disease caused by *Pectobacterium carotovorum* and *P. atrosepticum* has been posing a stiff challenge for successful potato cultivation. Bacterial isolates were evaluated for their antagonistic potential against the bacterial pathogens. Four bioactive isolates with strong inhibitory activity were selected under in vitro conditions. The isolates were identified as *Streptomyces* spp. based on the 16S rDNA sequence analysis. These isolates were tested for their ability to reduce in vivo soft rot symptoms on potato slices of cultivars Bintje, Yukon Gold, Russet

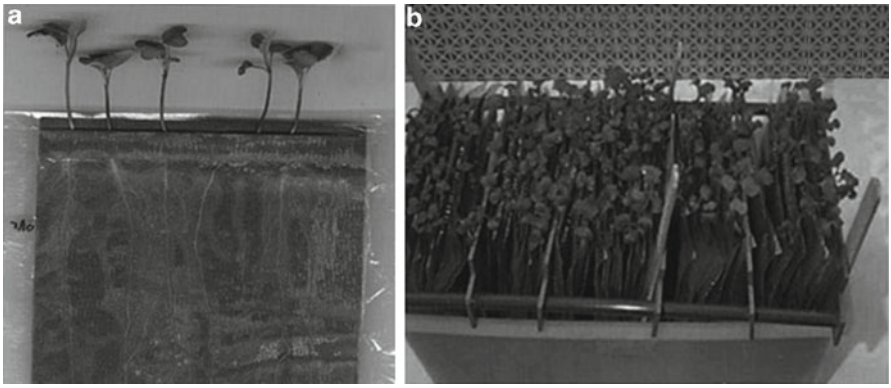

Fig. 4.10 Radish growth pouch assay used for determining the biocontrol potential of *Pantoea agglomerans* strain E278A against bacterial leaf spot disease caused by *Xanthomonas campestris* pv. *armoraciae*. (**a**) radish seedlings raised on growth pouches; (**b**) arrangement of growth pouches in holding racks (Courtesy of Han et al. 2000 and with kind permission of The American Phytopathological Society, MN, USA)

and Norland. Biomass inoculum and culture filtrates of the BCA isolates were used to treat the potato slices. The strain *Streptomyces* sp. OE7 reduced the symptom severity by 65–94 % caused by the two bacterial pathogens on potato slices. The strain OE7 was considered to have the potential for controlling the potato soft rot disease (Baz et al. 2012).

4.2.2.3 Root Treatments

A radish growth pouch assay was developed to demonstrate the effectiveness of *Pantoea agglomerans* strain E278Ar in inducing systemic resistance against the radish bacterial leaf spot disease caused by *Xanthomonas campestris* pv. *armoraciae* (*Xca*) (Fig. 4.10). The roots of radish seedlings were treated with suspensions E278Ar and challenged with *Xca* strain 704b at 7 days after root treatment with BCA. Disease severity on treated seedlings, was significantly reduced, compared with untreated control plants (Table 4.5). Root treatment with E278Ar reduced disease severity of bacterial leaf spot, when the foliage was sprayed with either a 10^8 or 10^6 CFU/ml suspension of the pathogen. The biocontrol agent essentially did not migrate into the foliage of radish plant after root treatment, indicating a spatial separation between the pathogen and BCA and induction of systemic resistance by the BCA in radish. The strain E278Ar was as effective as the synthetic resistance inducer 2,6-dichloroisonicotinic acid (INA), when applied as root treatment. The growth pouch developed in this study required less space and shorter period than the anthracnose bioassay (Han et al. 2000; Appendix 4.8). Two bacterial isolates obtained from root tips of tomato plants were identified as *Bacillus amyloliquefaciens* and *Paenibacillus alvei* were good rhizosphere colonizers and efficiently

Table 4.5 Suppression of radish bacterial leaf spot disease and population of the bacterial pathogen in leaves following root treatment with *Pantoea agglomerans* strain E 278Ar (Han et al. 2000)

Root treatment	Pathogen inoculation	Mean disease severity[a]	Pathogen population (Log CFU/g fresh weight)
Control	–	1.0	<1.5
Control	+	3.2	8.5
E278Ar	+	2.2	7.4
INA	+	2.3	6.9
$LSD_{0.05}$		0.5	0.9

[a]Mean disease severity ratings for five experiments; LSD (P = 0.05) as per Fisher's method

inhibited the mycelial growth of *Verticillium dahliae* in dual cultures. In glasshouse experiments, root dipping or soil drenching of egg plants with bacterial suspension of 10^7 CFU/ml resulted in significant reduction in disease severity expressed as percentage of diseased leaves (40–70 %), compared to the untreated controls under high pathogen inoculum level (40 microsclerotia/g of soil). In heavily Verticillium-infested potato fields, experiments with seed potatoes treated with a bacterial talc formulation (10^8 CFU/g) showed a significant reduction in symptom development expressed as percentage of diseased potato plants and significant increase (25 %) in yield of potato tuber over the control plot yield (Tjamos et al. 2004). In greenhouse assay, two bacterial isolates J-2 and B-11 were evaluated for their potential to protect beet root plants against *Sclerotium rolfsii,* causing root rot disease. Treatment of pathogen-infested soil with a biomass and culture filtrate of the bacterial isolates reduced the incidence of root rot disease ($P < 0.05$). The isolate J-2 was found to be more efficient in suppressing disease development. Both isolates were classified as belonging to the genus *Streptomyces* (Errakhi et al. 2009).

The efficacy of the chitinolytic rhizobacterial species *Serratia plymuthica* strain HRO-C48 was assessed under greenhouse condition to be applied against the soil-borne pathogens *Verticillium dahliae* and *Phytophthora cactorum* infecting strawberry crops. In soil naturally infested with *V. dahliae* and artificially inoculated with *P. cactorum*. Prior to planting in containers containing pathogen-infested soil, roots of strawberry plants were dipped in a suspension of *S. plymuthica* (2×10^9 CFU/ml) for 15 min. This root treatment reduced the number of wilted and necrotized plants. After 10 weeks, the average reduction of Verticillium wilt was 8.2 % and reduction of root rot was 3.6 %, compared with nontreated control. In addition, the yield was increased by 18.5 and 33.4 % in soils naturally infested by *V. dahliae* and in soils infested with *P. cactorum* respectively. A high biocontrol activity was correlated with a high increase in yield levels (Kurze et al. 2001).

A nonpathogenic *Agrobacterium vitis* strain F 2/5 was reported to be effective against grapevine crown gall pathogen *A. vitis* and the disease development (Burr and Otten 1999). However, this nonpathogenic strain F 2/5 was not effective on other host plants infected by *A. vitis*. In a later study, another nonpathogenic strain of *A. vitis* VAR03-1 was tested by soaking the roots of grapevine, rose and tomato for its protective ability against the crown gall pathogen *A. vitis, A. rhizogenes* and

Table 4.6 Biocontrol activity of nonpathogenic strains VAR03-1 and K84 against tumorigenic *Agrobacterium* spp. infecting tomato, rose and grapevine (Kawaguchi et al. 2008)

Treatment/experiment number	Tomato plants with tumors (%)[a]	Rose plants with tumors (%)[a]	Grapevine plants with tumors (%)[a]
VAR 03-1			
1	20.0[b]	10.0[b]	6.7[b]
2	26.7	0.0	0.0
3	33.3	5.0	0.0
4	NT	NT	3.4a/91.8
Mean/protective value	26.7a/63.6	5.0a/73.0	3.4a/91.8
K84			
1	46.7	5.0	66.7
2	13.3	5.0	20.0
3	6.7	0.0	13.3
4	NT	NT	46.7
Mean/protective value	22.2a/69.7	3.3a/82.2	36.7 a/12.0
Sterile distilled water			
1	40.0	20.0	53.3
2	80.0	25.0	40.0
3	100	10.0	33.3
4	NT	NT	40.0
Mean/protective value	73.3b	18.3b	41.7b

[a]Mean of three experiments with 15, 20 and 15 tomato, rose and grapevine plants respectively for each treatment

[b]Means followed by the same letter are not significantly different as per Fisher's test

A. tumefaciens infecting these crops. The soil was infested with these pathogens before planting the root-treated plants. Treatment with the strain VAR03-1 significantly reduced the number of plants with tumors and disease severity in grapevine, rose and tomato (Table 4.6). The efficacy of VAR03-1 was compared with the nonpathogenic strain K84 of *A. rhizogenes*. The inhibitory effects of VAR03-1 were almost equal to that of strain K84, but VAR03-1 protected grapevine more efficiently than K84. The strain VAR03-1 offered protection to a wide range of host plants against three tumorigenic *Agrobacterium* spp. (Kawaguchi et al. 2008; Appendix 4.9).

Pseudomonas fluorescens strain 1100-6 effective against *Agrobacterium vitis* causing crown gall disease in grapevine was transformed with the *gfp* gene encoding green fluorescent protein (GFP). The transformed strain *P. fluorescens* 1100-6-*gfp* was injected into three 1-year old potted grapevine cuttings, rooted in perlite and transferred to pasteurized potting mix. The disposition of genetically tagged strain 1100-6-*gpf* was evaluated after 6 months by PCR amplification of the *gpf* gene in extracts from grapevines inoculated with tagged strain at the time of planting. The extracts from the roots did not show the presence of detectable amplicons, indicative of the *gfp* gene. However, some vines (3/5) contained detectable populations of tagged 1100-6-*gfp* strain near the soil line. The *gfp*-gene sequence was detectable in

Table 4.7 Detection of genetically tagged *P. fluorescens* strain 1100-b-gfp from different regions of the grapevine plants (Eastwell et al. 2006)

	Root-dip inoculation[a]	
Sample	Inoculated(n=5 vines)	Non-inoculated (n=5 vines)
Roots	00b	00b
Stem below soil line	60a	00b
Stem above soil line	60a	30a
Soil extract	60a	60a
Pr>F	0.0097	0.0027

[a]values with the same letter in the same column are not significantly different (P=0.05) as per Duncan's multiple range test

stem sections cut from the vines up to 6-cm above the soil line. Extracts from the soil revealed detectable concentrations of the *gfp*-gene sequence in (3/5) containers as well as the stem extracts from plants that cohabited with an inoculated vine (Table 4.7). Observations on inoculated tissues using epifluorescence microscope revealed the presence of the BCA predominantly in the xylem tissues. The BCA was present in both the pith and xylem vessels at the point of inoculation, but only in xylem vessels at locations away from the site of inoculation. Occasionally the BCA persisted on the external surface of grapevines (Eastwell et al. 2006).

The biocontrol potential of *Bacillus subtilis* HS93, *B. licheniformis* LS674 and *Trichoderma harzianum* was assessed to reduce the development of Phytophthora blight caused by *P. capsici* and root rot disease caused by *Rhizoctonia solani* infecting pepper. Seed treatment and root drenching with bacterial suspension of HS93 with 0.5 % chitin was more effective against *P. capsici* and *R. solani* infection than the bacteria alone. The strain LS674 and *T. harzianum* reduced *Rhizoctonia* infection, but not *Phytophthora* infection. In the greenhouse assessments, seed treatment and root drenching with HS93 amended with chitin enhanced its biocontrol activity against *P. capsici*, but not against *R. solani.* Likewise, enhancement of biocontrol activity of LS 674 and *T. harzianum* was significant against *R. solani* by combining with 0.5 % chitin, but this combination did not have any effect on *P. capsici*. The results indicated the enhancement of biocontrol activity of the bacterial and fungal BCA, in the presence of chitin (Ahmed et al. 2003). A sequential screening procedure was developed to facilitate the selection of potent bacterial strains antagonistic against *Phytophthora capsici* causing *Phytophthora* blight disease of pepper. Bacterial strains (231) isolated from soils and roots of cucumber, pepper and tomato plants grown in different locations were first tested using a radial assay based on which 88 strains were pre-selected. These strains were screened by seedling assay with 2-week old pepper seedlings inoculated with a suspension of zoospores added at the rate 1,000 zoospores/g of potting mixture which caused complete death of seedlings without treatment with bacterial strains. The strains with 50 % control efficacy were selected for further test with 5-week old pepper plants. Four bacterial strains KJ1R5, KJ2C12, KJ9C8 and 11S16 were consistent in their biocontrol activity against *P. capsici*. These four strains were able to protect the pepper plants effectively against *Phytophthora* blight disease in the field trials also (Kim et al. 2008).

Bacterial strains *Pseudomonas putida* FC-6B, *Pseudomonas* sp. FC-7B, *P. putida* FC-8B, *Pseudomonas* sp. FC-9B and *Pseudomonas* sp. FC-24B isolated form used rockwool soilless substrates were evaluated for their ability to suppress Fusarium wilt disease of tomato. The pathogen *F. oxysporum* f.sp. *lycopersici* (5×10^4 chlamydospores/ml) was mixed with soil and distributed in pots. Tomato seedlings were removed carefully at 21 days after sowing. The roots of the seedlings were dipped in 100 ml of bacterial suspension (10^8 and 10^9 CFU/ml) for 10 min and planted in pots. *P. chlororaphis* MA342 was applied at 7.5×10^6 CFU/ml. Application of bacteria by root dipping significantly reduced wilt disease incidence by 20–55.0 %, by different treatments, compared with control. *Pseudomonas* sp. FC-9B (at 10^9 CFU/ml) reduced the disease incidence to the maximum extent. In addition, this strain significantly increased plant height and also plant biomass compared with the control (Srinivasan et al. 2009). The biocontrol efficacy of *Brevibacillus brevis* against *Fusarium oxysporum* f.sp. *lycopersici* infecting tomato was assessed in the glasshouse. Disease incidence was markedly reduced in pots coinoculated with pathogen and bacterial BCA, compared with pots inoculated with the pathogen alone. The carrier used to inoculate the seed with *B. brevis*, either carboxy-methyl cellulose (CMC) or vermiculite had no effect on the persistence of BCA on roots in the absence of the pathogen. The carrier had no influence on disease incidence (Chandel et al. 2010).

Aspergillus flavus present in most agricultural soils infects corn (maize) causing ear rot disease. In addition, it produced aflatoxins that are highly potent carcinogens and mutagens. The antifungal activity of *Pseudomonas chlororaphis* strain JP1015 and *P. fluorescens* strain JP2175 against *A. flavus* was determined using soil coculture assays, which were able to quantity fungal and bacterial populations over time. Growth of *A. flavus* was inhibited up to 100-fold by *P. chlororaphis* and up to 58-fold by *P. fluorescens* within 3 days, following soil coinoculation. A correlation was observed between bacterial inhibition of *A. flavus* growth in soil and the reduction of windborne spore dispersal. *P. chlororaphis* and *P. fluorescens* reduced the vegetative population (propagules) of *A. flavus* recovered from corn field soils. This growth inhibition greatly reduced fungal sporulation on the surface of the coinoculated soil. The inhibition of sporulation, in turn, reduced number of spores collected one meter downwind of the treated soil by 75- to 1,000-fold and 10- to 50-fold respectively by *P. chlororaphis* and *P. fluorescens*. Since *A. flavus* infection (contamination) of crops such as maize occurred primarily by windborne spore dispersal, reduction of pathogen growth and sporulation by the bacterial BCA, might be of significant importance for controlling the ear rot disease and aflatoxin contamination of grains (Palumbo et al. 2010).

Isolates (265) of *Streptomyces* spp. were evaluated for their potential to suppress the root and stem rot disease caused by *Sclerotium rolfsii* and bacterial wilt disease caused by *Ralstonia solanaceraum* infecting chilli pepper in Thailand. Three isolates were selected based on their effectiveness in inhibiting the pathogens growth and they were identified as *S. mycasofaciens* SS-2-243, *S. philanthi* RL-1-178 and *S. philanthi* RM-1-138. As *S. philanthi* RM-1-138 strongly inhibited the seed

germination and seedling growth, it was not tested further. Under greenhouse conditions, the strain RL-1-178 effectively suppressed the root rot and stem rot and its efficacy was approximately equal to that of *Trichoderma harzianum* and fungicide carboxin. The strains SS-2-243 and RL-1-178 were as effective as streptomycin sulfate in reducing the bacterial wilt disease of chilli pepper. *T. harzianum* had no effect on the bacterial wilt disease. The results showed the potential of *S. philanthi* RL-1-178 in suppressing both root and stem rot caused by *S. rolfsii* and bacterial wilt induced by *R. solanacearums* indicating its broad spectrum of biocontrol activity (Boukaew et al. 2011).

4.2.2.4 Stem Treatments

The antagonistic potential of *Rahnella aquatilis* strain HX2, isolated from vineyard soil, was evaluated using *Agrobacterium vitis* as the target pathogen capable of infecting grapevine and sunflower. The strain HX2 was cultured in liquid PDA medium and grown on a shaker at 180 rpm at 28 °C for 28 h. Pellets of bacteria were washed with distilled water and resuspended in buffered saline. The suspension of the strain HX2 was prepared in buffered saline. (2×10^8 CFU/ml). *Agrobacterium* cell suspension (2×0^8 CFU/ml) was mixed with two volumes of strain HX2. Sunflower stems were inoculated with a drop of mixture (3 µl) after making a longitudinal cut on the stem and the wounded sites were wrapped with parafilm. Gall formation was monitored for 15 days after inoculation. The crown gall tumor inhibition activity was calculated as per the formula

$$\left[\left(C - T\right)/C\right] \times 100$$

where C is the average weight of crown gall tumor in control and T is the average weight of the crown gall tumor in treated plants. Similar experiment was conducted to assess the effect of HX2 on disease suppression in grapevine also. The crown gall symptoms induced by *A. vitis* was reduced from 30 to 100 % by HX2 strain depending on the *Agrobacterium* strain (Chen et al. 2007).

Nonpathogenic strains of *Agrobacterium* sp. were isolated from graft unions of nursery stocks of grapevine in Japan. The nonpathogenic strains ARK-1, ARK-2 and ARK-3 were identified as *A. vitis*, based on classical diagnostic tests, sequence analysis and multiplex PCR procedure. Stems of grapevine seedlings were inoculated with cell suspensions of mixture of seven strains of *A. vitis* (Ti), strain VAR03-1 (reported earlier as a BCA) and one of the three newly identified nonpathogenic strains, as competitors to assay the suppression of tumor formation by *A. vitis*. A 1:1 cell ratio of pathogen/strains ARK-1, ARK-2 and ARK-3 reduced the tumor incidence, the strain ARK-1 being the strongest inhibitor of tumor formation. Strain ARK-1 established populations on roots of grapevine tree rootstock and persisted on roots for 1 year. The strains ARK-1, ARK-2 and ARK-3 did not produce a halo of inhibition against *A. vitis* (Ti) strain on YMA medium. Dead cells or culture

filtrates of strain ARK-1 did not have inhibitory effect on tumor incidence on grapevine stem. The results indicated that the new nonpathogenic strains might inhibit crown gall formation in grapevine plants through a mechanism different from that of VAR03-1 strain. The strain ARK-1 appeared to have the potential for effective suppression of grapevine crown gall disease (Kawaguchi and Inoue 2012).

4.2.2.5 Foliage Treatments

A greenhouse bioassay was developed to assess the biocontrol potential of *Pseudomonas syringae* strains TLP2 and Cit 7, *P. fluorescens* A 506, *P. syringae* pv. *tomato* DC 3000 (*Pst*) *hrp* mutants and nonpathogenic strains of bacteria from tomato leaves against tomato bacterial speck disease caused by *P. syringae* pv. *tomato, P. fluorescens* A 506, *P. syringae* TLP2, and *P. syringae* Cit 7 protected the tomato plants from moderate to high levels, when applied as foliage treatment. *Pst hrp* mutants could provide significant reductions in bacterial speck severity. *P. syringae* Cit 7 maintained an effective population size on tomato leaflets, when applied once a week. However, this strain did not prevent the formation of lesions on tomato fruits and the epiphytotic population size of the pathogen was also not affected by the *P. syringae* Cit 7. The results of greenhouse assays with multiple replications (minimum of three) could be the basis for predicting the superior efficacy of *P. syringae* over *P. syringae* TLP2 and *P. fluorescens* A 506 (Wilson et al. 2002).

Isolates of *Bacillus* spp., *Pseudomonas* spp. *Rahnella aquatilis* and *Serratia plymuthica* were evaluated for their ability to suppress the development of potato late blight disease caused by *Phytophthora infestans*. Russet Burbank potato plants (6-week old) grown in clay pots were used as test plants. One terminal and one primary leaflet at leaf position 4 in each of the three plants were treated with water or bacterial suspension applied at a concentration of 10^8 CFU/ml. Treated and adjoining (untreated) leaves were tagged and the plants were incubated for 24 h at 22 °C (day) and 17 °C (night) under a 16-h photoperiod. Drops of suspension containing zoospores of *P. infestans* were placed on tagged (treated and untreated) leaves of both control and treated potato plants. Then all plants were kept in a moist chamber (RH 100 %) for 48 h and transferred to growth room. After 6 days, the tagged leaves were removed scanned and analyzed with Assess (Lamari 2002) to estimate the infected area. Percentage protection provided by the bacterial isolates was calculated separately on treated (local protection) and untreated (systemic protection) leaves using the formula

$$\text{percentage of local protection} = 100 - \left[\text{BPu} / \left(\text{Pu}\right) \times 100\right]$$

where BPt is the infected area on leaves pretreated with a bacterial isolate and challenged with pathogen and Pt is the infected area on their counterparts from control

plants that were treated with sterile distilled water and challenged with the pathogen. The efficiency of protection offered was calculated using the formula

$$\text{percentage of local protection} = 100 - \left[\text{BPu} / \text{Pu} \times 100\right]$$

where BPu is the infected area on untreated leaves inoculated with *P. infestans* (from plants treated with a bacterial isolate) and 'Pu' is the infected area on corresponding untreated leaves inoculated with *P. infestaus* (from control plants). All bacterial isolates provided local protection of potato leaves against *P. infestans* ranging from 18 to 60 % reduction in infection rate. On the other hand, three isolates only could provide effective systemic protection against the late blight pathogen (Daayf et al. 2003). The biocontrol potential of *Serratia marcescens* strain B2 in suppressing the development of rice sheath blight disease caused by *Rhizoctonia solani* AG-1 IA was assessed in the greenhouse. Rice plants treated with bacterial suspension followed by challenge inoculation with *R. solani* exhibited reduced incidence of sheath blight disease, compared with control inoculated with the pathogen alone. The strain B2 survived in soil under glasshouse conditions for 4 weeks, when applied at a concentration of about 10^8 CFU/g soil indicating its persistence in the soil (Someya et al. 2005). The endophytic actinomycetes *Actinoplanes campanulatus*, *Micromonospora chalcea* and *Streptomyces spiralis* were evaluated for their efficacy in suppressing development of *Pythium aphanidermatum* and the disease caused under greenhouse conditions. These actinomycetes were more effective, when applied in combination of three than as individual strain. These glucanase-producing actinomycetes were equally effective as metalaxyl, when applied as a combined treatment, indicating that the actinomycetes could be used as replacement for the fungicide. In addition, they exhibited the growth promotional property as an added advantage (El-Tarabily et al. 2009).

Two strains *Pseudomonas fluorescens* and one strain of *Bacillus megaterium* were evaluated for their efficacy to inhibit the development of Septoria tritici blotch (STB) disease infecting wheat caused by *Mycosphaerella graminicola* (teleomorph). Among the total of 141 bacterial strains tested, seven bacterial isolates significantly reduced the percentage of leaf area covered with pycnidia up to 92 % compared to control in the preliminary screen. In the seedling tests, two strains of *P. fluorescens* (MKB21 and MKB91) and an unidentified strain MKB163 reduced the symptom intensity by 89, 92 and 90 % respectively. However, the strain MKB163 adversely affected the plant health causing severe chlorosis of the leaves. In the adult plant tests conducted in miniplots, *Bacillus megaterium*, reduced the disease intensity more effectively than the strains of *P. fluorescens* (MKB21 and MKB91). Suppression of STB in seedlings and adult plants by *B. megaterium* might be due to combination of different mechanisms (Kildea et al. 2008). Actinobacterial strains (11) isolated from different plants lentil, chickpea, pea, fababean and wheat were characterized and identified morphologically and also by using 16S rRNA gene sequencing procedure. All isolates possessed antifungal activity against

Phytophthora medicaginis, Pythium irregulare and *Botrytis cinerea*. Under green-house conditions two strains *Streptomyces* sp. BSA25 and WRA1 were the most effective and suppressed *Phytophthora* root rot disease of chickpea, when coinoculated with *Mesorhizobium ciceri* WSM 1666. *Streptomyces* sp. BSA 25 with rhizobial strain promoted vegetative growth of root and shoot dry weights by many folds compared to infected control. Combination of the bacterial BCA and the rhizobial strain provided protection to chickpea against the fungal pathogen and also promoted plant growth (Misk and Francoc 2011).

The pseudomonads obtained from cotton rhizosphere was assessed for their efficacy to suppress the development of cotton bacterial blight disease caused by *Xanthomonas campestris* pv. *malvacearcem* (*Xcm*). These strains along with *Pseudomonas aeruginosa* 7NSK2 and *P. fluorescens* CHA0 used as reference strains were grown on KB agar plates for 30 h at 26 °C. Suspensions of the test strains were prepared in 1 % methylcellulose solution and the concentration of bacterial cells was adjusted to OD 0.6 at 620 nm using a spectrophotometer. Acid-delinted and neutralized cotton seeds were disinfected with sodium hypochlorite solution followed by rinsing them in sterile distilled water. The seeds were dipped in bacterial cell suspensions for 45 min. Control seeds were treated with 1 % methylcellulose solution. Seeds, after air-drying for 30 min at 27 °C, were sown in pots containing sterilized sandy clay loam from cotton fields. The plants were inoculated by spraying the cells of the pathogen (*Xcm*) at a concentration of 10^6 CFU/ml. The plants were placed in the greenhouse at 31 °C (day) and 26° (night) and 95–100 RH. The percentages of leaf area infected were determined for all treatments after 15 days. All strains except two (13Q and EQ) significantly reduced the disease by inducing systemic resistance to the cotton bacterial blight disease (Fallahzadeh-Mamaghani et al. 2009).

4.2.2.6 Inflorescence Treatments

Basal kernel blight of barley caused by *Pseudomonas syringae* pv. *syringae* (*Pss*) affects the inflorescence at heading stage. *Pantoea agglomerans* (syn. *Erwinia herbicola*) strains isolated from asymptomatic barley kernels were tested for their efficacy to suppress the disease. *P. agglomerans* strains Eh454 and Eh239 were sprayed on barley cvs. B1202 and B2601 respectively at the soft dough stage, 3 days prior to inoculation with *Pss* strain Pss 552, including the heat-Killed strain Eh239 as control. The BCA strain Eh454 and Eh239 applied on cv. 1202 reduced basal kernel blight infection significantly by 91–100 % ($P=0.0026$), compared with positive control inoculated with Pss 552 alone. The heat-killed bacterial suspension Eh239D also reduced the infection by 52 %. The level of disease suppression was considerably less in cv. B2601 treated with the BCA strains. The effectiveness of the biocontrol was affected by the biocontrol strains, barley cultivar and time of application of the BCA strains. Application of the BCA strain prior to pathogen inoculation was effective in disease suppression, whereas no significant disease reduction could be seen, when the BCA strains were coinoculated with the pathogen (Braun-Kiewnick et al. 2000).

Search for potential biocontrol agents resulted in the isolation of 354 bacterial strains from wheat anthers. These strains were screened in two selection steps using an index of dominance (ID) assay. Twenty two strains (6 % of the total) were able to reduce the growth of *Fusarium graminearum* (teleomorph: *Gibberella zeae*) causing Fusarium head blight (FHB). These strains reduced the production of DON by *F. graminearum* on irradiated wheat grains by 60–100 %. The ability of bacterial strains to control FHB and to arrest DON production was evaluated under greenhouse conditions. Nine strains significantly reduced the FHB severity by 49–71 % (P = 0.05 %). The DON content in spikes produced by plants was significantly reduced by 32–100 %, compared with the control. Five strains decreased the mycotoxin content in the grains to considerable levels. Two of the effective strains were identified as *Brevibacillus* sp. BRC 263 and *Streptomyces* sp. BRC 87B that had potential for large scale application (Palazzini et al. 2007).

Pseudomonas fluorescens A 506 in the commercial product (BlightBan A 506) is recommended as a BCA for the control of fire blight of pear and apple. Addition of 0.1 mM $FeCl_2$ induced secretion of an antibiotic toxic to the fire blight pathogen *Erwinia amylovora* . Hence, the availability of iron to A 506 on the surfaces of pear and apples might significantly affect the effectiveness of protection provided by the BCA strain. An 'iron biosensor' construct of A 506 was developed by transformation with an iron-regulated promoter (*pvd*) fused to promoterless ice nucleation reporter gene (*inaZ*). The flowers of pear and apple were spray-inoculated with aqueous suspensions of A 506 (*pvd-inaZ*) in the screenhouse. This construct, A 506 (*pvd-inaZ*) could establish high populations on pear and apple flowers ranging from 10^4 to 10^6 CFU/flower. The floral stigma represents a unique microbial habitat, different from other aerial plant surfaces in that it offers an abundant, but transient supply of nutrients. The results indicated that bioavailable iron was in limited supply on the stigmatic surfaces of pear and apple flowers. Chelated forms of iron might be oversprayed on flowers colonized with A 506 or mixed with the inoculum to increase iron bioavailability to the BCA on the surface of pear and apple flowers and this may positively influence antibiotic production by A 506 and consequent fire blight suppression (Temple et al. 2004).

A procedure of physiological adaptation to increase colonization and survival of *Pseudomonas fluorescens* EPS62e in the phytosphere of rosaceous plants was developed to improve the effectiveness of this strain against the fire blight disease of pear and apple caused by *Erwinia amylovora* (*Ea*). This procedure consisted of osmo-adaptation (OA) and nutritional enhancement (NE). OA was based on saline stress and osmolyte amendment of the growth medium during inoculum preparation. NE consisted of addition of glycine and Tween 80 to formulation. NE and OA increased the growth rate and carrying capacity of EPS62e under high RH conditions and improved survival at low RH on flowers under controlled environmental conditions. NE did not promote growth or affect infection capacity of *Ea*. The effect of both methods was assessed under field conditions, by monitoring the population of EPS62e, employing quantitative PCR (Q-PCR) (for estimating total population) and CFU counting (for determining culturable population) methods. EPS62e colonized blossoms, following application under field conditions.

Table 4.8 Biocontrol efficacy of weakly virulent strains in suppressing the development of Pierce's disease in grapevine plants challenged with virulent strain of *Xylella fastidiosa* (Hopkins 2005)

Weakly virulent strain/ virulent strain	Symptomatic/ total plants	PD severity[a]
None/PD92-8	3/3	5
Syc 86-1/None	0/3	0
Syc 86-1/PD 92-8	2/3	1.0
PD 91-2/None	0/3	0
PD 91-2/PD 92-8	3/3	4.7
EB 92-1/None	0/3	0
EB 92-1/PD 92-8	0/3	0
EB 92-2/None	1/3	1.0
EB 92-2/PD 92-8	3/3	1.0

[a]Disease severity determined using a 0–5 scale

But the BCA was stressed, as indicated by a sharp decrease in culturable population compared to total population. However, after establishment in flowers and at the end of bloom, almost all total population became culturable. The physiological adaptation treatments increased the population levels of the BCA over those of nonadapted cells during the late stage of the flowering period. Adoption of the combination of NE and OA enhanced the effectiveness of the bacterial BCA against the fire blight disease, compared with EPS62e without physiological adaptation (Cabrefiga et al. 2011).

Naturally occurring avirulent or weakly virulent strains of *Xylella fastidiosa* (*Xf*), causative agent of grapevine Pierce's disease (PD) were evaluated for their ability to suppress the development of PD in grapevine. Weakly virulent strains of *Xf* PD-1 and Syc 86-1 inoculated into the lower internodes of Carignane grapevines protected the plants against a highly virulent strain of *Xf* (Hopkins 1992). In a later study, four weakly virulent strains PD91-2, EB92-1, EB 92-2 and Syc 86-1 inoculated by pin-pricking inoculation technique into the xylem vessels of grapevines plants. The highly virulent strain PD 92-8 was inoculated into the plants after 2 weeks. Development of symptoms in different treatments was observed for 6 months at an interval of 2 weeks. Infection of symptomatic plants was confirmed by culturing *Xf* from leaf petioles. Disease severity was scored after 6 months using a 0-5 disease rating scale. The strains Syc 86-1, EB 92-1 and EB 92-2 protected the grapevines against *Xf* effectively. These strains were isolated from sycamore and elderberry plants. Cuttings first inoculated with Syc 86-1 or EB 92-2 and challenged with virulent strain developed mild symptoms showing a severity rating of 1.0, whereas plants protected by EB 92-1 did not show any visible symptoms, following challenge inoculation with virulent strain PD 92-8. The strains PD 91-2 was effective, as all plants inoculated showed symptoms of PD in the greenhouse test (Table 4.8; Hopkins 2005; Appendix 4.10).

4.2.3 Field Evaluation of Biocontrol Activity

The bacterial biocontrol agents obtained from different substrates are evaluated for their efficacy in suppressing the development of the microbial plant pathogens and the disease(s) caused by them under in vitro and in vivo conditions. The laboratory tests are useful to eliminate ineffective ones from the large number of bacterial species/strains or isolates. The putative biocontrol agents (BCAs) that have direct inhibitory effects on the pathogen development may be selected by different laboratory tests. The putative BCAs that act on the pathogen through other mechanisms can be recognized by performing greenhouse tests. The natural conditions existing in fields are quite different from those provided in the greenhouse. Hence, the effectiveness of the BCAs selected through laboratory and greenhouse tests, has to be demonstrated under natural conditions, where the BCAs are expected to protect the crops susceptible to various bacterial pathogens. The bacterial BCAs selected in the laboratories alone or along with available commercial products are evaluated to determine the relative effectiveness of various BCAs which may be applied in different ways.

4.2.3.1 Soil Application

Bacterial biocontrol agents are applied to the soil either to inhibit the pathogen development/population and/or to protect the emerging seedlings from infection by pathogens. In general, soil application of BCA is both expensive and difficult to achieve uniform coverage of pathogen-infested areas for effective control of the target pathogen(s). The biocontrol agents may be applied as liquid cultures or formulated products on the soil at required concentrations. *Pseudomonas putida* 06909-rif/nal was applied in irrigation water for the control of *Phytophthora parasitica*. Application with every irrigation increased the BCA population as well as its biocontrol efficacy against *P. parasitica* over that of single yearly applications at the start of the irrigation season (Steddom and Menge 1998, 1999). A commercial field fermentor (BioJect) was developed for culturing and delivering *P. putida* 06909-rif/nal through irrigation water. An amount of 120 l of bacterial inoculum at 5×10^8 CFU/ml could be produced after 12-h fermentation and this quantity was sufficient for two applications/day. Ten repetitive application of *P. putida* resulted, in population levels similar to that from a single application at 10-fold higher concentrations. The fermentor could treat large volumes of irrigation water with pure cultures of bacterial BCAs (Steddom and Menge 2001). The efficacy of *Pseudomonas fluorescens* SS101 and its surfactant (cyclic lipopeptide surfactant massetolide A)-deficient mutant mass A 10.24 to suppress the population and root infection of apple and wheat seedlings by *Pythium* spp. was assessed. Both parent (wild type) strain and the mutant effectively suppressed resident *Pythium* populations to an equivalent level in the presence or absence of plant roots and ultimately suppressed *Pythium* root infection to the same degree on all host plants. The split root plant assays were conducted using strain SS101 or mutant 10.24 in the orchard soil to study the role

of induced resistance in suppressing the disease development. Strain SS101 or the mutant 10.24 significantly reduced infection by *Pythium* spp. on the component of the wheat or root system cultivated in soil treated with the respective bacterial strain. Infection of wheat roots and Gala apple seedling roots was reduced to 11 and 15 % respectively as against 34 and 60–70 % in untreated soil following application of the wild-type and mutant strains of *P. fluorescens*. The results of split-root assays indicated that strain SS101 did not limit root infection by *Pythium* spp. via induced systemic resistance (Mazzola et al. 2007).

4.2.3.2 Seed Treatments

Seed treatments with biocontrol agents is the preferred method of application of BCAs, as this treatment mode is easier and less expensive, in addition to requirement of small quantities of the BCA are required. Further, seed bacterization may protect the young seedlings against the pathogens present in the seeds as well as in the soil. The plant growth-promoting rhizobacteria (PGPR) have been employed to protect the plants against seedborne, soil borne and airborne pathogens. The PGPR strains have been shown to induce systemic resistance in cucumber to bacterial and fungal diseases. Seven PGPR treatments including single-strain treatments and mixtures of *Bacillus pumilus* strain INR7, *Curtobacterium flaccumfaciens* strain ME1 and *B. subtilis* strain GB03 were evaluated for their efficacy. In 1996 and 1997, all PGPR treatments significantly reduced the severity of angular leaf spot (*Curtobacterium flaccumfaciens*) and anthracnose (*Colletotrichum orbiculare*) diseases. Mixtures of PGPR strains provided higher level of protection against the diseases. As use of methyl bromide has to be phased out in several countries, concerned with adverse impact of the chemical on the environments, application of PGPR strains may effectively compensate the reduced plant growth often observed with methyl bromide fumigation (Raupach and Kloepper 2000).

Bacterial isolates obtained from wheat rhizosphere were evaluated for their efficacy against *Microdochium nivale* and *Fusarium culmorum* causing snow mold and seedling blight diseases respectively by treating the seeds with the isolates that were found to be effective under greenhouse conditions. Wheat seeds were treated with different bacterial cultures at a concentration at 10^9–10^{10} CFU/ml of bacterial cells. Seeds preinoculated with *F. culmorum* were treated with 300 ml of bacterial suspensions/kg of seeds and air-dried. Seeds naturally infected with *M. nivale* were also treated in a similar manner with bacterial isolates. Treated and dried seeds were stored for up to 2 weeks at 20 °C in paper bags until sown in the field. Three bacterial isolates were closely related to *Pseudomonas fluorescens* and one isolate was identified as *Pantoea agglomerans*. All these four isolates significantly suppressed the development of both diseases to a level that was equivalent to that of fungicide (Panoctine 400) treatment. Disease-suppressive effects were found to be repeatable in the field experiments conducted during 1997–2000. Seed treatments with naturally occurring effective bacterial isolates resulted in significant disease suppression of seedling blight and snow mold diseases as well as enhancement of grain yield

(Johansson et al. 2003). *Rhizobium* isolates (14) were tested for their efficacy in suppressing chickpea wilt disease caused by *Fusarium oxysporum* f.sp. *ciceris*. Chickpea seeds were soaked in the bacterial suspension (10^8 cells/ml) to coat the seeds uniformly with test *Rhizobium* isolates. They were sown in respective plots at the rate of 60 seeds/plot and replicated thrice. Non-bacterized chickpea seeds were sown in control plots. Number of wilted plants was recorded in each treatment at 12 weeks after sowing. The isolates Pch43 and Rh4 were the most effective and reduced the disease incidence to less than 8 % as against 48.7 % infection in control plots. Further, the *Rhizobium* isolates enhanced plant growth and yield under field conditions (Arfaoui et al. 2006). Weakly virulent strains of *Xylella fastidiosa* (*Xf*). were inoculated into two grapevine cultivars by the pin-pricking technique in the vineyard. Infection of plants with Pierce's disease (PD) occurred by natural transmission through the vector insects (glassy-winged sharp shooters) probably *Homalodisca coagulata* (glass-winged sharpshooters) and *Oncometopia nigricans*. Observations on disease incidence were made at 6-month intervals for 2 years. The *Xf* strain EB92-1 provided excellent control of PD in the cv. Cabernet Sauvignon for 4 years in central Florida. Inoculation with benign strains of *Xf* like EB92-1 seems to provide an effective opportunity to contain the incidence of PD and subsequent spread by the natural vectors (Hopkins 2005).

4.2.3.3 Treatment of Planting Materials

The effectiveness of *Rahnella aquatilis* strain HX2 in reducing the incidence and severity of grapevine crown gall disease caused by *Agrobacterium vitis* K308 was assessed under field conditions. One-year old cuttings of Muscat Hamburg grapevine free of crown galls with 2–3 nodes were soaked in a mixture of the HX2 (BCA) and K 308 (pathogen) (1:1) for 10 min. Positive control cuttings were treated only with K308. Crown gall formation and weight of tumor were assessed at 8 months after planting. Tumor inhibition activity was calculated based on the formula:

$$\text{Inhibition of tumor formation}\,(\%) = \left[(\mathrm{C}-\mathrm{T})/\mathrm{C}\right]\times 100$$

where 'C' is the average weight of crown gall tumor of the control and 'T' is the average weight of the crown gall tumor of treatments. The strain HX2 efficiently controlled the pathogenic strain *A. vitis* K 308, under field conditions. The antibiotic substances secreted by strain HX2 might have an important role in disease suppression. Treatment with strain HX2 decreased the tumor formation as well as disease severity, as reflected by the percentage of inhibition of tumor formation in infected grapevine plants. The strain HX2 could be detected in the grapevine rhizosphere, under field conditions up to 90 days after BCA application to the basal portions of the cuttings (Chen et al. 2007). A nonpathogenic strain of *Agrobacterium vitis* VAR 03-1 was tested, under field conditions for its efficacy in reducing the incidence and intensity of grapevine crown gall disease caused by *A. vitis* which can infect rose, tomato and sunflower as well. A mixed cell suspension (about 10^8 cells/ml)

Table 4.9 Efficacy of nonpathogenic strains VAR 03-1 and K 84 against *Agrobacterium vitis* infecting grapevine under field conditions (Kawaguchi et al. 2008)

Treatment	No. of replications	Plants with tumors (%)[a]	Protective value (%)
Strain VAR 03-1	1	0.0	
	2	0.0	
	3	0.0	
	Mean	0.0a	100.0
K 84	1	21.4	
	2	7.1	
	3	7.1	
	Mean	11.9b	15.0
Sterile distilled water	1	20.0	
	2	14.3	
	3	7.7	
	Mean	14.0b	–

[a]Means of three replications of 14 grapevine seedlings per treatment; means followed the same letter are not significant statistically as per Fisher's protected least significant difference test

containing four tumorigenic strains of *A. vitis* was used to infest the soil in concrete frame plots. After 2 weeks, the roots of 1-year old grapevine seedlings were pruned and soaked in nonpathogenic strains VAR 03-1 and K 84 in suspensions (about 10^9 cells/ml) for 1 h in distilled water at 25 °C. The strain VAR03-1 inhibited the development of tumors completely and the protective value of VAR 03-1 was 100 %. The strain K 84 was ineffective in reducing the formation of tumors in treated plants and it was not significantly different from control plants treated with distilled water (Table 4.9). The results of the field experiment strongly indicated that treatment of grapevine roots with the suspension of VAR 03-1 would be suitable for practical exploitation. In addition, strain VAR 03-1 might be a promising agent for the control of crown gall disease in rose and tomato as well (Kawaguchi et al. 2008).

4.2.3.4 Shoot Treatments

The effectiveness of the bacterial BCA *Paenibacillus lentimorbus* isolate CBCA-2 in suppressing the development of pistachio panicle and shoot blight caused by *Botryosphaeria dothidea* was assessed under field conditions. Pistachio trees were selected in a 30-years old orchard and the shoots were pruned. The bacterial cells (10^8 CFU/ml), culture fluids alone, washed bacterial cells in potato dextrose broth (PDB) and unwashed cells suspensions of *P. lentimorbus* and distilled water (control) were applied, using household sprayer directly into wound surfaces immediately after pruning until run-off. Each pruning wound was inoculated with a suspension of *B. dothidea* (100 µl) pycnidiospores (10^4/ml) at 5 h after treatment application. Observations on disease development were made at 8 months after inoculation by taking stem pieces from different treatments and isolating the pathogen in PDA containing lactic acid. Treatment with bacterial cells and culture filtrate reduced infection by *B. dothidea* significantly ($P < 0.05$). Bacterial cell suspension provided

maximum protection and reduced the infection rate from 14.8 (in control) to 2.2 % in 2001 and from 13.5 (in control) to 3.7 % in 2002. The results indicated that *P. lentimorbus* had the potential for effective control of the pistachio panicle and shoot blight disease and it could be integrated into practices adopted for management of the disease (Chen et al. 2003).

4.2.3.5 Foliar Treatments

Foliar application of bacterial biocontrol agents has been adopted more widely against foliar pathogens which are more numerous and widespread, as they can spread through wind even from distant locations. The bacterial strains/species that have shown promise under greenhouse conditions are tested under field conditions. *Pseudomonas syringae* strains TLP2 and Cit 7 and *P. fluorescens* strain A 506 were evaluated for their biocontrol potential against tomato bacterial speck disease pathogen *P. syringae* pv. *tomato* (*Pst*). The suspensions of the BCA (1×10^8 CFU/ml) were applied weekly to tomato foliage using a CO_2-powered backpack sprayer until leaves were visibly wet. The suspension of cells of *Pst* was applied once. The BCAs were applied 8–10 times during the season. The strain Cit 7 was found to be the most effective in reducing the bacterial speck severity of 28.3 %, as compared to 18.0 and 14.9 % reduction obtained with strains A 506 and TLP2 respectively. The *hrpS* mutant of *P. syringae* pv. *tomato* DC 3000 was also able to reduce the disease severity significantly under field conditions (Wilson et al. 2002). The biocontrol efficacy of three bacterial strains, *P. fluorescens* strains MKB21 and MKB91 and *Bacillus megaterium* strain MKB 135 was assessed in reducing the intensity of Septoria tritici blotch (STB) of wheat in small-scale plots. The plots were separated from one another by 1 m, incorporating a guard row of the spring barley cv Fractal. The plants were sprayed with a pycnidiospore suspension (10^6 spores/ml) of *Mycosphaerella graminicola* or a 0.2 % Tween solution (control) using a handle-held sprayer. The plots were covered with clear polythene bags immediately to provide high relative humidity favoring disease development. The bags were removed after 48 h and sprayed with cell suspensions of bacterial strains (10^7 CFU/ml). The plots were covered with polythene bags again for 24 h. Eight flag leaves from each plot were selected for evaluation of disease intensity at 28, 32, 36 and 40 days post-inoculation. The area under disease progress curve (AUDPC) was calculated and compared. Of the three strains of bacteria tested, only *B. megaterium* strain MKB 135 significantly reduced disease severity as indicated by AUDPC values in field trials conducted in 2004 and 2006. The reduction in disease severity by MKB 135 may be due to the result of a combination of different mechanisms of biocontrol activity (Kildea et al. 2008).

4.2.3.6 Inflorescence Treatments

The strains (4) of *Pantoea agglomerans* (syn. *Erwinia herbicola*) were evaluated under field conditions for their potential in suppressing the development of barley

basal kernel blight disease caused by *Pseudomonas syringae* pv. *syringae* (*Pss*). The BCA strains Eh236, Eh239, Eh454 and Eh460 were sprayed on barley cvs. B 2601 and B 1202, prior to the expected infection window for the pathogen *Pss* attack at the late milk stage. Overhead sprinkler irrigation for 5 h in the morning and evening was provided for favoring natural disease development. The BCA strains were sprayed as suspensions (10^9 CFU/ml). Percentage of infection was calculated after harvest as per the formula:

$$\text{percentage of kernel blight} = (a / b) \times 100$$

where 'a' is the number of blighted kernels per sample and 'b' is the total number of kernels per sample. The efficacy of *P. agglomerans* to decrease the kernel blight infection percentages depended on the susceptibility of barley cultivars. On cv. B1202, reduction of the disease ranged from 30 to 58 %, depending on the BCA strain used. In the cv. B2601, the disease was reduced by 45–68 %, when compared to untreated control. Blight reduction due to strain combinations was 42–48 % in cv. B2601 and 34–64 % in cv. B1202. The results indicated the need for selecting BCA strain to achieve maximum reduction in disease incidence (Braun-Kiewnick et al. 2000).

Pseudomonas fluorescens A506, the active ingredient of the commercial product BlightBan A506 and *Pantoea agglomerans* (syn. *E. herbicola*), were tested under field conditions for their ability to protect apple blossoms from infection by *Erwinia amylovora,* the causative agent of fire blight disease. The strains E325 and C9-1 of *P. agglomerans* and *P. fluorescens* A 506 were applied to open blossoms on enclosed and nonenclosed trees in order to minimize or allow honeybee-dispersal of the bacteria. Dispersal of antagonistic bacterial strains was less frequent on blossoming apple trees surrounded by plastic enclosures, presumably because the trees minimized the presence and activity of honey bees. All BCA strains were able to colonize the flower stigmas and multiplied over a 48- or 72-h period to a size approaching or exceeding 10^6 CFU/flower. The assessment of comparative effectiveness of antagonistic strains based on floral populations of *E. amylovora* and blossom blight incidence showed that A 506 and C9-1 were equally effective and more efficient than other strains (Pusey 2002). In another study, the comparative ability of suppression of apple fire blight disease was determined using *P. agglomerans* Eh252 (capable of producing antibiotic) and a near-isogenic antibiotic-deficient derivative, strain 10:12. The BCA strains were applied at 30 and 70 % bloom to pear or apple trees. Aqueous suspensions of freeze-dried cells of *E. amylovora* (*Ea*) were applied at full bloom. Both Eh252 and 10:12 reduced the growth of *Ea* on blossoms compared with control trees treated with water. In three of seven field trials Eh252 was more effective in decreasing the fire blight incidence than the mutant strain. Overall, Eh252 reduced the disease incidence by 55 ± 8 % and 10:12 by 30 ± 6 % (Stockwell et al. 2002).

Four bacterial strains *Pseudomonas fluorescens* A 506 and *Pantoea agglomerans* strains C9-1 and E325 and preparations of *Bacillus subtilis* QST 713 containing bacterial endospores and lipopeptide metabolites were assessed for their biocontrol

potential against fire blight disease of apple under field conditions in Michigan, New York and Virginia states of United States of America. The bacterial strains in individual tests were not consistent in their biocontrol activity as reduction in blossom infection in treated trees ranged from 9.1 to 36.1. The bacterial antagonists to be successful, colonization of a large proportion of flower stigma and growth to a large population size (10^5 to 10^6 CFU/blossom) are important requirements among others. The product Serenade containing lipopeptides produced by *B. subtilis* QST713 was slightly more efficient in controlling blossom blight than the other antagonists and more consistent from year to year and between locations. However, Serenade was found to be less effective, compared with streptomycin. Integration of biological control preparations with streptomycin might be a desirable approach to reduce the use of streptomycin, while achieving better fire blight disease control (Sundin et al. 2009).

Appendix 4.1: Media Used for Culturing Bacterial Biocontrol Agents

A. **General media**

1. King's medium B (Yoshida et al. 2001)
   Protease peptone No.3 20.0 g; K_2HPO_4-1.5 g; $MgSO_4$ 1.5 g; glycerin 10.0 ml; distilled water 1,000 ml.
2. Peptone-PD medium (Yoshida et al. 2001)
   Peptone 5.0 g; potato 200.0 g; dextrose 20.0 g distilled water 1,000 ml.
3. CY agar medium (Bull et al. 2002)
   Casitone 1.0; yeast extract 0.1 %; $CaCl_2$. H_2O 0.1 %; agar 1.5 %; cycloheximide 50 µg/ml; mystatin 50 µg/ml; vancomycin-10 µg/ml; distilled water 1,000 ml.
4. MGY agar medium (Steddom and Menge 2001)
   Mannitol. 10.0 g; L-glutamic acid 2.0 g KH_2PO_4-0.5 g; NaCl 0.2 g; Yeast extract-0.25 g; agar 18 g; distilled water 1,000 ml. Amended with different concentrations of rifampicin (1–50 mg/l).
5. API 50 CHB/E medium (Gacitúa et al. 2009)
   Ammonium sulfate 2.0 g; yeast extract 0.5 g; tryptone 1.0 g; disodic phosphate-3.22 g; monopotassium phosphate 0.12 g; Oligo element solution 10 ml; Phenol red 0.17 g; demineralized water. pH 7.4–7.8 with a known cloudy pattern of 1×10^6 CFU/ml called "MacFarland."

B. **Specific media for bacterial biocontrol agents**

1. *Pseudomonas* spp. (Mazzola et al. 2007; Lee et al. 2010)
   King's medium B amended with rifampicin 100 µg/ml
2. Nutrient broth (Kurze et al. 2001; Janisiewicz et al. 2005)

Peptone	6.75 g
NaCl	5.0 g
Yeast extract	1.50 g
Hydrolyzed protein	1.75 g
Distilled water	1,000 ml
Ptt	7.2

3. Basic broth medium for *Stenotrophomonas maltophila* (Zhang et al. 2001)

Yeast extract	0.5 g
$(NH_4)_2SO_4$	1.0 g
$MgSO_4$. $7H_2O$	0.3 g
KH_2PO_4	1.36 g
Distilled water	1,000 ml
Adjust to H to	7.0

4. King's B agar for fluorescent pseudomonads (Postma et al. 2000)
   Proteose peptone No.3 20.0 g
   (Difco Laboratories)

KH_2PO_4	1.5 g
$MgSO_4$. $7H_2O$	1.5 g
Glycerol	15 ml
Agar	13.5 g
Cycloheximide	100 mg
Detionized water	1,000 ml
Ground under UV	

5. Bennett's medium for *Streptomyces* spp. (Errakhi et al. 2007)
   Beef extract (Merck, Germany)-1 g/l; glucose 10 g/l; peptone-2 g/l; yeast extract 1 g/l; agar (Difco U8A) 15 g/l; distilled water-1,000 ml.
6. Pigment producing medium (PPM) for *Pseudomonas chlororaphis* (Liu et al. 2008)
   Tryptone – 22 g; glucose 20 g; KNO_3 – 5 g; distilled water – 1,000 ml; ampicillin – 100 µg/ml; tetracyclines –125 µg/ml; gentamycin – 30 µg/ml
7. CYagar for myxobacteria (Bull et al. 2002)
   Casitone 0.3 %, Yeast extract – 0.1 %; $CaCl_2H_2O$ – 0.1 %; agar 2 % water 1,000 ml.
8. Luria-Bertani Broth (Chen et al. 2007)
   Tryptone – 10 g; Yeast extract – 5 g; Nacl – 10 g; distilled water –1,000 ml.
9. Martin broth (Chen et al. 2007)
   Dextrose – 10 g; peptone – 5.0 g; KH_2PO_4 – 1.0 g; $MgSO_4.7H_2O$ – 0.5 g; Bengal red – 0.03 g; streptomycin sulfate – 30 µg/ml
10. Gause No.1 synthetic medium for actinomycete,
    Starch soluble – 20 g; KNO_3 – 1 g; KH_2PO_4 – 0.5 g; $MgSO_4.7H_2O$ – 0.5 g; NaCl – 0.0 g; $FeSO_4$ – 0.01 g; $K_2Cr_2O_7$ – 0.1 g; distilled water – 1,000 ml.

Appendix 4.2: Detection of *Pseudomonas fluorescens* (Pf) and *Fusarium oxysporum* f.sp. *cubense* (Foc) in Banana Roots Using FITC Technique (Mohandas et al. 2004)

A. **Production polyclonal antibodies (PAbs)**

i. Prepare suspension of *Pf* (1.024×10^8CFU/ml) and Foc (9.2×10^4CFU/ml) and mix them individually with phosphate buffer saline (PBS, pH7.4) + Freund's complete adjuvant (1:1 v/v).
ii. Inject two different rabbits intramuscularly with 1 ml of Pf or Foc mixture at 1 week interval for 4 weeks.
iii. Purify the antiserum by affinity column chromatography procedure (Sigma).
iv. Perform double diffusion test to confirm the presence of Pf-and Foc-specific antibodies.
v. Conjugate the antibodies (0.353 mg/ml) with fluorescein isothiocyanate (FITC) and determine the concentrations spectrophotometrically at 280 nm.

B. **Detection of Pf and Foc by FITC-conjugated antibodies**

i. Inoculate 2-month old hardened tissue cultured banana plants with Pf (50 g) alone in charcoal base (1.024×10^8 CFU/ml) or Foc (50 g) grown on sorghum seeds (9.2×10^4 CFU/ml) or coinoculate with 50 g each of Pf and Foc and maintain controls without inoculation with either Pf or Foc.
ii. Collect root samples from different treatments; and wash them thoroughly with distilled water.
iii. Take thin sections with sharp razor blade; place them on clean glass slides; place drops of conjugated antibodies and air-dry for 20 min to fix them firmly.
iv. Dip the slides into phosphate buffered saline (pH7.2) + bovine serum albumin (BSA, 1 % w/v) to block non-specific protein binding sites; remove the slides and drain the excess solution.
v. Treat with 10–20 µl of FITC-conjugated Pf-and Foc-antibodies separately in a humification chamber under dark condition for 1 h and wash off the excess antibody with PBS + 0.1 % BSA solution.
vi. Examine the slides under a fluorescence microscope at 20 sections for each treatment and record the observation and repeat the experiment three times.

Appendix 4.3: Quantitative Detection of Pyrrolnitrin-Producing Bacteria by Real-Time PCR Assay (Garbeva et al. 2004)

A. **Extraction of DNA from bacterial species**

i. Extract bacterial cells from soil samples (10 g) using suspension in 95 ml 0.1 % tetra sodiumpyrophosphate (NaPP: $Na_2P_2O_7.12H_2O$) containing 10 g gravel (2–4 mm diameter) by shaking for 25 min at 250 rpm;

ii. Serially dilute by 10-folds in 0.8 % NaCl and streak 100 µl of 10^{-2} and 10^{-3} dilutions on appropriate medium.
iii. Harvest the bacterial cells from overnight culture into 1 ml 0.88 % NaCl; lyse the cells by bead beating (1 g 0.11 mm diameter beads in 1 ml) four times for 30 s each; extract the lysate with phenol-Tris-HCl (pH 8.0) and chloroform/isoamyl alcohol (24:1) followed by precipitation with 96 % ethanol in the presence of 5 M NaCl; wash the DNA pellets with 70 % ethanol; vacuum-dry and dissolve in 50 µl sterile Milli-Q water.

B. **Amplification by PCR**

i. Perform PCR reactions in a thermal cycler using 50 µl reaction volumes containing 0.2 M each of primer PRND1 and PRND2, 3.75 mM $MgCl_2$, 200 µM of each dNTP and 0.25 µgT4 gene 32 protein (Boehringer, Mannheim, Germany) using 5 U Ampli*Taq* Stoffel fragment in 1×Stoffel buffer; follow the thermal cycling schedule as follows: denaturation at 95 °C for 2 min, followed by 30 cycles of 95 °C for 1 min, 67 °C for 1 min and 72 °C for 1 min and final extension at 72 °C for 10 min.
ii. Purify the PCR amplicons using the High-Pure PCR product purification kit (Boehringer-Manheim, Aluere, NL) and digest with restriction enzymes in 15 µl volumes containing 1 µg of DNA, 1× reaction buffer and 10 U of each enzyme; incubate for 2 h at 37 °C
iii. Separate the digests on 2 % agarose gels and analyze with the Molecular Analyst software (Version 1.61, Bio-Rad, NL)

C. **Real-time PCR assay**

i. Design the PCR primers with Primer Express software (PE-Applied Biosystems, UK) targeting the sequence within *prnD* locus of the pyrrolnitrin producer *Burkholderia cepacia.*
ii. Label the probe at the 5′-base with fluorescent reporter dye FAM (6-carboxy-fluorescein) and at the 3′-base with quencher dye TAMRA (6-carboxy-tetramethyl rhodamine).
iii. Perform the assay in 50 µl reaction volumes with reagents from the qPCR™ CoreKit(Eurogentec) use primer and probe at concentrations as per the manufacturer's recommendations in automated ABI prison™ 7700 sequence detector (PE Biosystems).
iv. Adopt the following thermocycling conditions:
Initial cycle 50 °C for 2 min, 95 °C for 10 min and 40 cycles each at 95 °C for 15S and 60 °C for 1 min.
v. Maintain a negative control without template DNA for each set of reaction.
vi. Measure the fluorescence intensities of each dye using the ABI Prism 7700 sequence detector at every temperature step and cycle during the reaction; data acquisition and analysis are to be handled by sequence detector version 1.6 software (PE Biosystems).

Appendix 4.4: Identification of *Pseudomonas fluorescens* Using Polymerase Chain Reaction (PCR) (Scarpellini et al. 2004)

A. **Extraction of template DNA**

i. Grow the isolates in tryptic soy broth (Difco Laboratories, MD, USA) at 30 °C for 48 h.
ii. Prepare the template DNA by boiling 200 µl of bacterial suspension in MilliQ (OD_{600} = 0.6) in safe-lock Eppendorf tubes for 10 min; cool the tubes immediately on ice; and centrifuge at 20,000 ×*g* for 10 min at 5 °C.
iii. Use one microliter of template DNA suspension for each reaction.

B. **PCR amplification**

i. Perform PCR reactions in a volume of 50 µl containing ≈50–100 mg of bacterial genomic DNA solution, 5 µl of 10×PCR buffer, 200 µM of each dNTP, 2 mM $MgCl_2$, 0.5 µM of each primer and 0.5U of *Taq* polymerase (Amersham-Pharmacia).
ii. Amplify 16S region with primer set 16SF-16SR (16SF-5′AGAGTTTGATCC TGGCTCAG-3′; 16SR-5′-CTACGGCTACCTTGTTA-3′) using the following thermal profile: n:2 min at 94 °C; 5 cycles consisting of 94 °C for 45 s, 55 °C for 1 min, 72 °C for 2 min; 35 cycles consisting of 92 °C for 45 s, 60 °C for 45 s, 72 °C for 2 min; final extension of 72 °C for 2 min and final cooling at 4 °C.
iii. Amplify 16S-23S intergenic spacer (ITS1) region with the primer set 16 F945 and 23R458 (16 F945-5′-GGGCCCGCACAAGCGGTGG-3′;23R458-5′-CTTTCCCTCACGGTAC-3′) using the following thermal profile: 5 min at 94 °C: 30 cycles consisting of 94 °C for 1 min, 55 °C for 1 min, 72 °C for 2 min and final extension for 72 °C for 2 min.
iv. Amplify 16S-specific region for *P. fluorescens* using primer set 16SPS EfluF and16SPER(16SPSEfluF-5′EGCATTCAAAACTGACTG-3′;16SPSER-5′-AATCACACCGTGGRTAACCG-3′).
v. Analyze the 7 µl of PCR product by electrophoresis on 1 % agarose gel in TAE buffer and strain with ethidium bromide (0.2 µg/ml).

Appendix 4.5: Assessment of Antagonistic Activity of Bacterial Species in Agar Plates

A. **Dual culture method (Daayf et al. 2003).**

i. Recover the bacterial species/isolates from natural substrates; culture on nutrient agar and incubate for 2–3 days at room temperature.
ii. Prepare bacterial suspensions by adding 100 µl of sterile, distilled water to the developing colony in the plate and mixing.

iii. Prepare four replicates of 5 µl aliquots of the bacterial suspension; place the aliquots at 90 °C apart on the perimeter of each of two petridishes each (9 cm diameter) of clarified V8-potato dextrose agar and incubate the plates overnight at room temperature.
iv. Place a 5 mm-plug of the fungal pathogen (*Phytophthora infestans*) at the center of the plate and incubate for about 7 days.
v. Measure the diameter of fungal colonies in the treatments, when the control colony attains full growth (about 85 mm) reaching the periphery of the plate.
vi. Calculate the percentage of relative inhibition in different treatment using the formula.
Percentage of relative growth = B/P × 100 where B growth of the fungus challenged with bacteria and P is the growth of the fungus in the control.
vii. Repeat the assessment twice and calculate the mean.

B. **Plate antagonism assay (Melnick et al.** 2008**)**

i. Place two mycelial plugs of the fungal pathogen (*Phytophthora capsici*) 5 cm apart on V8 agar in petriplates (10 cm).
ii. Streak the test bacterial isolates between the plugs containing the fungus at 2.5 cm from each plug; control plates do not have the challenging bacterial species.
iii. Maintain four replicates for each treatment.
iv. Incubate the plates at room temperature.
v. Measure the radial growth of the fungus at 1, 2 and 3 days after incubation.
vi. Repeat the experiment twice and calculate the mycelial growth inhibition at different periods of incubation.

Appendix 4.6: Assessment of Biocontrol Potential of Bacterial Isolates Against Fungal Pathogens Infecting Soybean and Tomato (Benítez and Mc Spadden Gardener 2009)

Seedling Lesion Bioassay

i. Surface-sterilize the seeds and germinate them on water agar (WA, 7.5 g agar/l) at room temperature in the dark and after 4 days, transfer the emerging seedlings (3 seedlings/plate) to petriplates containing WA.
ii. Place pathogen plug (5 mm) at the center of each plate.
iii. Prepare the bacterial cell suspension from 24-h old cultures in 1/10× TS broth; centrifuge; wash twice with sterile water; treat the seedlings with bacterial cell suspension (10^7 cells) with 100 µl aliquot for each seedling and maintain triplicates for each treatment including controls with and without bacterial isolates.
iv. Express the disease severity as the percentage of the seedling root length marked by a lesion.

Appendix 4.7: Assessment of Effects of Seed Bacterization of Tomato with *Lysobacter capsici* PG4 on Incidence of Fusarium Wilt Disease Under Greenhouse Conditions (Puopolo et al. 2010)

i. Grow the pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) on PDA for 7 days in petriplates; flood the plates with 10 ml of sterile distilled water (SDW); shake well in a shaker for 30 min at 100 rpm; scrap of the fungal growth and filter through sterile glass wood to separate the conidia.
ii. Adjust the conidial concentration to 4×10^3 conidia/ml; mix aliquots (5 ml) of the suspension with sandy soil (20 g) and transfer to pots.
iii. Dip the tomato seeds coated with mixture of the bacteria strain (1×10^9 cells/ml) and methyl cellulose (1 %), and dry the seeds overnight in a laminar flow cabinet.
iv. Place one seed coated with BCA in each pot at 1 cm depth at 2 days after inoculation of FORL and place the pots in the greenhouse at 21 °C, relative humidity 70 %.
v. Remove the plants carefully after 3 weeks; wash the roots; observe the presence of browning and lesions and calculate the percentages of symptomatic plants in each treatment.
vi. Maintain suitable control treatment and analyze the data statistically.

Appendix 4.8: Effect of Root treatment with *Pantoea agglomerans* E278Ar on Development of Radish Bacterial Leaf Spot Caused by *Xanthomonas campestris* pv. *armoraciae* (Han et al. 2000)

i. Grow the bacterial BCA in L-broth for 20 h at 30 °C with rifampicin (50 μg/ml) on a rotary shaker at 200 rpm; centrifuge at 3,840 ×*g* for 10 min and discard the supernatant.
ii. Resupend the BCA cells in autoclaved 0.25 ×*g* modified Hoagland's solution and 200 μl of 1×10^9 CFU/ml suspension; pipette the suspension directly onto the roots of 3-day old radish seedlings at least 5 cm below the crown to reduce the movement of the BCA to the foliage.
iii. Use 5–10 pouches for each treatment at 5 seedlings/pouch; treat the control plants with Hoagland solution alone and arrange the pouches in a completely randomized design.
iv. Grow the bacterial pathogen in sucrose-peptone broth containing 20 g sucrose/l and 5 g Bacto peptone/l amended with streptomycin (50 μg/ml); centrifuge at 3,840 ×*g* for 10 min; spray a suspension of 20×10^8 CFU/ml prepared in sterile tap water with 0.02 % Silwet L-77 (Lehle seeds, Texas, USA) as a wetting agent

to run- off level onto radish seedlings and spray the control plants with the wetting agent alone.

v. Determine the disease severity using a 1–6 rating scale.

Appendix 4.9: Assessment of Biocontrol Efficacy of Nonpathogenic Strains of *Agrobacterium* spp. Against Tumorigenic *Agrobacterium* spp. Under Greenhouse Conditions (Kawaguchi et al. 2008)

i. Prepare cell suspensions of nonpathogenic strains (VAR03-1 and K84) from 48 h old slant cultures grown on potato semisynthetic agar (PSA) and adjust the concentration to $OD_{600} = 1.0$ (10^9 cells/ml).
ii. Prepare cell suspensions of tumorigenic strains and adjust the concentrations to $OD_{600} = 1.0$ (10^8 cells/ml).
iii. Infest the soil with pathogenic strains by pouring cell suspensions to the soil (500 ml/kg).
iv. Pull out young healthy plants carefully; rinse the roots in water to clean all soil particles; prune the roots and soak them in a cell suspension of nonpathogenic strains for 1 h or in sterile water (control) at 25 °C.
v. Plant the seedlings in pots containing soil infested with different pathogenic species; maintain the required final concentration of pathogen cells at 5×10^7 cells/g soil at 8 cm depth.
vi. Calculate the protective value as per the formula,

$$\text{Protective} = 100\% - \frac{Ax100}{C} \text{ where}$$

'A' is the percentages of plants with tumors in treatment and 'C' is the percentage of plants with tumors in control.

Appendix 4.10: Suppression of Grapevine Pierce's Disease (PD) by Using Weakly Virulent Strains of the Pathogen *Xylella fastidiosa* (Xf) (Hopkins 2005)

i. Grow the virulent and weakly virulent strains of Xf on PD3 medium for 4–6 days (log phrase); scrap off the bacteria from the medium; prepare a suspension in succinate-citrate-phosphate (SCP) buffer containing disodium succinate (1.0 g/l), trisodium citrate (1.0 g/l), K_2HPO_4 (1.5 g/l), KH_2PO_4(1.0 g/l), pH 7.0 and adjust the concentration of strains to 10^7–10^8 CFU/ml ($OD_{600} = 0.25$).

ii. Prepare four rooted green cuttings of grapevine cv. Carignane with a minimum of six nodes for each treatment.
iii. Place drops (0.02 ml) of the suspension of weakly virulent strains on opposite sides of both second and third internodes from the stem base; pierce three to five times through each of the four drops with a dissecting needle.
iv. After 2 weeks, inoculate drops (0.02 ml) of the virulent strain into the opposite sides of both fourth and fifth internodes from the stem base and place the plants in the greenhouse for 6 months at 18–23 °C (night) and 28–33 °C (day).
v. Observe the symptoms at 2 week interval for 6 months and determine the disease severity using the 0–5 scale where 0 = no symptoms; 1 = marginal necrosis (MN) or other symptoms on the basal leaf; 2 = MN in one-third or less of leaves; 3 = one-third to one half of leaves with MN; 4 = one-half to three-fourths of leaves with MN and death of growing point; and 5 = 100 % of leaves with symptoms or dead plant.

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Chapter 5
Mechanisms of Action of Bacterial Biological Control Agents

The existence of natural suppressive soils has been recognized in the Salinas valley (California, United States), the Chateaurenard region near Cavaillon (France), the Canary Islands and the Broye Valley (Switzerland). Crops grown on suppressive soils do not suffer much from certain disease(s), due to reduction in disease incidence and/or severity, in spite of the presence of the pathogen(s) in those soils. In contrast, the conducive soils allow the development of the pathogens and the diseases induced by them. The phenomenon of disease suppression in certain soils showed two common characteristics: (i) loss of suppressiveness, when the soil was pasteurized and (ii) transferability of suppressiveness to conducive soils (opposite of suppressiveness). These two characteristics indicated the involvement of the activities of soil microorganisms that are sensitive to heat or other adverse conditions. The suppressiveness of the soil associated with the presence of different species of bacteria and fungi may be predominantly, due to the production of various kinds of antimicrobial compounds, in addition to aggressive colonization of available plant surfaces, competition for nutrients and stimulation of natural plant defense systems. Some of the microorganisms have been shown to promote the growth of the treated plants. The various mechanisms of the biocontrol activities of plant-associated bacterial species are discussed in this chapter.

The bacterial biocontrol agents (BCAs) may act directly or indirectly by different mechanisms such as antibiosis, competition for nutrients, colonization of specific sites required for establishing infection by the pathogens and inducing resistance to the pathogens by activating host plant defense systems. Furthermore, the plant growth-promoting rhizobacteria (PGPR) are known to enhance plant growth, in addition to the protection of the plants against microbial plant pathogens. The mechanisms of biocontrol activities of plant-associated bacterial species are discussed to have an insight into the three-way interactions among plants, bacterial biocontrol agents and plant pathogens.

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5.1 Types of Antagonism

Antagonism exhibited by bacterial biocontrol agents predominantly depends on the ability to produce compounds that are inhibitory to bacterial and fungal plant pathogens infecting various crops or adversely affect the pathogen development in other ways (Table 5.1).

5.1.1 Pseudomonas *spp.*

5.1.1.1 Metabolites-Mediated Antagonism

Antibiotics

Root colonization is a process by which bacteria introduced on seeds, vegetatively propagated plant parts, or into soil are distributed along roots. Plant-associated bacteria have the ability to colonize and persist in the rhizosphere and they are collectively called as rhizobacteria. The plant growth-promoting rhizobacteria (PGPR) form an important group of bacterial biocontrol agents that are being employed for the management of crop diseases in various ecosystems. The PGPR species are classified under the genera *Pseudomonas, Bacillus, Azospirillum, Rhizobium* and *Serratia*. *Pseudomonas* strains with antagonistic potential play clear colony phase variation, exhibiting opaque and translucent colonies (Phase I). The important biocontrol traits such as motility and production of antifungal metabolites, proteases, lipases, chitinases and biosurfactants are correlated with phase I morphology and these characteristics are absent in bacteria with phase II morphology. Phase variation is based on structural changes at the DNA level. Phase variation as a regulatory system can influence the production of diverse traits such as production of proteases and lipases, pili, outer membrane proteins, fimbriae and surface lipoproteins (van den Broek et al. 2003). Although the PGPRs have multiple mechanisms of biocontrol activities, production of different kinds of antibiotics seems to be the principal mechanism of action against crop pathogens. These BCAs secrete phloroglucinols, phenazines, pyoluteorin, pyrrolnitrin, rhamnolipids. Several antibiotics of lesser importance are also produced by the different genera of PGPR. The activities of these metabolites against microbial pathogens are described.

Production of antibiotics by bacterial species with biocontrol potential is demonstrated in five steps. Diffusible or volatile secondary metabolites secreted by bacterial strains in vitro are purified and identified chemically. Then the antibiotic compound of significance is detected and quantified in the rhizosphere or natural substrates into which the putative bacterial BCA is introduced. The HPLC process of purification has been applied for this purpose. The structural and the principal regulatory genes controlling the expression of the antibiotic compound are identified and characterized. In the case of intrinsically poor biocontrol strains, they can be transformed by

Table 5.1 Production of antibiotics by bacterial biocontrol agents effective against microbial plant pathogens

Bacterial biocontrol agent/ antibiotics produced	Microbial plant pathogens	References
I. *Pseudomonas* spp.		
A. Pholoroglucinols 2,4-diacetylphloro-glucinol (2,4-DAPG)	*Gaeumannomyces graminis* var. *tritici*	Raaijmakers and Weller (1998) and de Werra et al. (2009)
	Fusarium oxysporum f.sp. *pisi*	Landa et al. (2002)
	Pythium ultimum var. *sporangiferum*	de Souza et al. (2003a, b)
	Rhizoctonia solani and *sclerotium rolfsii*	De La Fuente et al. (2004)
	Aphanomyces cochlioides	Bechewich and Hearth (1998) and Islam and Fukushi (2010)
	Xanthomonas oryzae pv. *oryzae*	Velusamy et al. (2006)
B. Pyoluteorin	*Pythium ultimum*	Howell and Stipanovic (1980)
	Rhizoctonia solani	Brodhagen et al. (2004)
C. Pyrrolnitrin	*Botrytis cinerea* *Gaeumannomyces graminis* var. *tritici*	Hammer and Evensen (1993), Tazawa et al. (2000), and Ajouz et al. (2009)
D. Aerugine	*Colletotrichum orbiculare* *Phytophthora capsici* and *Pythium ultimum*	Lee et al. (2003)
E Phenazines	*Gaeumannomyces graminis* var. *tritici*	Thomashow et al. (1990) and Pierson and Thomashow (1992)
	Fusarium oxysporum f.sp. *ciceris* and *F. udum*	Anjaiah et al. (2003)
	Fusarium oxysporum f.sp. *radicis-lycopersici*	Chin-A-Woeng et al. (2001a, b)
	Rhizoctonia solani	Rosales et al. (1995)
	Verticillium longisporum	Debode et al. (2007)
	Pythium myriotylum, P. spendens	Perneel et al. (2007, 2008)
	Sclerotinia sclerotiorum	Athukorala et al. (2010)
II. *Bacillus* spp.		
A. Surfactins	*Sclerotinia sclerotiorum* *Fusarium graminearum*	Athukorala et al. (2009)
B. Bacillomycin D	*S. sclerotiroum*	Athukorala et al. (2009)
	F. graminearum	
	Alternaria alteranta *Fusarium graminearum*	Zhao et al. (2010)
C. Fengycins	*S. sclerotiroum*	Athukorala et al. (2009)
	F. graminearum	
D. Azalomycin F	*Xanthomonas campestris* pv. *campestris*	Kim et al. (2003)
	Botrytis cinerea	
	Fusarium oxysporum	
	Pythium sp.	
	Rhizoctonia solani	

(continued)

Table 5.1 (continued)

Bacterial biocontrol agent/ antibiotics produced	Microbial plant pathogens	References
D. Iturins	*S. sclerotiroum*	Athukorala et al. (2009)
	F. graminearum	
	Colletotrichum truncatum	Huang et al. (2005)
	Macrophomina phaseolina	
	Phomopsis sp.	
	Rhizoctonia solani Sclerotinia sclerotiorum	
	Botrytis cinerea	Pyoung et al. (2010)
	Fusarium solani	
	Rhizoctonia solani	
	Phytophthora capsici	
III. *Paenibacillus* spp.		
Polymyxins	*Pseudomonas* spp.	Wiese et al. (1998)
Antifungal peptides	*Rhizoctonia solani*	Chen et al. (2010)
IV. *Burkholderia*		
Pyrrolnitrin	*Rhizoctonia solani*	Lievens et al. (1989)
Pyoluteorin		Baligh et al. (1999)
V. *Serratia*		
Pyrrolnitrin	*Sclerotinia sclerotiorum*	Rite et al. (2002)
Prodigiosin	*Penicillium expansum*	Levenfors et al. (2004)
VI. *Pantoea*		
Herbicolins	*Erwinia amylovora*	Ishmaru et al. (1988)
Pantocins		Wright et al. (2001)
VII. *Streptomyces* spp.		
Geldanamycin	*Rhizoctonia solani*	Beauséjour et al. (2001)

integrating the desired antibiotic biosynthetic genes that are not present in the original strains. Finally, the expression of the desired antibiotic biosynthetic genes has to be demonstrated in the rhizosphere through the use of easily detectable reporter genes that are fused to the structural genes for antibiotic biosynthesis (Haas and Défago 2005).

Fluorescent *Pseudomonas* spp. are found in abundance in the rhizosphere of plants and their ability to suppress the development of fungal and bacterial pathogens and the diseases induced by them in a wide range of crops in different ecosystem has been demonstrated. In addition, the pseudomonads exert beneficial stimulatory effect on plant growth, resulting in increased yield of agricultural and horticultural produce. These bacterial species have been intensively studied, because of the benefits provided to crop cultivation. These bacterial species secrete several antibiotics, siderophores and hydrogen cyanide (HCN) which are involved in their biocontrol activities. The antibiotics produced by *Pseudomonas* spp. belong to six classes, phloroglucinols, phenazines, pylouteorin, pyrrolnitrin, cyclic lipopeptides (all of which are diffusible) and volatile hydrogen cyanide (HCN). The complete genomic analysis to determine the sequences involved in different activities of bacterial biocontrol agents including the biosynthesis of various secondary metabolites has

been performed. In *Pseudomonas fluorescens* strain Pf-5, nearly 6 % of the 7.07 Mb genome is devoted to the biosynthesis of secondary metabolites, including antibiotics toxic to soilborne fungi and oomycetes that infect plant roots and two siderophores involved in iron acquisition. Three orphan gene clusters, for which the encoded natural product was not known earlier, were also identified in the genome of Pf-5 strain. The product synthesized from one of the orphan clusters was identified using the genomesotopic approach, employing a combination of genomic sequence analysis and isotope guided fractionation. One of the orphan gene clusters in Pf-5 was shown to be involved in the biosynthesis of orfamide A, member of a new group of bioactive cyclic lipopeptides with a putative role in biological control of microbial pathogens, causing plant diseases (Loper and Gross 2007).

Pholoroglucinols

Among the phloroglucinols, 2,4-diacetylphloroglucinol (2,4-DAPG) is well known. This phloroglucinol PhlD is a polyketide responsible for the production of monoacetyl phloroglucinol (MAPG) and PhlA, PhlC, and PhlB are required to convert MAPG to 2,4-DAPG (Bangera and Thomaslow 1999). Two major phenotypic groups of 2,4-DAPG producers have been distinguished based on the production of antifungal compounds: one group can synthesize 2,4-DAPG, HCN and pyoluteorin and another group synthesizes only 2,4-DAPG and HCN. The abundance of 2,4-DAPG-producing *Pseudomonas* spp. has been correlated with the natural suppression of wheat take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (Raaijmakers and Weller 1998). The fluorescent pseudomonads form an important group of plant growth-promoting rhizobacteria (PGPR) involved in the biological control of several crop diseases. They suppress the development of root and seedling diseases. *Pseudomonas fluorescens* CHA0 suppressed tobacco black root disease (Stutz et al. 1986), wheat take-all disease (Keel et al. 1992) and tomato wilt and crown and root rot disease (Tamietti et al. 1993; Duffy and Défago 1997). Suppression of sugar beet damping-off disease by *Pseudomonas* spp. (Shanahan et al. 1992) and wheat take-all disease by *P. fluorescens* strains Q2-87 and Q8rl-96 (Pierson and Weller 1994; Raaijmakers and Weller 1998) was also demonstrated. Although much progress has been made in the understanding of the biosynthesis and regulation of 2,4-DAPG production by *Pseudomonas* strains, adequate attention does not appear to have been paid to investigate the responses of fungal pathogens to 2,4-DAPG and the mode of its action on the target pathogens. Mostly, the effect of 2,4-DAPG was assessed on the mycelial growth. The fungal pathogens are known to produce different spore forms and structures that are resistant to chemicals and other adverse environmental conditions. Hence, it is essential to understand the variations in the sensitivity of different phases of the life cycle of the pathogen to a specific biocontrol trait for assessing the potential of the biocontrol agents possessing such traits.

Inconsistent performance of most bacterial biocontrol agents under field conditions from site to site and from year to year tends to be the disappointing reality. To overcome this obstacle, it is necessary to understand the sources of variability in the BCA performance. *Pseudomonas fluorescens* CHA0 produces the antibiotic

2,4-diacetylphloroglucinol (2,4-DAPG) that has a key role in its mechanism of biocontrol activity. The strain CHA0 carrying a translational *phlA'-lac* fusion was used to monitor the expression of the *phl* biosynthetic genes governing 2,4-DAPG production in vitro and in the rhizosphere. The reporter gene expression was greater in the rhizosphere of maize and wheat (monocots) compared with the gene expression in bean and cucumber (dicots). Similar variations were observed at cultivar levels also. Plant age had significant impact on gene expression. Root infection by *Pythium ultimum* stimulated bacterial gene expression on both cucumber and maize and this was dependent on differences in rhizosphere colonization on these plant species. The enhanced gene expression may probably be attributed to an indirect effect of disease (causing greater release of root exudates) rather than to a direct signaling from the pathogen (Notz et al. 2001).

Strains of *Pseudomonas fluorescens* produce DAPG that could inhibit growth of diverse group of different groups of organisms including fungi, bacteria, protists and nematodes (Bender and Rangaswamy 1999). The responses of *Pythium* spp., a ubiquitous pathogen infecting several crops to 2,4-DAPG were studied. The variation in sensitivity of 14 *Pythium* isolates obtained from multiple hosts to 2,4-DAPG was assessed. Variation within and among *Pythium* spp. to 2,4-DAPG was observed. Different propagules of *P. ultimum* var. *sporangiferum* exhibited significant differences in their sensitivity to 2,4-DAPG. Zoospores were the most sensitive, followed by zoosporangia, the mycelium being the most resistant structure. The activity of 2,4-DAPG was influenced by pH levels and the low pH favored its activity. Level of acetylation of phloroglucinols was found to be a major determinant of their activity. Ultrastructural alterations in hyphal tips of *P. ultimum* var. *sporangiferum* exposed to 2,4-DAPG were assessed, using the transmission electron microscope (TEM). Different stages of disorganization in hyphal tips of the pathogen were observed. Localized alteration (proliferation or disruption) in plasma membrane organization, development of an extensive network of smooth membranous vesicles, degenerated cytoplasm bordered by a retracted plasma membrane and hyphal senescence accompanied by vacuolization and degeneration of its content were frequently observed alterations in the fungal pathogen. It appeared that 2,4-DAPG did not affect the cell wall structure and composition of hyphal tips of the pathogen, since B(1,3)-1, B(1,4)- and B(1,6)-glucans were present at the same concentrations in hyphal tips both in the presence or absence of 2,4-DAPG, as revealed by immunolocalization experiments using the primary antibody (de Souza et al. 2003a, b).

The genes required for the biosynthesis of 2,4-DAPG by *P. fluorescens* Q2-87 have been cloned and the biosynthetic locus includes *phlA, phlC, phlB* and *phlD*. A rapid polymerase chain reaction (PCR)-based assay targeting the *phlD* gene essential in phloroglucinol biosynthetic pathway was developed. The limited distribution of *phlD* among microbes makes it an ideal marker gene for 2,4-DAPG-producing *Pseudomonas* spp. present in the soil and other environmental samples to be detected (Fig. 5.1) (McSpadden Gardener et al. 2001). Likewise, the genes required for biosynthesis of phenazines, pyrrolnitrin and pyoluteorin have also been cloned and sequenced from different *Pseudomonas* spp. (Kirner et al. 1998; Mavrodi et al. 1998; Nowak-Thompson et al. 1999). Strains of *Pseudomonas fluorescens* were

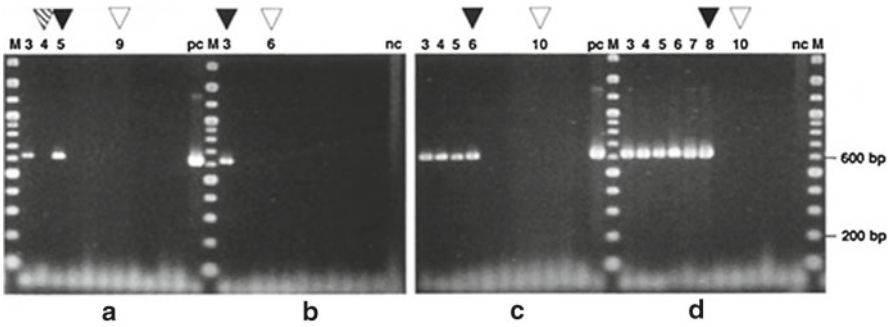

Fig. 5.1 Detection of *phlD* sequences in *Pseudomonas* spp. in the rhizosphere samples, grown in *Pseudomonas*-selective media using whole cell template for PCR amplification at different dilutions (*A, B, C* and *D*). Presence of *phlD*$^+$ bacteria in the serial dilution culture is indicated by 629- bp DNA product. *Lane M*: 100-bp DNA size standard; *numbers at the top* of the figure indicate the dilutions in the series (Courtesy of McSpadden Gardener et al. 2001 and with kind permission of The American Phytopathological Society, MN, USA)

screened for the production of 2,4-DAPG by employing a PCR-based screening procedure that used primers Phl2a and Phl2b. A fragment of 745-bp characteristic of the biosynthetic genes was amplified. The results of HPLC, HNMR and IR analysis also confirmed the ability of the strains to produce 2,4-DAPG. The purified antibiotic 2,4-DAPG was able to suppress the development of the rice bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* in in vitro assays. This antibiotic preparation suppressed the development of bacterial blight disease up to 59–64 % in greenhouse and also in field evaluation (Velusamy et al. 2006).

The isolates (>300) of 2,4-DAPG-producing fluorescent *Pseudomonas* spp. isolated from soils suppressive to Fusarium wilt or take-all disease were analyzed by whole-cell repetitive sequence-based PCR (rep-PCR) with the BOXAIR primers. The isolates belonging to seven genotypes A, D, E, L, O, P and Q were indentified. Fourteen isolates representing the different genotypes were evaluated for their ability to colonize the rhizosphere of pea plants. Population densities of genotypes D and P significantly increased faster and remained above 10^6 CFU/g of roots over the experimental duration of 15 weeks. The genetic profiles generated by the rep-PCR or RFLP analysis of the gene *phlD* indicated the rhizosphere competence of the 2,4-DAPG-prdoucing strains introduced into the rhizosphere (Landa et al. 2002). Strains of *Pseudomonas fluorescens* produce 2,4-DAPG which has a broad-spectrum of activity against many fungal plant pathogens as well as against the multidrug-resistant human pathogen *Staphylococcus aureus,* due to its bacteriolytic activity (Kamei and Isnansetyo 2003). However, the sensitivity of fungal pathogens *Pythium* spp. to 2,4-DAPG has been shown to vary significantly (Notz et al. 2001). In a later study, the sensitivity of 76 plant-pathogenic and/saprophytic strains of *Fusarium oxysporum* to 2,4-DAPG produced by *P. fluorescens* was assessed. *F. oxysporum* strains (17 %) including *F. oxysporum* f.sp. *melonis* (strain Fom38 and Fom1127) and *F. oxysporum* f.sp. *cubense* (strains Focub 1, 2 and 13) were relatively tolerant

to high concentrations of 2,4-DAPG. Some tolerant strains (18) were able to metabolize 2,4-DAPG. In two tolerant strains, deacetylation of 2,4-DAPG to less fungitoxic derivatives monoacetylphloroglucinol and phloroglucinol occurred. Fusaric acid produced by *F. oxysporum* strain might directly affect 2,4-DAPG biosynthesis by repressing the expression of the biosynthetic gene *phlA*. It was postulated that the presence of sublethal concentration of 2,4-DAPG may trigger fusaric acid production in *F. oxysporum* strains to repress 2,4-DAPG synthesis in *P. fluorescens* to prevent further accumulation of 2,4-DAPG in their habitat to toxic concentrations. Fusaric acid-mediated repression of 2,4-DAPG synthesis in *Pseudomonas* spp. was found to be strain-dependent, since fusaric acid blocked the 2,4-DAPG biosynthesis in strain CHA0, but not in strain Q21-87. The results suggested the need for the consideration of the fact that plant pathogens are dynamic organisms capable of rapidly adapting to and coping with adverse conditions like the presence of antagonistic microorganisms in the same habitat (Duffy et al. 2004; Schouten et al. 2004).

The 2,4-diacetylphloroglucinol (2,4-DAPG)-producing *Pseudomonas* spp. have been isolated from the rhizospheres of different crops grown in soils from diverse geographic regions (Keel et al. 1996). *P. fluorescens* UP61 was found to be effective against soilborne pathogens *Sclerotium rolfsii* infecting beans and *Rhizoctonia solani* infecting tomato. This strain produced three antibiotics viz., 2,4-DAPG, pyrrolnitrin and pyoluteorin contributing to its biocontrol activity. Molecular techniques such as 16S rDNA, RFLP, RAPD and rep-PCR assays and partial sequencing of the *phlD* gene governing the biosynthesis of 2,4-DAPG revealed the similarity of the strain UP61 with other biocontrol agents isolated from other geographical locations that have been shown to produce these antibiotics (De La Fuente et al. 2004). It is desirable to estimate the genotypic diversity of the 2,4-DAPG-producing *Pseudomonas* spp. that offers an enormous resource for identifying strains that are highly rhizosphere-competent and superior for biological control of crop diseases. A simple and rapid method was developed to detect the presence and assess the genotypic diversity of *ph1D*$^{+}$*Pseudomonas* strains directly in the rhizosphere samples without the need for prior isolation or enrichment on nutrient media. Denaturing gradient gel electrophoresis (DGGE) of 350-bp fragments of *phlD*$^{+}$ allowed discrimination between genotypically different *phlD*$^{+}$ reference strains and indigenous isolates. The DGGE analysis of the *phlD* gene allowed identification of new genotypic groups of specific antibiotic-producing *Pseudomonas* with different abilities to colonize the rhizosphere of sugarbeet seedlings (Bergsma-Vlami et al. 2005).

Pseudomonas fluorescens CHA0 produces antifungal antibiotics 2,4-DAPG and pyoluteorin (PLT) effective against several fungal pathogens and it promotes growth of plants also. Plant growth promotion may be due to solubilization of inorganic phosphates by production of organic acids especially gluconic acid which is one of the important acids produced by *Pseudomonas* spp. The role of gluconic acid produced by the strain CHA0 in phosphate solubilization was studied by producing mutants deficient in the genes encoding glucose dehydrogenase (*gcd*) and gluconate dehydrogenase (*gad*) required for conversion of glucose to gluconic acid and 2-ketogluconate respectively. The ability of strain CHA0 to acidify the environment and to solubilize mineral phosphate was found to be strongly dependent on its

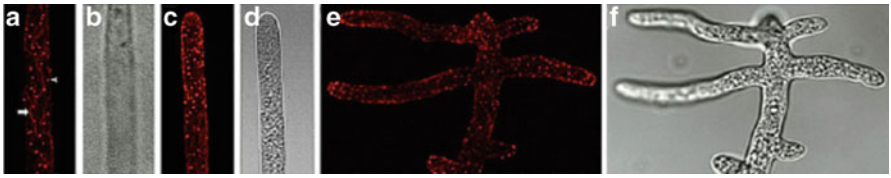

Fig. 5.2 Differential interference contrast (DIC) micrographs (**b**, **d** and **f**) and their corresponding confocal images (**a**, **c** and **e**) showing F-actin organization in control (**a**–**d**) and excessively branched and curled hyphae of *Aphanomyces cochlioides* by *Pseudomonas fluorescens* ECO-001; (**a**) and (**c**): normal organization of F-actin network; (**e**): disruption of F-actin in a *P. fluorescens*-hyperbranched hyphae; (**d**): DIC micrograph corresponding to (**c**); and (**f**): DIC micrograph corresponding to (**e**) (Courtesy of Islam and Fukushi 2010 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

ability to produce gluconic acid. The results indicated that formation of gluconic acid by CHA0 inhibited the production of PLT entirely, while production of DAPG was partially inhibited. In contrast, production of antifungal compounds by the mutant deficient in *gcd* was enhanced resulting in improved biocontrol activity against wheat take-all disease caused by *Gaeumannomyces graminis* var. *tritici.* The mutant was unable to produce gluconic acid. The study provided evidence to link the production of gluconic acid and loss or partial inhibition of ability to produce DAPG and PLT by the strain CHA0 (de Werra et al. 2009).

Pseudomonas fluorescens strain ECO-001 isolated from *Plantago asiatica* inhibited the polar growth of *Aphanomyces cochlioides,* causing damping-off disease of beet root and spinach on potato dextrose agar (PDA) medium by inducing excessive branching and curling of the hyphae. The antibiotic 2,4-DAPG secreted from the strain ECO-001 was found to be responsible for the observed abnormalities of the hyphae of *A. cochlioides.* The mechanism of action of 2,4-DAPG was not clearly understood, although its inhibitory effect on fungal pathogens has been widely reported. Tips of growing hyphae of Peronosporomycetes (earlier known as Oomycetes) contain an apical cap of the fine F-actin filaments adjacent to the plasma membrane, extending back along the hyphae and merge with subapical pattern of cortical actin cables and plaques (Bachewich and Heath 1998). To elucidate the mechanism of growth inhibition by ECO-001, a technique was developed to detect localized changes in the cortical filamentous actin (F-actin) organization by rhodamaine-conjugated phallodin (RP). Confocal laser scanning microscopic (CLSM) observations revealed that both ECO-001 and synthetic DAPG severely disrupted the organization of F-actin in *A. cochlioides* hyphae in a similar manner (Fig. 5.2). A known inhibitor of F-actin polymerization, latrunculin B also caused similar growth inhibition, excessive branching and induced disruption of F-actin in the pathogen hyphae. This investigation suggested that DAPG produced by ECO-001 might have direct effect on growth and development of *A. cochlioides* through disruption of cytoskeletal F-actin network. This report appears to be the first to indicate that disruption of cytoskeleton of a eukaryotic *A. cochlioides* by the metabolite DAPG secreted from a bacterial BCA (Islam and Fukushi 2010) (Appendix 5.1).

Pseudomonas fluorescens Pf-5 secretes a suite of secondary metabolites which are toxic to plant pathogens causing seed- and root rot diseases in several crops. The strain Pf-5 produces an array of toxic metabolites including pyoluteorin, pyrrolnitrin, 2,4-DAPG and hydrogen cyanide. Pyoluteorin and 2,4-DAPG are synthesized by polyketide synthase complexes and secreted from cells of Pf-5. Pyoluteorin production is governed by a biosynthetic gene cluster comprising of nine structural genes whose predicted functions entail the biochemical transformations required for pyoluteorin biosynthesis from acetate and proline precursors (Nowak-Thompson et al. 1999). Evidences strongly indicated that pyoluteorin served as an autoregulator, positively influencing its own production in *P. fluorescens* Pf-5. Exogenous application of pyoluteorin enhanced pyoluteorin production and exogenous pyoluteorin enhanced transcriptional activity of three pyoluteorin biosynthetic genes. Further, coinoculation with pyoluteorin-producing cells induced expression of pyoluteorin biosynthetic gene. In this respect, pyoluteorin functioned similar to established signaling molecules. Exogenous application of pyoluteorin repressed 2,4-dicetylphloroglucinol (2,4-DAPG) production by the strain Pf-5, as in the related strain *P. fluorescens* CHA0. Pyoluteorin and 2,4-DAPG mutually inhibited one another's production in Pf-5. The operation of pyoluteorin autoregulation in the rhizosphere was detected on cucumber seedlings in pasteurized soil with cross-feeding experiments. In the rhizosphere, expression of a pyoluteorin biosynthetic gene by a pyoluteorin-deficient mutant of Pf-5 was enhanced by pyoluteorin produced by coinoculated cells of Pf-5. This study showed that pyoluteorin has the ability to function as an intercellular signal between distinct populations of bacterial cells coinhabiting the rhizosphere. This finding appears to be the first to establish that pyoluteorin, an antibiotic could function as a single molecule in a natural environment (Brodhagen et al. 2004).

The communication mechanism between antagonistic *Pseudomonas* spp. and between rhizosphere bacterial communities has been studied to gain insight into the interactions between microorganisms that have a bearing on disease suppression and plant growth. Quorum sensing, the regulation of gene expression in response to the intracellular concentration of N-acylhomoserine lactones (AHLs), is a highly conserved mechanism utilized by a diverse range of Gram-negative bacteria (Narayanasamy 2008). The performance of *Pseudomonas* biocontrol agents is likely to be improved by applying mixtures of strains which are complementary in their capacity to suppress plant diseases. A combination of *P. fluorescens* CHA0 with *P. fluorescens* Q2-87 was studied in respect of the effect of these strain on each other's expression of a biocontrol trait. In both strains, production of 2,4-DAPG is a crucial factor contributing to the suppression of root diseases. DAPG acts as signaling compound, inducing the expression of its own biosynthetic genes. Dual cultural assays in which the two strains were grown in liquid medium physically separated by a membrane revealed that Q2-87 strongly induced *phlA* expression in a Δ*phlA* mutant of strain CHA0. Likewise, *phlA* expression on a Q2-87 background was induced by DAPG produced by CHA0. When coinoculated onto the roots of wheat seedlings grown under gonotobiotic conditions, the strains Q2-87 and CHA0, but not their respective DAPG-negative mutants, were able to enhance *phlA* expression

in each other. The results implicated that the positive cross-talk between two nonrelated pseudomonads might stimulate each other in the expression of an antimicrobial compound necessary for biocontrol activity (Maurhofer et al. 2004). In contrast, negative cross-communication among strains of *Pseudomonas aureofaciens* 30–84 in respect of expression of phenazine gene was observed. The presence of negative signal producing strains in a mixture with strain 30–84 reduced the ability of strain 30–84 to suppress the take-all disease pathogen *Gaeumannomyces graminis* var. *tritici* in vitro. The results suggested that cross-communication among members of the rhizosphere community and strain 30–84 might control secondary metabolite production and pathogen suppression (Morello et al. 2004).

A novel approach was adopted to monitor, in the rhizosphere, the expression of genes substantially contributing to the biocontrol efficacy of fluorescent pseudomonads. The biocontrol potential of *P. fluorescens* CHA0 is primarily determined by the production of antifungal compounds like 2,4-DAPG. The expression of the genes governing the synthesis of these compounds depended on abiotic and biotic environmental factors such as elements present in the rhizosphere. A new method for the in situ analysis of antifungal gene expression was employed, involving flow cytometry combined with green fluorescent protein (GFP)-based reporter fusions to the *phlA* and *prnA* genes essential for production of 2,4-DAPG and pyrrolnitrin respectively in strain CHA0. Expression of *phlA-gfp* and *prnA-gfp* in CHA0 cells harvested from the rhizosphere of a set of plant species, as well as from the roots of healthy, leaf pathogen-attacked and physically-stressed plants were analyzed. Levels of *phlA* and *prnA* expression varied significantly in the rhizosphere of different plant species. Physical stress and leaf pathogen infection lowered *phlA* expression levels in the rhizosphere of cucumber plants (de Werra et al. 2008).

Pseudomonas fluorescens strains CHA0 and Pf-5 produce the antibiotics 2,4-diacetylphloroglucinol (2,4-DAPG) and pyoluteorin (PLT) with broad-spectrum antifungal and antibacterial activity (Haas and Keel 2003). The DAPG and PLT biosynthetic genes are located in the DAPG locus *phlABCD* and PLT locus *pltLABCDEFG* respectively. The role of the sigma factor RpoN (δ^{54}) in the regulation of the antibiotic production and biocontrol activity in *P. fluorescens* CHA0 was investigated. The *rpoN* mutant was defective for flagella and displayed drastically reduced in swimming and swarming motilities. However, the *rpoN* mutant produced DAPG in greater concentrations (several folds) and showed higher level of expression of the biosynthetic gene *phlA,* compared with the wild-type strain and the mutant complemented with a single copy of $rpoN^+$. In contrast, loss of RpoN function led to marked reduction in PLT production and *plt* gene expression, suggesting that RpoN may control the balance of the antibiotics DAPG and PLT in the strain CHA0. In the natural soil, the *rpoN* mutant was found to be less efficient in suppressing the root rot disease in cucumber caused by *Pythium ultimum*, although the mutant was not significantly impaired in its root colonization capacity even at early stages of root infection by *Pythium* spp. (Table 5.2). The results confirmed that RpoN was the major regulator of the biocontrol activity in *P. fluorescens* CHA0 (Péchy-Tarr et al. 2005).

Table 5.2 Impact of R_{poN} on the suppression of *Pythium* damping-off and root rot of cucumber by Pseudomonas fluorescens CHA0 in natural soil (Péchy-Tarr et al. 2005)

Bacterial strain tested	*Pythium* added	Surviving plants/ flask (%)	Colonization of roots by *P. fluorescens* (Log_{10} CFU/g of roots)
None	–	100a	ND
CHA0 C wild type	–	99a	6.67 ± 0.37
CHA 250 (ΔrpoN)	–	100a	6.55 ± 0.15
CHA 251 (ΔrpoN alt Tn 7 :: $rpoN^+$)	–	98ab	7.19 ± 0.24
CHA0/pME 8013 (PrpoN – $rpoN^+$)	–	97ab	6.62 ± 0.10
CHAO/ pME 8014 ($Plac^-$ $rpoN^+$)	–	100a	6.53 ± 0.02
None	+	63c	ND
CHA0	+	92ab	7.56 ± 0.43
CHA 250	+	73c	7.20 ± 0.73
CHA 251	+	90b	7.30 ± 0.15
CHA0/pME8013	+	95ab	7.44 ± 0.16
CHA0/pME3014	+	93ab	7.57 ± 0.48

x – Data represent the means from six individual repetitions; means followed by the same letter in a column are not statistically different (P = 0.05) as per Fisher's least significant difference
ND not detected

Pseudomonas fluorescens CHA0 produces two potent antibiotics 2,4-DAPG and pyoluteorin (PLT) which have been shown to be primarily responsible for its biocontrol activity against several plant pathogens. Regulation of DAPG and PLT biosynthesis is complex and consists of several levels of pathway-specific and global control. DAPG strongly represses PLT biosynthesis and vice-versa, pointing to a mechanism of mutual feed back control that might help *P. fluorescens* to keep production of these compounds at balanced levels (Baehler et al. 2005). The exoproducts of the strain CHA0, the two proteins MVaT and MvaV were characterized and their potential as novel regulators of the biocontrol activity of the strain CHA0. In *mvaT* and *mvaV* in frame-deletion mutants of strain CHA0, PLT production was enhanced by about 4-and 1.5-fold respectively, whereas DAPG production remained as in wild-type strain. On the other hand, in a double mutant with disrupted *mvaT* and *mvaV*, PLT production was enhanced up to 20-fold, while DAPG biosynthesis was entirely repressed. The effects of mutations on antibiotic biosynthesis were confirmed by following expression of *gfp*-based reporter fusions to the corresponding biosynthetic genes. Biocontrol activity was almost entirely abolished in double mutant. The results indicated that MvaT and MvaV could act together, as further regulatory elements in the complex network, controlling expression of biocontrol traits of *Pseudomonas* spp. (Baehler et al. 2005).

Pseudomonas fluorescens strain KD does not produce 2,4-diacetylphloroglucinol (2,4-DAPG) which is the principal antibiotic involved in the biocontrol activity of several strains of *Pseudomonas* spp. However the strain effectively protected

cucumber plants against the damping-off disease caused by *Pythium ultimum*. The type III secretion system (TTSS) is employed by bacteria for pathogenic or symbiotic interaction with plant and animal hosts. The presence of TTSS genes in *P. fluorescens* KD was detected. Despite the presence of pathogenic attribute, the strain did not behave as a phytopathogen. Inactivation of *hrcV* strongly reduced the biocontrol potential of the strain KD against damping-off disease in cucumber. The reduced biocontrol efficacy was not due to a lower ecological fitness of *hrcV* mutant, because the mutant persisted in the potting mix and colonized the plant roots to the same level as that of the wild-type strain, regardless of whether the pathogen was present or not. The expression of the operon containing *hrcV* in the strain KD was strongly stimulated in vitro and in situ by *P. ultimum,* but not by cucumber (Rezzonico et al. 2005).

Phenazines

The biocontrol efficacy of *Pseudomonas chlororaphis* PCL 1391, *P. aeruginosa* PNA1 and 7NSK2 and *Pseudomonas* CMR12a in suppressing the development of *Verticillium longisporum,* infecting cauliflower, was assessed using a microplate assay. *P. chlororaphis* PCL 1391 was the most effective in suppressing the microsclerotia of *Verticillium* by the activity of phenazine-1-carboxamide (PCN) (Appendix 5.2). A seven-gene operon phzABCDEFG is responsible for the synthesis of phenazine-1-carboxylic acid (PCA) and *phzH* encodes for an asparagine synthetase like enzyme that is responsible for the conversion of PCA to PCN. The involvement of PCN in *P. chlororaphis* PCL 1391-mediated reduction of microsclerotia viability was studied by using the mutants deficient in *phzB* and *phzH* genes. A *phz B* mutant (PCL 1119) was phenzine-deficient and a *phzH* mutant (PCL1121) produced PCA instead of PCN. PCL 1119 was as effective in inhibiting microsclerotia germination as the wild-type, whereas PCL 1121 was more effective, when compared to the wild-type. In addition, involvement of PCA in the suppression of germination of microsclerotia could be demonstrated. The mutants of *P. chlororaphis* PCL 1391 and *P. aeruginosa* 7NSK2 overproducing PCA were more effective in inhibiting microsclerotia germination and formation of secondary microsclerotia, when compared to wild-type strain. The strains of *Pseudomonas* produced biosurfactants that might facilitate adhesion of the bacteria on the surface of fungal pathogens and act synergistically with the antibiotics to increase the effectiveness of biocontrol by the bacterial BCA (Debode et al. 2007).

Pseudomonas chlororaphis strain PCL 1391 produces phenazine-1-carboxamide (PCN) crucial for its biocontrol activity against tomato wilt pathogen *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL). In addition, the strain PCL 1391 produces hydrogen cyanide (HCN), chitinase and protease. The expression of the biosynthetic operon for PCN was shown to be under regulation of quorum sensing (Chin-A-Woeng et al. 2001a, b). Additional genes contributing to the regulation of phenazine biosynthesis were identified. Screening for PCL 1391 transposon mutants for increased PCN production resulted in the identification of the *psrA* gene. The transcriptional activity of *psrA* was profiled along with the quorum-sensing regulatory genes and

phz biosynthetic genes during growth. In addition to the already identified *phzI/phzR* and *gac* regulatory genes, the *psrA* gene also significantly contributed to the regulation of PCN biosynthesis. In addition, the repressing effect of *psrA* expression on quorum-sensing genes, *phz* biosynthetic genes and on itself was also demonstrated in this study by Chin-A-Woeng et al. (2005). *Pseudomonas chlororaphis* GP72 isolated from the rhizosphere of green pepper plants produced the phenazines mainly phenazine-1-carboxylic acid (PCA) and 2-hydroxy-phenazine (2.0H-PHZ) which showed broad spectrum of antifungal activity against plant pathogen (Liu et al. 2007). The alternative sigma factor RpoN functions in concert with specialized transcriptional activators (enhancer-binding proteins) to control the expression of genes coding for very diverse functions in response to environmental stimuli. In pseudomonads the features that depend on RpoN include motility and diverse metabolic functions, such as transport and metabolism of various nitrogen and carbon sources (Köhler et al. 1989). The effect of *rpoN* gene chromosomal inactivation on production of antifungal compound was assessed by inoculating the parental strain GP72 and the *rpoN* mutant GP72N were inoculated in King's Medium B (KMB) and Pigment Producing Medium (PPM). The parental strain produced significantly higher quantities of PCA than the mutant in both media tested. Complementation of the *rpoN* gene in mutant GP27N restored the ability to synthesize PCA as effectively as the parental strain and its motility. The results showed that PCA biosynthesis was partially repressed by the deletion of RpoN in *P. chlororaphis* GP 72 strain. The results suggested that RpoN might be involved as a positive regulator in the regulation of PCA biosynthesis in *P. chlororaphis* GP 72 (Liu et al. 2008a, b).

The putative role of phenazines produced by *Pseudomonas aeruginosa* PNA1 effective against *Pythium splendens* (infecting bean) and *P. myriotylum* (infecting cocoyam) was investigated. The biological activity of PNA1 was attributed to the production of phenazine-1-carboxylate (PCA) and phenazine-1-carboxamide (PCN), since its tryptophan autotrophic mutants FM13 deficient in phenazine production was unable to protect the plants against the oomycete pathogens. Exogenous supply of tryptophan restored the biocontrol activity of FM13 strain, as reflected by the reduction in disease severity in cocoyam plants. (Tambong and Höfte 2001). Two *Pseudomonas* strain isolated from the rhizosphere of cocoyam plants exhibited excellent biocontrol activity in vivo against *Pythium myriotylum* to a level similar to that of *P. aeruginosa* PNA1. They were designated *Pseudomonas* CMR5C and CMR12a. These strains produced phenazines and surfactants. Strain CMR5C formed pyrrolnitrin and pyoluteorin also (Perneel et al. 2007). In a later study, the phenazine mutant FM29 and rhamnolipid (surfactant) mutant PNA1-Rhl of *P. aeruginosa* PNA1 applied separately or with sterile volcanic soil, no suppressive effects were observed. The wild-type strain PNA1 significantly suppressed root rot disease, compared with the mutant strains. When both mutants were applied concurrently, the biocontrol efficacy exceeded that of parental strain PNA1. Bean seeds developed less reduction in pre-emergence damping-off caused by *P. splendens,* when treated with a mixture of purified PCN and rhammolipids. Microscopic observations revealed substantial vacuolation and disintegration of hyphal cells of *Pythium,* after incubation with liquid medium amended with both PCN and rhamnolipids (Perneel et al. 2008).

Interactions between two bacterial biocontrol strains of *Pseudomonas chlororaphis* PCL 1391 and *P. fluorescens* WCS 365 with *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL), causing tomato foot and root rot disease, were visualized in confocal laser scanning microscope (CLSM), using different autofluorescent proteins as markers. Tomato seedlings were bacterized with the strains PCL1391 and WCS 365 and planted in sand system infested with FORL. The bacterial strains reached root surface earlier and multiplied faster than the pathogen. The bacteria and fungal hyphae colonize the same niches on the tomato root, namely, the intercellular junctions. This might be due to chemotaxis toward and utilization of exudates compounds. By colonizing these sites and utilizing the exudates nutrients, the bacteria prevent colonization and penetration of the root tissue by FORL. The strain PCL 1391 produced phenazine-1-carboxamide (PCN) which altered the growth and morphology of fungal hyphae both in vitro and in vivo (greenhouse condition). The lack of PCN production in the strain PCL119 resulted in a delay in the appearance of morphological alterations of hyphae. In the case of the strain WCS 365, induced systemic resistance (ISR) is considered to play a major role in the mechanism of biocontrol activity of this strain. However, no differences between the effects on the FORL by WCS 365 and PCL1391 that could be related to ISR, were observed. Probably the effects of ISR by WCS 365 were more or less compensated for by the antibiosis effect of the strain PCL 1391. The extensive root colonization by both bacterial strains may represent a new mechanism in biocontrol by these *Pseudomonas* strains (Bolwerk et al. 2003).

Pseudomonas chlororaphis strain PCL 1391 and *P. fluorescens* WCS 365 were evaluated for their efficacy in suppressing the development of three races of *Colletotrichum lindemuthianum* in bean plants. Disease development was suppressed most effectively by the strain PCL 1391, whereas the strain WCS 365 exhibited no significant difference compared to the positive control. Combination of the two strains was no better than *P. chlororaphis* alone. Both strains proved to be excellent colonizers of bean roots and their combined treatment resulted in increased total bacterial populations on the root tips. Yet the population of *P. fluorescens* was reduced in the presence of *P. chlororaphis. P. chlororaphis* adversely affected the growth, sporulation and conidial germination of all the three races of *C. lindemuthianum*. In contrast, there was no effect on these characteristics of the pathogen races following treatment with *P. fluorescens*. The antibiotic effect of *P. chlororaphis* on the pathogen was considered to be due to its ability to produce phenazine-1-carboxamide which was detected using thin layer chromatography (TLC) technique. The results indicated the effectiveness of *P. chlororaphis* in protecting the bean plants against the races of anthracnose pathogen tested (Bardas et al. 2009). *Pseudomonas chlororaphis* strain PA 23 provided effective protection to canola and sunflower against *Sclerotinia sclerotiorum.* The strain PA23 produced the non-volatile antibiotics phenazine and pyrrolnitrin as well as the volatile antibiotics nonanal, benzothiazole and 2-ethyl-1-hexanol. In the greenhouse, the role of the nonvolatile antibiotics on root colonization and biocontrol ability of PA23 against *S. sclerotiorum* on sunflower was studied. Application of the strain PA23 alone or in combination with phenazine- and pyrrolnitrin-deficient *Tn* mutants resulted in significantly higher

(P = 0.05) root bacterial number and suppression of Sclerotinia wilt disease. The bacterial population decreased considerably and it appeared to be negatively correlated with the number of antibiotics produced by PA23. The role of phenazine or pyrrolnitrin was not clearly discernible from the results. The strains producing at least one antibiotic were able to maintain relatively higher population than non-producers. However, an increase in bacterial number increased at 6 weeks after sowing, in the case of strains producing at least one antibiotic. Combination of PA23 with certain mutants led to reduced root colonization and biocontrol potential of the strain PA23 (Athukorala et al. 2010).

The phenazine biosynthetic locus consists of seven genes (*phzABCDEFG*) arranged in a single operon in *Pseudomonas chlororaphis*. A pLAFRI cosmid clone (p06phz) which carried the biosynthetic genes of *P. chlororaphis* 06 was introduced into *Rhizobium etli* USDA 9032 by triparental mating with pRK2013 as helper plasmid. Thin-layer chromatography analysis with Silica Gel G chromatography showed that *R. etli* carrying the *Phz* genes produced a yellow-colored compound that had an Rf value similar to the hydroxyl phenazine-1-carboxylic acid. *P. chlororaphis* 06 produced two more compounds with Rf values similar to those for 2-phenazine carboxylic acid and 2-hydroxyl phenazine which were not detected in the *R. etli* transconjugants. The phenazine-producing *R. etli* transconjugants could inhibit the mycelial growth of *Botrytis cinerea* and *Fusarium oxysporum* in the plate assay like *P. chlororaphis* 06. In contrast, *R. etli* carrying the cloning vector alone did not inhibit the pathogen growth. The consequences of phenazine production on the symbiotic performance of the phenazine-producing *R. etli* were assessed. The transconjugant grew poorly on YEM broth and the phenazine production inhibited the viability of the cells. Black bean inoculated with phenazine-producing strain produced pale green leaves indicative of nitrogen deficiency. Numerous brownish small nodules produced, did not exhibit acetylene reduction activity, indicative nitrogen fixation. The engineered strain, although produced the antibiotic, lacked the capacity for nitrogen fixation and its growth was inhibited by phenazine, making the utility of the engineered strain very much limited as a biocontrol agent (Krishnan et al. 2007).

Bacterial isolates (105) obtained from rhizosphere soils, roots, stems and leaves of winter wheat, grown in irrigated and rainfed fields, exhibited antagonistic activity against *Gaeumannomyces graminis* var. *tritici* in vitro tests. These isolates were identified as *Pseudomonas* spp. by amplified ribosomal DNA restriction analysis. Based on biocontrol assays, 13 strains, were selected, because of their aggressive colonization of wheat rhizosphere and effective suppression of wheat take-all disease. Three of the thirteen strains, HC9-09, HC13-07 and JC 14–07 (all stem endophytes) had genes for the biosynthesis of phenazine-1-carboxylic acid (PCA), but none possessed genes for the production of 2,4-diacetylphloroglucinol, pyoluteorin or pyrrolnitrin. In addition, production of PCA by these strains was corroborated by high-pressure liquid chromatography (HPLC) analysis of 2-day old cultures. HPLC quantitative time-of-flight 2 mass-spectrophotometry analysis of extracts of roots of spring wheat colonized by HC9-07, HC13-07 or *P. fluorescens* 2–79 revealed that all three strains were able to produce PCA in the wheat rhizosphere.

Ability to produce PCA by strain HC 9–07 resulted in the loss of biocontrol activity indicating the initial role played by PCA in suppressing take-all disease development. Analysis of DNA sequences within the key phenazine biosynthesis gene *phzF* and of 16S rDNA indicated that strains HC9-07, HC 13–07 and JC 14–07 were similar to the well-established PCR producer *P. fluorescens* 2–79 (Yang et al. 2011).

Pseudomonas strain LBUM223 inhibited in vitro growth of several fungal pathogens such as *Botrytis cinerea*, *Phytophthora cactorum* and *Sclerotinia sclerotiorum* and it carried phenazine biosynthetic genes involved in the production of phenazine-1-carboxylic acid (PCA) (Paulin 2007). The ability of the strain LBUM 223 to suppress the potato common scab disease caused by *Streptomyces scabies* was assessed. The pathogen produced thaxtomins, involved in inducing disease symptoms on tubers. The involvement of PCA in repressing thaxtomin biosynthesis genes *txtA* and *txtC* and control of potato common scab disease was investigated, using a mutant deficient in PCA production (LBUM *223 phzC*$^{-}$). Seed piece tuber treatment with LBUM 223 before planting in pathogen-infested soil suppressed the disease development. A relationship was demonstrated between PCA-producing ability of the strain LBUM 223 and its capacity to inhibit the growth of *S. scabies* and its ability to repress the biosynthesis genes *txtA* and *txtC* in pathogen. The mutant LBUM 223 *phzC*$^{-}$ was less efficient in reducing the growth of *S. scabies* and it could not repress the thaxtomin biosynthetic genes. The results suggested that production of PCA by the strain LBUM 223 might have a key role in limiting pathogen development, repressing the expression of pathogenicity genes *txtA* and *txtC* and consequently in limiting the surface area of progeny tubers covered with common scab lesions (St-Onge et al. 2011). The antagonistic potential of *Pseudomonas* sp. LBUM300 in suppressing the development of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), causing tomato bacterial canker disease was determined. *Pseudomonas* sp. LBUM300 produced both DAPG and HCN on *Cmm* under in vitro and in planta conditions. Nonsynthesizing isogenic mutants of the producer strains were generated to dissect the role of individual metabolite on *Cmm* biological control. Novel specific quantitative PCR TaqMan assays allowed quantification of *Cmm* in tomato plants and rhizospheric soil. *Pseudomonas* sp. LBUM223 and LBUM300 strains significantly repressed *Cmm* growth in vitro, while their respective non-producing mutants showed less or no significant antagonistic activity. In planta, only *Pseudomonas* sp. LBUM 300 could reduce disease development and *Cmm* rhizospheric population. The results suggested that production of both DAPG and HCN contributed to the antagonistic ability of *Pseudomonas* sp. LBUM 300 strain. Simultaneous production of DAPG and HCN by LBUM 300 makes it a desirable candidate for further advancement in commercialization for the biological control of tomato bacterial canker disease (Lanteigne et al. 2012).

Pseudomonas CMR12a was effective against *Rhizoctonia solani* causing root rot disease of bean and it produced phenazines and cyclic lipopeptides (CLPs). The involvement of phenazines and CLPs in the biocontrol activity of the strain CMR 12a was investigated, using two different anastomsis groups AG2-2 (intermediately aggressive) and AG4HGI (highly aggressive). The wild-type strain CMR12a reduced drastically the disease severity caused by both *R. solani* AGs.

A CLP-deficient and a phenazine-deficient mutant of CMR12a could still protect the bean plants with less efficiency, compared with wild-type strain. The biocontrol activity was lost entirely, when the mutant was deficient in production of both antibiotics. Washing of bacterial cells before application resulted in significant reduction in disease suppressive ability of the wild-type strain, indicating that removal of metabolites produced during growth on plate was necessary for retaining higher level of biocontrol efficacy. The observations under the microscope revealed pronounced branching of hyphal tips of both *R. solani* AGs in the presence of CMR12a strain. Phenazine-deficient mutant induced more branched and denser mycelium, whereas CLP-deficient mutant and mutants deficient in both CLPs and phenazine did not alter the vegetative growth of the pathogen. The results indicated that phenazine and CLPs might have role in the suppression of development of Rhizoctonia root rot disease of bean (D'aes et al. 2011). In *Pseudomonas aeruginosa* PAO1, two genes *PhzM* and *PhzS* were characterized. They code for enzymes that modify phenazine into its related derivatives. The gene *phzM* is located upstream of *phZA1B1C1D1E1F1G1* operon and it is involved in the production of pyocyanin (Mavrodi et al. 2001). The purified pyocyanin from *P. aeruginosa* TO3 was evaluated for its antifungal activity against *Macrophomina phaseolina.* Purified pyocyanin inhibited the growth of the pathogen. Using a well-diffusion method, the effect pyocyanin on disease suppression and biofilm formation by the rhizobial strain Cal2 on radicles of groundnut (peanut) was assessed. Pyocyanin suppressed disease more effectively at high concentration. However, at lower concentration pyocyanin, increased colony-forming units of Ca12 on radicles of seedlings were observed. Application of pyocyanin-producing pseduomonads together with rhizobia contributed to the enhancement of modulation ability and sustained the growth and productivity of groundnut even in the presence of the destructive pathogen *M. phaseolina* (Khare and Arora 2011).

Rhizospheric bacterial antagonists isolated from cabbage of rhizosphere were evaluated for their efficacy in suppressing the development of blackrot disease caused by *Xanthomonas campestris* pv. *campestris* (*Xcc*). Of the three strains, TO7 was the most effective in suppressing the disease symptoms than the other two strains SA3 and CA9. The strain TO7 was tested in the field and it was very effective in controlling black rot disease in cabbage, when applied through root dip method. Based on the nucleotide homology and phylogenetic analysis, TO7 was identified as nearest homolog of *Pseudomonas fluorescens*. The strain T07 produced 2,4-diacetylphloroglucinol (2,4-DAPG) which was analyzed in different physiological conditions through qRT-PCR analysis of *phlD* gene, followed by its subsequent effect on antagonistic activity. The TO7 culture of mid log and extended stationary growth phase with shaking had a maximum *phlD* expression. Growth temperature and pH exerted direct effect on *phlD* expression with maximum production at 16 °C and at alkaline pH 8.5. The results showed that *P. fluorescens* strain T07 had potential for effective suppression of the black rot disease of cabbage and its mode of action was through production of the antibiotic 2,4-DAPG (Mishra and Arora 2012).

Pyrrolnitrin

Many fluorescent and non-fluorescent *Pseudomonas* spp. have been reported to produce the antibiotic pyrrolnitrin (PRN). *P. fluorescens, P. chlororaphis* and *P. aureofaciens* are able to secrete PRN antibiotics that are effective against fungal pathogens such as *Botrytis cinerea* (Hammer and Evensen 1993) and *Gaeumannomyces graminis* var. *tritici* (Tazawa et al. 2000) and *Sclerotinia sclerotiorum* (Fernando et al. 2005). Intensification of research on the biocontrol potential of various microorganisms belonging to diverse classes has been made to find alternative methods for managing microbial plant pathogens which developed resistance to currently applied fungicides or other chemicals. However, it may be possible to have similar loss of efficacy of biological control agents. For example, about 17 % of strains of *Fusarium oxysporum* were found to be naturally tolerant to the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) produced by *Pseudomonas* spp. Likewise, some isolates of *Botrytis cinerea* could also tolerate 2,4-DAPG (Schouten et al. 2004, 2008).

In order to assess the sensitivity of *Botrytis cinerea* to pyrrolnitrin, the baseline sensitivity distribution of *B. cinerea* populations to pyrrolnitrin-producing strain PhZ24 of *Pseudomonas chlororaphis* was assessed in vitro. The efficacy of the strain PhZ24 was assessed in in vitro confrontation assay on TSA medium containing Tryptic Soy broth (3 g) and agar (15 g) against 12 selected isolates of *B. cinerea*. The inhibition zones were measured (in mm) from 5 days after inoculation. The efficacy of the strain PhZ24 against *B. cinerea* isolates on tomato plants was determined. The EC_{50} values of 166 isolates of *B. cinerea* based on spore germination ranged from 0.0038 to 0.0318 μg/ml of pyrrolnitrin. The most resistant isolate was separated from the most sensitive isolate by a factor of 8.4 arrived at by dividing the EC_{50} value of the least sensitive isolate by the EC_{50} value of the most sensitive isolate. The wide variation of sensitivity suggested that *B. cinerea* might have the potential to evolve to some level of resistance to pyrrolnityrin. However, all isolates (12) tested against the strain PhZ24 displayed a similar level of sensitivity, whatever their EC_{50} values were in vitro. In addition, on tomato plants, protection level offered by the bacterial BCA was between 96 and 100 %, indicating high potential of the bacterial strain PhZ24. No significant differences in the responses could be recorded between the isolates of *B. cinerea*. This may be possibly, because *P. chlororaphis* PhZ24 is also able to produce another antibiotic phenazine-1-carboxylic acid (PCA) (Ajouz et al. 2009).

Botrytis cinerea, causative agent of gray mold diseases of tomato and a range of plant species, exhibits high genetic variability and it is considered a high-risk pathogen in terms of development of resistance to fungicides (Leroux 2004). Mutants of *B. cinerea* differing in their level of resistance to pyrrolnitrin [3-chloro-4 (2´-nitro-3´-chlorophenyl)-pyrrole] were selected. Development of resistance to pyrrolnitrin in four strains of *B. cinerea* was consistently associated with a dramatic loss of aggressiveness on tomato and apple fruits (Ajouz et al. 2010). The behavior of two near-isogenic lines of *B. cinerea* with differing level of resistance to pyrrolnitrin on artificially inoculated petioles and stems of tomato plants was compared by

using histopathological procedures. Differences in the aggressiveness between the pyrrolnitrin resistant mutant BCG20P and wild-type parent BC1GO were not related to the early stages of infection. The conidial germination, proliferation of germ tube and colonization of petioles were similar in the mutant and wild-type isolates. Both isolates produced mycelium within tomato petiole tissues, but the mutant generally failed to spread further to the stem. The absence of mycelium in the stem in the case of slow-growing mutant might be due to the limited induction of reactive oxygen intermediates involved in the HR reaction of the plant. Development of resistance to pyrrolnitrin in *B. cinerea* sounds a caution on the possibility of the pathogen developing resistance to the biocontrol agents that primarily suppress the development of pathogens by producing antibiotics (Ajouz et al. 2011a, b).

Pseudomonas fluorescens strain MM-B16 isolated from a mountain forest soils in Korea was identified based on the physiological and biochemical characteristics and 16S rDNA sequence analysis. The culture filtrates of strain MM-B16 were assayed for their antifungal properties, using the pathogen *Colletotrichum orbiculare* infecting cucumber, *Phytophthora capsici* and *Pythium ultimum* infecting pepper (chilli) in in vitro tests. The active compound present in the culture filtrates was identified as a thiazoline derivative, aerugine [4-hydroxy-methyl-2-(2-hydroxyphenyl)-2-thiazoline]. The antibiotic was effective against the pathogens of cucumber and pepper, but not against yeasts and bacteria. Aerugine was relatively more effective against *C. orbiculare* and *P. capsici,* compared with level of protection against *P. ultimum.* However, the efficacy of aerugine was less than the fungicides metalaxyl and chlorothalonil (Lee et al. 2003). *Botrytis cinerea* isolates (204) were tested to establish a baseline sensitivity to pyrrolnitrin antibiotic. The pathogen isolates exhibited a range of sensitivity to pyrrolnitrin with an 8.4-fold difference in EC_{50}, using reduction in spore germination by 50 % as the basis. The less sensitive isolates showed in sensitivity to other antibiotics also. The efficacy of the pyrrolnitrin-producing *Pseudomonas chlororaphis* ChPhzS24 strain was tested in vitro and on tomato plants by inoculating *B. cinerea* isolates with differing sensitivity to pyrrolnitrin determined on the EC_{50} values. Although the *B. cinerea* isolates had different EC_{50} values, no significant differences in the sensitivity of pathogen isolates could be observed to the ChPhzS24 strain. The results indicated that the pathogen isolates differed in their sensitivity to the purified antibiotic, but not to the BCA strain which could suppress the pathogen development through other mechanisms, in addition to the antibiotic produced by the BCA strain concerned (Ajouz et al. 2011a, b).

Strains of *Pseudomonas chlororaphis* PA23, *Pseudomonas* strain DF41 and *Bacillus amyloliquefaciens* BS6, capable of inhibiting infection of canola petals by *Sclerotinia sclerotiorum* in both greenhouse and field evaluation, *Bacillus thuringiensis* BS8, *B. cereus* L and *B. mycoides* S showing inhibitory effects in in vitro tests were examined for the presence of antibiotic-specific genes by applying PCR assay and Southern blotting. Thirty primers were employed to amplify antibiotic biosynthetic genes encoding phenazine-1-carboxylic acid (PCA). 2;4-DAPG, pyoluteorin and pyrrolnitrin and the zwittermicin A self-reliance gene. The PA sequence showed that it had >90 % identity with phenazine biosynthetic genes from three strains of

P. chlororaphis and it also showed 93 % similarity to the *phzCD* sequence from the positive control strain *P. fluorescens* 2–79. The PCR product (1,050-bp) amplified from *P. chlororaphis* PA23 by primers Prn AF/PrnAR exhibited high similarity (>90 %) to pyrrolnitrin biosynthetic genes *prnABCD* of *P. fluorescens* and *P. chlororaphis*. The presence of pyoluteorin and 2,4-DAPG biosynthetic genes could not be detected in *P. chlororaphis*, as there was no PCR-amplified product by employing specific primers. Production of phenazine and pyrrolnitrin by *P. chlororaphis* was confirmed by HPLC technique. *Pseudomonas* spp. DF41 and *B. amyloliquefaciens* BS6 lacked the genes for biosynthesis of any of the antibiotics tested. *B. thuringiensis* BS8, *B. cereus* L and *B. mycoides* possessed the zwittermicin A self-resistance gene (Zhang et al. 2006).

Pseudomonas chlororaphis strains DF 190 and PA23, *Bacillus cereus* strains DFE4 and *B. amyloliquefaciens* strains DFE16 were effective against canola blackleg disease caused by *Leptosphaeria maculans* (anamorph: *Phoma lingam*). These antibiotic-producing strains were evaluated for their ability to induce systemic resistance (ISR) by split-inoculation (SPI) of a cotyledon different from that of the pathogen inoculated cotyledon. Application of the bacteria 24 or 48 h prior to pathogen inoculation was an important factor in the suppression/prevention of blackleg lesions. The strains PA23 and DF190 produced phenazines (phenazine-1-carboxylic acid, 2 hydroxyphenazine) and pyrrolnitrin and the strains DFE4 and DFE16 produced the lipopeptide antibiotics iturin A, bacillomycin D and surfactin. The strains PA23 and DFE4 were tested as representative producers of each set of antibiotics for the split inoculation of the extracts for induction of ISR. The assays showed a small, but significant reduction in disease severity via a systemic response. The local (SPI) inoculation of the extract (for direct antagonism) showed significantly higher reduction of disease severity which was also consistent with the SPI of the bacterial cells, establishing a more important role for the antifungal metabolites present in the culture extracts for the direct suppression of the development of blackleg symptoms. The localized inhibition of pycnidiospores by the bacteria could be due to successful colonization of the infection site which in turn most possibly act as a suitable delivery system for the antifungal metabolites (Ramarathnam et al. 2011).

The GacS/GacA system in known to control the expression of genes required for the synthesis of secondary metabolites such as antibiotics in several *Pseudomonas* spp. *A gacS* mutant lost the biocontrol activity against *Leptosphaeria maculans*, causative agent of canola blackleg disease. The biocontrol activity could be restored in the mutant PA23-314, when the *gacS* gene was added to the mutant. The phenazine mutant PA23-63 showed antifungal and biocontrol activity similar to that of the wild-type strain (Selin et al. 2010). The biocontrol activity of the mutant PA23-63, though lacked phenazine production, revealed that phenazines were not required for the biocontrol of *L. maculans*. The results showed that localized plant-defense-related enzyme activity at the site of inoculation was not induced by the bacterial strains of *P. chlororaphis*. Direct antifungal activity at the site of infection appeared to be the dominant mechanism mediating control of blackleg disease of canola (Ramarathnam et al. 2011).

Production of antibiotics by bacterial biocontrol agents may become an unacceptable attribute for their use against postharvest pathogens affecting fruits and vegetables, although they protect the treated produce against the pathogens effectively. *Pseudomonas cepacia* strain LT-4-12-W produced pyrrolnitrin and also reduced the in vitro growth and conidial germination of *Monilinia fructicola,* causing peach brown rot and *Botrytis cinerea* causing gray mold disease of apple and pear (Pusey and Wilson 1984; Janisiewicz and Roitman 1988). However, the production of antibiotics as a critical factor in biocontrol potential of these BCAs is not established unequivocally, since *P. cepacia* could be employed for the control of green mold disease of lemon caused by a pyrrolnitrin-resistant strain of *P. digitatum* (Smilanick and Denis-Arrue 1992). Likewise, use of the strain LT-4-12-W of *P. cepaciae* resulted in significant control of blue mold decay on oranges inoculated with pyrrolnitrin-resistant mutants of *Penicillium italicum* (Janisiewicz and Korsten 2002).

Penicillium digitatum causing green mold and *P. italicum* inducing blue mold diseases of citrus could be controlled by the application of *Pseudomonas syringae* strains, ESC-10 and ESC-11, the principal ingredients of the commercial product BioSave. These strains produced syringomycin E. Although the purified syringomycin could inhibit the growth of a variety of fungi, the presence of syringomycin was not detected in the fruit wounds treated with *P. syringae*, raising doubt as to the role of antibiotic in disease control (Bull et al. 1997) and suggesting the operation of a different mechanism not dependent on the production of syringomycin. The *syrB* mutants of strains ESC-10 and ESC-11 were generated by disrupting the *syrB* biosynthesis gene by a *lacZ* reporter gene coding for β-galactosidase activity. In cultures inoculated with the *syrB* mutant of strain ESC-10, ß-glucosidase activity was higher in media containing albedo tissue after 4 days of incubation. The reporter-gene system has been used to determine the production of syringomycin E by these strains in wounds on lemons or oranges (Bull et al. 1998). However, it may not be feasible for the antibiotic-producing BCAs to be registered for postharvest use on food products, because of the concern relating to the introduction of antibiotics into human food which may have an adverse effect on the resistance of humans to antibiotics. In addition, inhibition of microbial pathogens by a single chemical compound may prove to be ineffective, when a strain of the pathogen resistant to the antibiotic develops.

Hydrogen cyanide (HCN) is a volatile antibiotic produced as a secondary metabolite by Gram-negative bacteria *P. fluorescens, P. aeruginosa* and *Chromobacterium violaceum* (Askeland and Morrison 1983). HCN is highly toxic to most aerobic microorganisms, because of its ability to block the cytochrome oxidase pathway event at very high dilutions (pmol). Suppression of disease development is attributed to the action of HCN on certain oomycetes. Development of tobacco black rot disease caused by *Thielaviopsis basicola* was suppressed by *P. fluorescens* CHA0 that produced HCN, in addition to other antibiotics and siderophores. The mutants of wild type strain CHA0 deficient in synthesis of HCN, antibiotics, exoenzymes could not protect the tobacco plants against infection by *T. basicola.* HCN was considered to be primarily responsible for the biocontrol of tobacco black rot disease

(Voisard et al. 1989). In *P. fluorescens* strains Q2-87 and CHA0, the *hcnABC* gene encode for HCN synthetase required for HCN production (Haas and Défago 2005). In order to detect the HCN producers in a mixed population of pseudomonads, primers targeting *hcnAB* genes were designed with MultiAlin from the consensus of the *hcn* sequences between *P. fluorescens* strain CHA0 and *P. aeruginosa* strain PAO1 were designed. A single amplicon of about 570-bp in length was obtained for all HCN^+ strains using the PCR procedure developed in this investigation. No amplicon was generated from the two negative HCN pseudomonads. The HCN^+ bacterial strains could be sensitively detected in samples where they were in a low, percentage of total pseudomonad community and/or where the numbers were low regardless of their proportion to the numbers the total community which is an important feature of environmental biodiversity analysis (Svercel et al. 2007).

Cyclic Lipopeptides

Various bacterial genera have been shown to produce peptide antibiotics exhibiting antagonistic activity against a wide range of fungal plant pathogens. Cyclic lipopeptides (CLPs) have been detected in the cultures of both Gram-positive and Gram-negative bacteria. *Pseudomonas* spp. are known to produce CLPs commonly. An antifungal cyclic peptide effective against *Rhizoctonia solani* was isolated from *P. fluorescens* strain 96.578. The production of the CLP tensin was high in liquid media with glucose, mannitol or glutamate as growth substrate. When applied to sugar beet seeds, the strain 96.578 produced tensin during seed germination (Nielsen et al. 2000). Strains of *P. fluorescens* have been reported to produce three types of CLPs viz tensin (Henriksen et al. 2000), amphisin (Sorensen et al. 2001) and viscosinamide. The CLP antibiotics have either 9 or 11 amino acids in the peptide ring. They possess antimicrobial and biosurfactant properties (Nielsen et al. 2002). The low molecular weight biosurfactants include glycolipids and lipopeptides such as rhamnolipids and surfactin. Rhamnolipids secreted by strains of *Pseudomonas aeruginosa* were effective against *Pythium aphanidermatum, Plasmopara lactucae-radicis* and *Phytophthora capsici*. Purified rhamnolipids caused cessation of motility and lysis of entire zoospore populations within <1 min (Stanghellini and Miller 1997). Rhamnolipid B produced by *P. aeruginosa* B5 inhibited the mycelial growth of *Phytophthora capsici* and spore germination of *Colletotrichum orbiculare* in in vitro assays. The diseases caused by these pathogens were suppressed in pepper and cucumber respectively following application of purified rhamnolipid B (Kim et al. 2000). *Pseudomonas fluorescens* strain SS 101 (biovar II) isolated from wheat rhizosphere exhibited zoosporicidal activities rendering the zoospores of *Pythium* spp. *Phytophthora infestans* and *Albugo candida* inactive. Application of cell suspension of strain SS101 to soil or hyacinth bulbs protected the plants against root rot disease caused by *Pythium intermedium*. Two genes involved in surfactant production by *P. fluorescens* SS101 were identified by random Tn5 mutagenesis followed by anchored-PCR and subsequent sequencing of the Tn5 flanking regions. The surfactants produced by strain SS101 were isolated by reverse-phase high-pressure liquid chromatography (RP-HPLC). The principal constituent was identified as a

cyclic lipopeptide (1,139-Da) containing nine amino acids and a ten-carbon hydroxyl fatty acid by mass spectrometry and nuclear magnetic resonance analysis. The results suggest that the biosurfactants produced by the strain SS101 might play a key role in the biocontrol activity against *P. intermedium* (de Souza et al. 2003a, b).

Cyclic lipopeptides (CL) produced by bacterial species have been reported to be responsible for their biocontrol activities against fungal pathogens. A derivative of *Bacillus subtilis* BBG100 that over-produced the CLP mycosubtilin exhibited enhanced activity against *Pythium* spp. infecting tomato seedlings (Leclére et al. 2005). Application of viscosinanmide-producing *P. fluorescens* strain DR54 to sugar beet substantially increased plant emergence and root length in soil infested with *Pythium ultimum* (Thrane et al. 2000). Production of CLPs with antifungal and biosurfactant properties by *Pseudomonas fluorescens* strains in bulk soil and sugar beet rhizosphere was investigated. When sugar beet seeds were coated with CLP-producing strains and subsequently germinated in non-sterile soil, the strain DR54 maintained a high and constant viscosinamide level in young rhizosphere for about 2 days, while strains 96.578 and DSS73 produced higher concentrations of the CLPs tensin or amphisin. All three CLPs were present in detectable levels for several days in rhizosphere. The results suggested that production of CLPs might occur only in specific habitats like rhizosphere of germinating sugar beet seeds rather than in the bulk soil (Nielsen and Sørensen 2003). In another study, production of an antifungal polyketide 2,3-deepoxy-2,3-didehydrorhizoxin (DDR) by *Pseudomonas* sp. effective against wheat seedling blight disease caused by *Fusarium culmorum*, was observed (Johansson and Wright 2003).

Strains of *Pseudomonas fluorescens* have been reported to produce surface-active compounds called as surfactants with inhibitory effects on zoospores of *Phytophthora infestans* and *Pythium* spp. included in Oomycetes (de Souza et al. 2003a, b; De Bruijn et al. 2007). The biosurfactant produced by *P. fluorescens* strain SS101 was identified as massetolide A, a cyclic lipopeptide with 9-amino acid peptide ring linked to 3-hydroxydecanoic acid (de Souza et al. 2003a, b). Application of the strain SS101 suppressed effectively the Pythium root rot infection of flower bulb crops (de Boer et al. 2006). The role of massetolide A in the biocontrol activity of the strain SS101 was investigated. The gene *massA* governing the biosynthesis of the surfactant massetolide has been identified in *P. fluorescens* SS101. The capacity of the wild-type strain and its mutant or the cyclic lipopeptide surfactant massetolide A to inhibit hyphal growth of different *Pythium* spp. was assessed in vitro. Strain SS101 only marginally suppressed in vitro growth of *Pythium* spp. and growth was not inhibited in the presence of the mutant 10.24. The results indicated that *P. fluorescens* SS101 was very effective in controlling diverse *Pythium* populations infecting different crops grown in various soils. Further, production of the cyclic lipopeptide massetolide A did not have significant role in suppression of root rot disease (Mazzola et al. 2007). *Pseudomonas fluorescens* SS101 effectively prevented infection of tomato leaves by the late blight pathogen *Phytophthora infestans* and also restricted the expansion of existing lesions and sporangial production. As the sporangia form an important primary

and secondary inoculum source for *P. infestans*, destructive effect of strain SS101 on both lesion area and sporangia formation might lead to a reduction in rate of disease development. This investigation showed that massetolide A-deficient mutant 10.24 was significantly less effective in its biocontrol activity, compared with wild-type strain. Application of massetolide A on tomato leaves and roots effectively protected treated plants against *P. infestans*. These findings showed that the CLP massetolide A was an important component of the biocontrol efficacy of strain SS101. The purified preparation of massetolide A produced significant control of the tomato late blight disease both locally and systemically via induced resistance indicating the multifunctional capacity of the cyclic lipopeptide produced by *P. fluorescens* SS101 (Tran et al. 2007).

The biosurfactants are capable of affecting cell surface of plant pathogenic fungi (Raaijmakers et al. 2006) and also have the ability to act on lipids creating pores on the membrane layer (Kim et al. 2004a, b). The role of biosurfactants in the *Pseudomonas*-mediated reduction in the viability of microsclerotia of *Verticillium longisporum* infecting cauliflower, was investigated. The biosurfactant deficient mutants of *Pseudomonas* (MR 12a) and *P. aeruginosa* PNA1 were less effective in the suppression of the viability of *Verticillium* microsclerotia, when compared with the wild-type strain. The biosurfactants were effective at a bacterial cell density of 2×10^9 CFU/ml. The results showed that biosurfactant production by BCA did not fully account for *Verticillium* microsclerotia suppression. Other mechanisms may also be involved in the suppression of microsclerotial germination. The biosurfactant mutant of *P. aeruginosa* PNA1 had residual effect on the formation of secondary microsclerotia. Likewise, the biosurfactant of *Pseudomonas* CMR12a had a residual effect on *Verticillium* germination and the formation of secondary microsclerotia. The residual adverse effects of the mutants were attributed to their ability to produce the antibiotic phenazine-1-carobxylic acid (PCA) (Debode et al. 2007). *Pseudomonas* sp. DF41 suppressed effectively the stem rot disease of canola caused by *Sclerotinia sclerotiorum*. DF41 strain produced a number of compounds including hydrogen cyanide (HCN), protease, alginate and lipopeptide (LP) molecules. All these compounds that might contribute to the biocontrol potential of DF41 were under the control of Gac. DF1 also produced autoinducers, suggesting that the bacterium employed quorum sensing as part of its lifestyle. Two mutants of DF41 strain with drastically reduced antifungal activity were generated by transposon mutagenesis technique. The *gacS* (DF41-469) mutant had an insertion in *gacS* forming part of the GacS/GacA regulatory system, while the *lp* (DF469-1278) mutant had an insertion in lipopeptide synthesis. The *gacS* and *lp* mutants could not protect canola plants against *S. sclerotiorum* in the greenhouse tests. Both mutants could not persist in canola phyllosphere. The loss of biocontrol activity of the mutants was considered to be due to the reduced synthesis of antifungal compounds and not due to a declining population size. It was concluded that the strain DF41 relied on the production of antifungal compounds (LPs) for suppressing the development of Sclerotinia stem rot disease of canola (Berry et al. 2010).

The possibility of using biosurfactants produced by bacterial biocontrol agents for suppressing the development of zoospore-producing oomycetes and the diseases caused by them especially in closed hydroponic cultivation systems was explored. The potential of a biosurfactant produced by *Pseudomonas koreensis* was assessed by applying a crude extract of the BCA. Assessment of the effect of the crude extract on *Pythium ultimum* in hydroponic tomato cultivation showed that incidence of the disease was significantly reduced. Application of the biosurfactant did not influence the indigenous microflora, when evaluated as sole carbon source utilization (Hultberg et al. 2010a). The efficacy of the biosurfactant from *P. koreensis* the strain 2.74 against the potato late blight pathogen *Phytophthora infestans* was assessed using a detached leaf assay. Development of disease on the treated leaves challenged with the pathogen zoospores was reduced significantly. The biosurfactant inhibited the motility of the pathogen zoospores, but caused only a minor reduction in mycelial growth rate. No adverse effect on the rate of sporangia production in pure culture was observed due to treatment with the bacterial biosurfactant (Hultberg et al. 2010b). Use of antagonistic microorganisms for the control oomycetes infecting crops in hydroponic systems is a sustainable approach, since the biosurfactans and biosurfactant-producing microorganisms have been found to be potentially useful components of a sustainable biocontrol strategy. Three modes of supplying the biosurfactant-producing strain to a recirculating hydroponic cultivation system infected with the zoospore producing pathogen, *Pythium ultimum* were evaluated. *Pseudomonas koreensis* strain 2.742 was added as washed cells, in its spent KB broth or in a minimal medium adapted from the nutrient solution and compared with control treatments. When a high concentration of washed cells of the strain 2.74 was added to the plant cultivation system, the infection was reduced up to 50 %. Addition of purified biosurfactant also protected the tomato plants as effectively as the washed cells of the BCA strain. The spent broth induced phytotoxic symptoms. The results indicated the effectiveness of the bacterial strain as well as the purified biosurfactant for the management of disease caused by zoospore-producing oomycetes (Hultberg et al. 2011).

Pseudomonas aeruginosa PNA1 isolated from chickpea rhizosphere has been shown to be effective against *Pythium* spp. and other pathogenic fungi. *P. aeruginosa* PNA1 produces rhamnolipid biosurfactants, in addition to phenazines. The biosurfactants and the antibiotics produced by the strain PNA1 might act synergistically. Phenazines and surfactants might interact with the mycelium and zoospores respectively, the two important asexual propagules of *Pythium* spp. (de Souza et al. 2003a, b). The role of biosurfactants in the biocontrol activity of *P. aeruginosa* PNA1 was investigated in vivo via mutant analysis and by using purified surfactants. A rhamnolipid-deficient and a phenazine-deficient mutant of PNA1 were used either separately or jointly in the greenhouse. The experiments on cocoyam (*Xanthosoma sagittifolium*) and bean (*Phaseolus vulgaris*) demonstrated the importance of biosurfactants for the biocontrol of *P. myriotylum* and *P. splendum,* infecting cocoyam and bean respectively. Lysis of zoospores was caused by the surfactants, due to intercalation with the zoospore plasma membranes which are not protected by a cell wall as in the case of other fungal cells. When the mutants were concurrently introduced in the

soil, the biocontrol activity was restored to the wild-type levels. When bean seeds were treated with a mixture of purified phenazine-1-carboxamide (PCN) and rhamnolipids, pre-emergence damping-off was more effectively reduced, than when the seeds were treated with either of these compounds alone. The results indicated the synergistic activity of PCN and rhamnolipids, resulting in more effective disease suppression (Perneel et al. 2008).

Antibiotics have been demonstrated to play a significant role in crop disease management by employing the bacterial biocontrol agents. In the recent years, new insights and concepts in biological control of plant pathogens by bacteria producing antibiotics in different substrates have been developed. Assays performed in vitro provided data on broad-spectrum activity of most bacterial BCAs Different lines of evidences supported the role and functions of antibiotics in in situ interactions between the bacterial BCAs and microbial plant pathogens. Culture filtrates or purified antibiotics provided similar levels of suppression of pathogen growth or disease development. Inactivation of antibiotic production by mutagenesis led to a reduction in the biocontrol capacity of the bacterial strain. It was possible to enhance the level of antibiotic production in the wild-type strain via introduction or modification of antibiotic biosynthetic or regulatory genes. Another line of evidence was provided by introduction of antibiotic biosynthetic genes in heterologous, non-producing strains and subsequent evaluation of their ability to control plant diseases. Reporter gene systems and bio-analytical techniques have clearly shown the presence of antibiotics secreted by bacterial BCAs in the spermosphere and rhizosphere of a range of host plants. Despite the availability of several evidences of the presence of antibiotics in the substrates of interaction of BCA-pathogen, the question as to whether the amount of antibiotic produced, is sufficient to inhibit the growth or metabolic activity of the pathogen in situ is yet to be addressed.

Furanones

Fungi, bacteria and actinomycetes have been reported to produce 3-(1-hydroxyalkyl)-2 (5H)-furanones with antimicrobial properties (Nagnuma et al. 1992; Ordentlich et al. 1992; Braun et al. 1995). *P. aureofaciens* strain 63–28 produced three 3-(alkyl)-2 (5H)-furanone derivatives which inhibited the radial growth of *Pythium ultimum* and induced abnormal appearance of the hyphae (Paulitz et al. 2000). *Pseudomonas jessenii* produced two secondary metabolites viz (i) 3-[(1R)-hydroxy-octyl]-5-methylene-2(SH)-furanone (4,5 didehydraacaterin) and (ii) 3-[(1R)-hydroxyhexyl]-5-methylene 2(5H)-furanone that induced hyperbranching and swelling of hyphae of *Aphanomyces cochlioides* and *Pythium aphanidermatum*. Treatment of the hyphae of these fungal pathogens with the two furanones produced by *P. jessenii* caused characteristic accumulation of plaques at the hyphal tips, similar to the effects induced by an actin assembling inhibitor. Staining with rhodamine-phalloidin which binds to plasma membrane-associated filamentous-actin (F-actin) revealed that tip-specific actin filaments were remodelled into a plaque-like form at an early stage of encounter (up to 24 h) with the furanones. At later stages of encounter (48 h), the plaques were eliminated reflecting the disorganization of actin arrays in the morphologically

abnormal hyphae of both pathogens, as observed under confocal laser scanning microscope (CLSM) and transmission electron microscopic (TEM). The results suggested that the mechanism of action of furanones on fungal pathogens may be through interference in morphogenesis and growth establishment and maintenance of cell polarity and subcellular organization of the pathogens and these alterations may be mediated by F-actin (Deora et al. 2010).

Volatile Organic Compounds

The bacterial biocontrol agents *Pseudomonas fluorescens* Q8r1-96, *P. fluorescens* B-4117 and *Serratia plymuthica* IC1270 have been shown to significantly suppress the tumor development induced by *Agrobacterium vitis* in tomato seedlings which were treated with the BCAs prior to pathogen inoculation (Khmel et al. 1998; Raaijmakers and Weller 2001; Ovadis et al. 2004). The mutants deficient in 2,4-diacetylphloroglucinol (2,4-DAPG) and pyrrolnitrin (Prn) production were also as effective as the wild-type strains, indicating that the antibiotics produced by these BCAs are not involved in tumor development suppression. In order to identify the compounds of BCA origin involved in the biocontrol activity against *A. vitis,* the volatile organic compounds (VOCs) produced by the three strains of BCAs were evaluated for their efficacy in inhibiting the growth of *A. vitis* and suppressing tumor development in tomato. The VOCs produced by strains of *P. fluorescens* and *S. plymuthica* inhibited the growth of *A. tumefaciens* and *A. vitis* strains in vitro. A derivative of *P. fluorescens* Q8r1-96 tagged with a *gfp* reporter and *P. fluorescens* B-4117 and *S. plymuthica* IC1270 marked with rifampicin resistance stably persisted in tomato tissues for at least 1 month. Solid-phase micro-extraction-gas chromatography–mass spectrometry analysis revealed that dimethyl disulfide (DMDS) was the major headspace volatile produced by *S. plymuthica* and it was able to suppress the growth of *Agrobacterium* effectively in vitro. In addition, DMDS was emitted by tomato plants treated with *S. plymuthica*. On the other hand, *P. fluorescens* strains produced 1-undecene as the main volatile compound and the volatiles including DMDS were produced in relatively low quantities (Table 5.3) (Dandurishvili et al. 2010) (Appendix 5.3).

The mechanisms underlying the VOCs action could be related to the ability of some volatiles to interfere with the levels and ratios of phytohormones (Zhang et al. 2007) which are known to be important factors in crown gall formation. Volatiles produced by some rhizobacterial strains trigger growth promotion and induce systemic resistance in plants through cytokinin and ethylene signaling pathways (Ryu et al. 2004; Farag et al. 2006). Inhibition of ethylene-signaling pathway might be effected by PGPR-by producing the enzyme 1-aminocylopropane-1-carboxylate (ACC) deaminase which can degrade ACC-the immediate precursor of ethylene in plants. This may result in lower plant ethylene levels and consequently suppression of crown gall formation. The possibility of DMDS and/or other VOCs being involved in inducing systemic resistance to crown gall disease in tomato has to be investigated (Narayanasamy 2008).

Table 5.3 Inhibition of growth of *Agrobacterium* spp. by strains of *Pseudomonas fluorescens* and *Serratia plymuthica* (Dandurishvili et al. 2010)

	Antagonist strain				
Pathogen strain[b]	B-4117	Q8r 1-96	Q8r 1–96 mutant (DAPG$^-$)	IC 1270	IC 1270 mutant (Prn – 1)
At C58	16–20[a]	6–10	nz[b]	6–10	nz
At Sh-1	11–15	6–10	nz	11–15	1–5
Av S4	11–15	6–10	1–5	11–15	nz
AvTm4	11–15	6–10	nz	11–15	nz

[a]Growth inhibition zone (mm)
[b]At *Agrobacterium tumefaciens*, AV *A. vitis*, nz no inhibition zone

Bacteriocin-Mediated Antagonism

Bacteriocins secreted by bacterial species are proteinaceous compounds with deleterious effects on closely related bacterial competitors in the same substrate/environment. These toxic metabolites constitute a structurally and functionally diverse group within the antimicrobial compounds. *Pseudomonas* spp. colonizing rhizosphere have been reported to produce bacteriocins. The bacteriocin LipA produced by *Pseudomonas* sp. strain BW11M1 is a novel type of antibacterial protein and it exhibits homology to mannose-binding lectins mostly found in monocots (Parret et al. 2003). The biocontrol agent *P. fluorescens* strain Pf-5 has two *llpA*-like genes designated *llpA1-p$_f$-5* land *llpA2p$_f$-5*. Recombinant *Escherichia coli* cells expressing *llpA1pf-5* or *llpA2pf-5* acquired bacteriocin activity and secreted a 31-kDa protein cross-reacting with $LlpS_{BWIIMI}$ antibodies. Analysis of antimicrobial spectrum showed that $LlpA1_{pf\text{-}5}$ and $LlpA2p_{f\text{-}5}$ were able to inhibit *P. fluorescens* strains. Bacteriocin production by BCAs may contribute to their rhizosphere competence. Thus the recombinant strains equipped with bacteriocin genes may be able to perform better as plant growth-promoting inocula by out-competing rhizosphere inhabiting pseudomonads (Parret et al. 2005).

Enzyme-Mediated Antagonism

Strains of *Pseudomonas* spp. may be able to suppress crop diseases by producing antibiotics, lytic enzymes and siderophores, by depriving pathogens of nutrients and habitation sites or by inducing systemic defence reactions in the host plants (Leong 1986; Cook 1993). Chitinase, a hydrolytic enzyme capable of degrading fungal cell-wall components is one of the mechanisms implicated in biocontrol (Ordentlich et al. 1998). Genetically engineered BCAs with multicopy chitinase genes have been demonstrated to have greater pathogen suppressive capacity (Sundheim et al. 1988; Simi 1994). Inhibition of *R. solani* was significantly enhanced, when *P. fluorescens* transformed with chitinase gene *chiA*. The extent of inhibition of mycelial growth was correlated with level of suppression of damping-off disease of cotton (Simi 1994). Likewise, *Pseudomonas* strain NRR1 B-15135 expressing heterogenous

chitinase genes from *Serratia marcescens* possessed greater level of biocontrol capacity against radish Fusarium wilt. However, no improvement in the disease suppressive effect could be observed against wheat take-all disease (Sundheim et al. 1988). Antagonism of *Pseudomonas* spp. against microbial plant pathogens has been observed only in a few cases. Involvement of a glucanase β-1,3-glucanase produced by *P. cepacia* in suppressing the development of *Rhizoctonia solani*, *Sclerotium rolfsii* and *Pythium ultimum* was able to degrade and lyse fungal mycelia. The bacterial enzymes chitinases and β-1,3-glucanase are encoded by a single gene (Siddique 2005).

Necrosis-inducing plant pathogenic pseudomonads produce two groups of cylic lipodepsipeptides (LDPs) that are toxic to plants and microorganisms. One group contains nonapeptide lactones like syringomycins (SRs), syringtoxins (STs), syringostatins and pseudomycins. The other group with lipopeptides includes tolaasins, syringopeptins (SPs) and fuscopeptins. *Pseudomonas syringae* pv. *syringae* (*Pss*) secreted two main LDPs, syringomycin E (SRE) and syringopeptin 25A (SP_{25A}) together with at least four types cell wall-degrading enzymes (CWDEs). In antifungal bioassays, the purified toxins SRE and SP_{25A} interacted synergistically with chitinolytic and glucanolytic enzymes purified from the same bacterial strain or the fungal BCA *Trichoderma atroviride* strain P1. The synergism between LDPs and CWDEs occurred against fungal pathogens *Verticillium dahliae, Botrytis cinerea*, *Penicillium expansum* and *Phytophthora infestans* as well as *P. syringae* itself, with a level dependent on the enzyme used to permealize the microbial cell wall. The in vivo biocontrol assays using *P. syringae* alone or in combination with *T. viride*, including a *Trichoderma* endochitinase knock-out mutant indicated that the synergistic interaction between LDPs and CWDEs was involved in the antagonistic mechanisms of biocontrol activity may be more effective in suppressing development of crop disease (Fogliano et al. 2002). *Pseudomonas fluorescens* strain P5 showed strong inhibitory effect on *Gaeumannomyces graminis* var. tritici causing wheat take-all disease and *Rhizoctonia solani* causing damping-off disease of cotton. The strain P5 was transformed with the 6.5-kb chitinase gene fragment from the bacterial BCA *Serratia marcescens* strain M90-3. The biocontrol potential of the wild type strain P5 and the transformant P5-1 was compared by both in vitro and in vivo tests. Inhibition of mycelial growth of *G. graminis* var. *tritici. R. solani,* infecting cotton (damping-off disease) and *R. solani* infecting rice (sheath blight disease) by the transformant P5-1 was significantly more than that of the wild-type strain P5. Both the wild-type and transformant strains suppressed the development of wheat take-all, cotton damping-off and rice sheath blight diseases. However, the transformant P5-1 protected the cotton and rice plants more effectively against *R. solani* than the wild-type strain (Table 5.4) (Xiao-Jing et al. 2005).

Groundnut (peanut) seed endophyte *Pseudomonas aeruginosa* strains GSE 18 and GSE19 reduced the incidence of stem rot disease caused by *Sclerotium rolfsii* and the seedling mortality by 54 and 58 % respectively. In dual cultures, these strains reduced the mycelial growth of *S. rolfsii*. Cell-free culture filtrates of the strains GSE18 and GSE19 inhibited the activity of the cell wall-degrading enzymes (CWDEs) polygalacturonase and cellulase produced by *S. rolfsii* to the maximum

Table 5.4 Biocontrol efficacy of wild-type and transformed strains of *Pseudomonas fluorescens* in suppressing development of diseases in wheat, rice and cotton (Xiao-Jing et al. 2005)

		BCA strain	
Disease/effect of BCA	Control	P5	P5-1
A. Wheat take-all			
Disease index	42.3 ± 5.9a	26.4 ± 3.3b	21.2 ± 3.6b
Control effect (%)	–	37.6	49.9
B. Rice sheath blight			
Lesion length (cm)	6.21 ± 0.12a	4.02 ± 0.18b	3.14 ± 0.15c
Control effect (%)	–	35.3	49.4
C. Cotton damping-off			
Dead plants(%)	80.1 ± 4.6a	64.2 ± 2.7b	55.3 ± 3.2c
Control effect (%)	–	19.8	31.0

Values are mean ± standard deviation for 12 replicates; for each disease, values followed by the same letter are not statistically significant ($P < 0.01$)

extent of 55 and 50 % respectively at 6 days after inoculation. As the strains GSE18 and GSE19 were tolerant to thiram, a commonly applied seed dressing fungicide, the potential of *P. aeruginosa* strains for the biocontrol of stem rot disease has to be exploited (Kishore et al. 2005a, b). In another study, the strong antagonistic activity of *P. aeruginosa* strain GRC against *Sclerotinia sclerotiorum* causing stem rot disease of groundnut was observed. Scanning electron microscopic (SEM) observations revealed morphologic abnormalities such as perforation, lysis and fragmentation of hyphae of *S. sclerotiorum* induced by *P. aeruginosa*. The strain GRC secreted extracellular chitinase. The role of the chitinase in suppressing the mycelial growth was demonstrated through *Tn5* mutagenesis. Seed bacterization with strain GRC improved seed germination and reduced stem rot disease incidence in pathogen-infested soil by 97 %. In addition, plant growth, number of nodules, pods and grain yield/plant were significantly increased in comparison to control (Gupta et al. 2006).

The biocontrol efficacy of a bacterial species may be enhanced by the presence of another bacterial species producing fungal cell wall-degrading enzymes. *Pseudomonas fluorescens* strain LRB3W1 suppressed the development of many plant diseases including cabbage Fusarium yellows disease caused by *Fusarium oxysporum* f.sp. *conglutinans* (FOC) by producing antibiotics (Someya et al. 2007b) A mycolytic bacterial species *Serratia marcescens* strain B2 inhibited several fungal pathogens, causing diseases such as cucumber damping-off, gray mold disease and rice sheath blight (Someya et al. 2005a, b). The strain B2 produced chitinases, a class of cell wall-degrading enzymes (Someya et al. 2001). *P. fluorescens* strain LR3W1 inhibited the mycelial growth of FOC, while *S. marcescens* strain B2 did not affect the pathogen growth. The disease suppressive effect of strain B2 was less than that of strain LRB3W1. But the combined effect of both bacterial strain was much more greater than strain LRB3W1 alone. Colonization of each bacterial strain was not influenced by one another. Treatment of bud cells of FOC with 2,4-DAPG from strain LRB3W1 and fungal cell wall-degrading enzyme from strain B2 resulted in an additive adverse effect. The results indicated the synergistic action of strain B2

leading to enhancement of effectiveness of suppression of disease caused *F. oxysporum* f.sp. *conglutinans* in cabbage (Someya et al. 2007a).

Pseudomonas chlororaphis strain PA-23 was effective in suppressing the development of the canola stem rot disease caused by *Sclerotinia sclerotiorum.* The fungal pathogen produces ascosporic inoculum from germinating sclerotia and the ascospores infect the flowers of canola. Chitinase and β-1,3-glucanase are the key enzymes involved in the lysis of pathogen cell walls. The ability of the strain PA-23 to induce the activities of these two enzymes was assessed by spraying plants twice at 30 and 50 % bloom followed by challenge inoculation with *S. sclerotiorum.* The BCA-treated canola plants had significantly higher chitinase and β-1,.3-glucanase activities. On the other hand, the activities of these enzymes were less in healthy control, ascospore-inoculated control and PA-23-treated plants. β-1,.3-glucanase attained the peak at 4 days after inoculation (DAI) for chitinase and at 6 days of β-1,.3-glucanase. The enzymatic activities declined slowly with increase in time (Fernando et al. 2007). The impact of treatment with the root nodulating *Sinorhizobium fredii* KCC5 and *Pseudomonas fluorescens* LPK2 on plant growth and suppression of the development of the pigeonpea wilt disease caused by *Fusarium udum* was studied. Both bacterial strains showed chitinase and β-1, 3-glucanase activities and also produced siderophore, indoleacetic acid (IAA), and solubilized insoluble phosphate. In addition, the bacterial strains could degrade and digest cell wall components resulting in hyphal perforations, empty cell (halo) formation, shrinking and lysis of fungal mycelia along with significant degeneration of conidia. The mycelial growth of the pathogen *F. udum* was drastically arrested. Furthermore, *P. fluorescens* LPK2 produced volatile cyanogens (HCN) toxic to the pathogen. In addition to disease suppression, bacterial inoculants, promoted seed germination, and enhanced number of nodules formed, shoot and root length, shoot and root weight, and number of pods, when they were applied with half-dose of chemical fertilizers. The results showed that the strains LPK2 and KCC5 exhibited synergism, aggressive colonization of the roots, enhancement of plant growth and greater efficiency in suppressing the development of pigeonpea wilt disease (Kumar et al. 2010).

5.1.1.2 Competition for Nutrients and Space

Siderophore Production

Bacterial BCAs present in different substrates compete for the available nutrients with the microbial plant pathogens and other microorganisms. Availability of essential micronutrients such as iron is a crucial factor for all microorganisms. Iron becomes a limiting factor in the rhizosphere depending on the soil pH. In highly aerated (oxidized) soil, iron present in ferric form is insoluble in water and in low concentration ($<10^{-18}$ M). Hence, it may not be available to the microorganisms. The iron-binding ligands known as siderophores with high affinity for iron are produced by all microorganisms to sequester iron from the micro-environment.

Ability to produce siderophores confers to bacteria competitive advantages to colonize plant tissues and to exclude other microorganisms from the same ecological niche. The siderophores may be of two types: catechol type or hydroxamate type (Neilands 1981). The fluorescence of pseudomonads is attributed to the presence of an extracellular diffusible pigment called pyoverdin (Pvd) or pseudobactin. This pigment has high affinity for Fe^{3+} ions and it is a siderophore (iron-carrier) of the producer strain. In iron-depleted media, Pvd-producing *Pseudomonas* spp. are able to inhibit the growth of bacteria and fungi with less potent siderophores. Under certain conditions, Pvd functions as a diffusible, bacteriostatic or fungistatic antibiotic. It may be possible for a sessile producer of potent siderophore like Pvd might compete at a distance with other microorganisms that have less efficient iron-uptake systems (Kloepper et al. 1980).

The importance of production of siderophore as a mechanism of biological control of *Pseudomonas fluorescens* strains A1, BK1, TL3B1 and B10 against the fire blight pathogen *Erwinia amylovora* was demonstrated by Kloepper et al. (1980). Synthesis of siderophore in vitro in *Pseudomonas* spp. was correlated with the capacity to inhibit germination of chlamydospores of *Fusarium oxysporum* (Sneh et al. 1984; Elad and Baker 1985). Under greenhouse conditions, *P. putida* strain B10 suppressed Fusarium wilt and take-all diseases. However, the suppressive effect of the bacteria was lost, when the soil was amended with iron which repressed siderophore production in this strain (Kloepper et al. 1980). In another study, *Pseudomonas* strain B324 with characteristics intermediate between *P. fluorescens* and *P. putida* was inhibitory to all seven isolates of *Pythium ultimum* var. *sporangiferum* infecting wheat, in addition to induction of strong growth-promoting effect on wheat plants under iron-limiting conditions (Becker and Cook 1988). As it can be expected, the mutants deficient in the production of siderophores like pyoverdin showed reduced biocontrol potential in suppressing the development of plant diseases as in the case tobacco black root rot disease by strain *P. fluorescens* CHA0 (Keel et al. 1989).

Bacterial isolates (216) obtained from paddy field soil samples were tested for their siderophore production and effectiveness in inhibiting mycelial growth of *Alternaria* sp. (leaf spot), *Fusarium oxysporum* (root rot), *Magnaporthe grisea* (blast) and *Sclerotium* sp. (stem rot) infecting rice. In dual culture technique, siderophore-producing rhizobacteria showed strong antagonistic activity against all the four rice pathogens to varying degrees ranging from 10.4 to 37.5 %. *Pseudomonas aureofaciens* strain AR1 was the most effective producer of siderophore and it secreted hydroxymate type siderophore. Siderophore in the strain AR1 reached the peak after 15 days at an optimal temperature of 30 °C, yielding 99.96 ± 0.46 μg/ml of siderophore. This study showed selection of bacterial isolate capable of producing siderophores, in addition to their ability to function via other mechanisms of biocontrol activity to achieve greater effectiveness in disease management (Chaiharn et al. 2009). *Pseudomonas aeruginosa* (PN1 ~ PN10) strains (10) isolated from rhizosphere of chir-pine were evaluated for their antagonistic activities against the root rot pathogen *Macrophomina phaseolina* and plant growth properties in vitro and in vivo. *P. aeruginosa* PN1 produced siderophore, indole-acetic acid (IAA), cyanogen and solubilized

phosphorus, in addition to chitinase and β-1, 3-glucanase. In dual culture assay, the mycelial growth of *M. phaseolina* was inhibited by 69 %. The culture filtrate was less effective in inhibiting the mycelial growth of the pathogen. *P. aeruginosa* PN1 increased the plant growth and biomass in pot experiment containing the pathogen-infested soil. PN1 showed the strong chemotaxis toward root exudates resulting in effective root colonization. The BCA strain exhibited strong antagonistic property against *M. phaseolina,* suppressed the development of disease in chir-pine and improved growth of chir-pine seedlings (Singh et al. 2010).

The genetic diversity of siderophore-producing bacteria of tobacco rhizosphere was assessed by applying amplified ribosomal DNA restriction analysis (ARDRA), 16S rRNA sequence homology and phylogenetic analysis. Bacteria belonging to 14 different genera generated 28 ARDRA patterns. Gram negative isolates were more frequently detected with more than 95 % total frequency. For Gram-positive bacteria, *Bacillus* and *Rhodococcus* were the only two genera with a 1.7 % total frequency. *Pseudomonas* and *Enterobacter* isolates were dominant in the tobacco rhizosphere environment with 44.5 and 24.7 % total frequency respectively. It was observed that 75 % of the isolates that had the high percentages of siderophore units (between 40 and 60 %) belonged to *Pseudomonas* spp. The G-229-21 strain of *Pseudomonas* could produce high-affinity carboxylate type siderophores under low iron-conditions and its siderophores strongly suppressed the development of *Phytophthora parasitica* var. *nicotianae* under low iron conditions (Tian et al. 2009). The diversity of endophytic siderophore-producing bacteria (SPB) associated with rice plants was studied. The presence of the SPB species was detected in grains, leaves and roots. The population of the heterotrophic siderophore producing bacteria was higher in mature rice plants. The amplified restriction DNA ribosomal analysis (ARDRA) was applied to generate patterns specific to different bacterial genera belonging to *Pseudomonas, Sphingomonas, Burkholderia* and *Enterobacter.* Among the bacterial isolates tested, only one isolate of *Pseudomonas* was able to inhibit *Azospirillum brasilense* and *Herbaspirillum seropedicae*. *Pantoea ananatis* was the most common species associated with roots at tillering and leaves to subsequent stages (Loaces et al. 2011).

Pseudomonas fluorescens strain A506, a commercially available product (marketed as Blight Ban A506) is recommended for the control of fire blight disease affecting apple and pear trees. The strain A506 has to outcompete *Erwinia amylovora,* causative agent of fire blight disease, for sites and nutrients for epiphytic growth and subsequent infection of the host plant. The bioavailability of iron on floral surfaces is a crucial factor that influences the production of antibiotics effective against *E. amylovora.* An iron biosensor was used to examine the relative bioavailability of iron to A506 (*pvd-inaZ*) on surfaces of pear and apple flowers through the suppression or expression of an iron-regulated promoter at the transcriptional level. The strain A506 produced a pyoverdin siderophore on iron-depleted media, but not in media amended with 0.1 mM ferric chloride. Concentration of iron that suppressed pyoverdin production also stimulated antibiosis. The results indicated that chelated forms of iron could be oversprayed on flowers colonized by the strain A506 or mixed with the inoculum to increase iron bioavailability to the bacterial

BCA on the surface of pear or apple flowers (Temple et al. 2004). *P. fluorescens* strain CV6 effective against *Phytophthora drechsleri* causing cucumber root rot disease produced substantial amount of siderophore, in addition to antifungal compounds and indole-acetic acid (IAA). This strain also produced enzymes involved in disease resistance, indicating the multi-mechanisms of biocontrol activity of the BCA strain (Maleki et al. 2010).

Interactions between rhizosphere microorganism may have positive or negative effect on the extent of disease suppression achieved. Positive interaction between strains of BCAs may be exploited to enhance the effectiveness of biocontrol of plant diseases. *Pseudomonas putida* WCS 358 effectively suppressed radish Fusarium wilt disease caused by *F. oxysporum* f.sp. *raphani* by competing for iron through the production of the siderophore pseudobactin. Addition of iron to the nutrient solution for plants reduced the disease suppressive effect of the strain WCS358. The pseudobactin negative mutants of WCS358 could also suppress the wilt disease as efficiently as the parent wild-type strain, suggesting that the mutants might act on the pathogen through an alternative mechanism. *P. putida* strain RE8 did not rely on the production of pseudobactin for its disease-suppressive activity. The strain RE8 was found to induce systemic resistance in treated plants. When the mixture of the strains WCS 358 and RE8 was applied to the soil, the effectiveness of disease suppression was enhanced to higher level, compared to treatments with single strain. This additive effect on disease suppression could be the result of pseudobactin-mediated competition for iron combined with induced systemic resistance. The results indicated that the strains that could complement with each other by acting through different mechanisms, have to be selected to achieve more effective disease control (de Boer et al. 2003). *Pseudomonas corrugata* causes vein necrosis disease of tomato grown in hydroponic system. A rifampicin resistant mutant named as *Pseudomonas* sp. LSW25R was antagonistic to *P. corrugata* and inhibited the mycelial growth of several fungal pathogens such as *Botrytis cinerea, Rhizoctonia solani* and *Sclerotinia sclerotiorum*. Blossom-end rot (BER) is a physiological disorder affecting tomato fruits. In hydroponically-grown tomato BER was dramatically reduced by 51 %, following treatment with LSW25R. Analysis of leaves from treated plants revealed that calcium uptake was at higher rate, compared with control in the hydroponic system. LSW25R strain could successfully colonize the rhizosphere during cultivation due to its broad-spectrum of antifungal activity and endophytic colonization. Locations of colonization by the strain LSW25R were investigated, using scanning electron microscope (SEM). Tomato roots of 3-day old plantlets were examined. Clusters of the bacterial strain colonized the surface of the epidermis in high-density around the natural aperture of the root (Fig. 5.3a) and on the grooved lines of the epidermal cells in the root (Fig. 5.5b). Further, LSW25R colonized under the epidermal cells around natural apertures (Fig. 5.3c, d). In addition, the BER incidence was significantly reduced, presumably through enhancement of calcium uptake (Lee et al. 2010).

Pseudomonas syringae pv. *syringae* (*Pss*) 22d/93, an epiphyte isolated from soybean leaves showed high level of antagonistic activity against the closely related pathogen *P. syringae* pv. *glycinea* (*Psg*) causing soybean bacterial blight disease

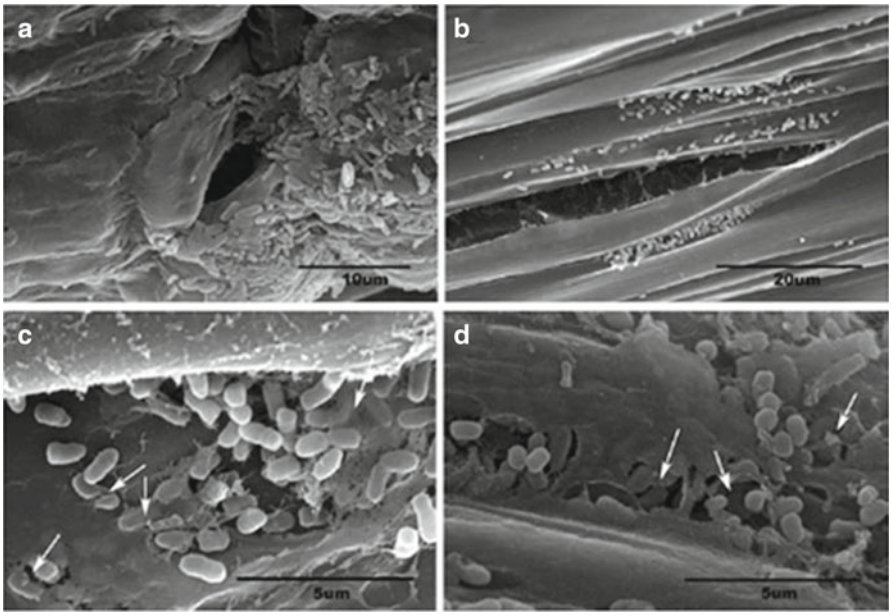

Fig. 5.3 Visualization of colonization by *Pseudomonas* sp. LSW25R of tomato radicle using scanning electron microscope (**a**) and (**b**): clusters of LSW25R localized on the surface of root around root apertures; (**c**) and (**d**): cells of LSW25R present under the epidermis of root (indicated by *arrows*) (Courtesy of Lee et al. 2010 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

(Völksch et al. 1996). The strain Pss22d/93 was able to effectively suppress the bacterial blight disease under field conditions also (Völksch and May 2001). In a later study, *Pss*22d/93 was found to produce significantly larger amounts of siderophores than the pathogen. Both the BCAs produced the same siderophore pyoverdin and achromobactin. However, the regulation of siderophore biosynthesis in pss22d/93 was very different from that of the pathogen. The epiphytic fitness of *Pss*22d/93 mutants defective in siderophore biosynthesis was determined, following spray inoculation of soybean leaves. At 10 days after inoculation, the population of the mutants was reduced by two orders of magnitude lower than that of the wild-type strain. But there was no significant difference in the biocontrol efficacy of the wild-type strain and the mutants of *Pss*22d/93, suggesting that the siderophores did not play any essential role in the biocontrol potential of the strain Pss22d/93. On the other hand, production of pyoverdin and achromobactin contributed significantly to the epiphytic fitness of *P. syringae* pv. *syringae* 22d/93, thus improving the effectiveness of its biocontrol activity against soybean bacterial blight pathogen (Wensing et al. 2010).

Erwinia amylovora, causing the fire blight disease of apple and pear, requires nicotinic acid (NiAc), nicotinamide ($NiNH_2$) and/or 6-hydroxynicotinic acid (6-HNiAc) as essential growth factors, when cultured on minimal medium in vitro (Paternoster

et al. 2009). It may be expected that an organism capable of eliminating or reducing the availability of $NiNH_2$ might effectively inhibit the growth of *E. amylovora* and suppress the development of fire blight disease. A total of 735 bacteria and 1,237 yeast isolates obtained from apple blossoms were pre-screened for nicotinic acid-degradation. The strain JAN degraded NiAc for its growth to the maximum extent and also showed the highest levels of biocontrol efficacy against *E. amylovora.* This strain was identified as *Pseudomonas rhizosphaerae* strain JAN, based on high 16S rRNA gene sequence homology. The JAN mutants M3G7 and M40E5 with impaired capacity to degrade NiAc and $NiNH_2$ showed strong reduction in their biocontrol activity against *E. amylovora.* M3G7 and M40ES were disrupted in *ntrC* and *ntrB* genes respectively. The biocontrol potential of the strain JAN and *P. fluorescens* A506, a commercial product used as a reference strain in experiments was assessed both in vitro and in the greenhouse. The efficacy of JAN on pear slices and apple hypanthia was comparable to that of strain A506. Under greenhouse conditions, JAN showed consistent high level of control across all three experiments, whereas the strain A506 was less effective in two of the three experiments. The results indicated that the bacterial BCA JAN could suppress the development of fire blight disease by effectively degrading the essential growth factor of the pathogen *E. amylovora* (Paternoster et al. 2010).

Competition between the biocontrol agents and the pathogens for available nutrients on or in the plant tissues occurs and such a competition may also result in the exclusion of less efficient pathogens from the same ecological niche. The efficacy of four strains of *Pseudomonas fluorescens* was assessed for suppressing the development of *Pythium ultimum* infecting pea and promoting the plant growth. All strains of the BCA reduced the number of lesions and the root and soil populations of *Pythium*, while strains SBW25 and CHA0 increased the number of lateral roots of treated plants. The strain SBW25 did not produce any antifungal metabolites and its biocontrol activity was related to its greater colonization ability and rhizosphere competence (Naseby et al. 2001). Application of *Erwinia herbicola* to the blossoms protected the plants against blossom blight phase of fire blight disease caused by *E. amylovora.* Preemptive and competitive colonization of stigmatic surfaces by *E. herbicola* reduced the rate of colonization by *E. amylovora* (Wilson et al. 1992). The efficacy of *Acidovorax avenae* subsp. *avenae* (AAA) (AAA99-2), *Pseudomonas fluorescens* A506 and an unidentified Gram-positive bacterium recovered from watermelon seed (WS-1) was assessed for suppressing infection of watermelon blossom by *A. avenae* subsp. *citrulli* (AAC), causing bacterial fruit blotch (BFB) disease of watermelon. In female blossoms treated with AAC, the pathogen population increased at 96 h post-inoculation. In contrast, pathogen population declined rapidly in blossoms treated with *P. fluorescens* and AAA. Reduction in AAC populations was faster for blossoms treated with *P. fluorescens* strain A506 than for AAA. At 48 h after inoculation, AAC could not be recovered from blossoms treated with either A506 or AAA strains indicating the complete displacement of the pathogen population by the BCA strains (Fessehaie and Walcott 2005).

Antifungal compounds produced by bacterial BCAs have to be delivered in the rhizosphere for effective suppression of soilborne pathogens and this can be

accomplished only by efficient colonization of plant root systems. Identification of root colonization traits will be useful for better understanding of root colonization process. Traits identified as important for root colonization include the motility, the presence of the O antigen of the lipopolysaccharide, synthesis of aminoacids and site-specific recombination. The root colonization gene *sss* which encodes site-specific recombinase has been employed to improve root colonization of wild-type strains of *Pseudomonas* spp. (Dekkers et al. 1998). The efficiency of root colonization by *Pseudomonas fluorescens* WCS 365 and its mutant PCL 1206 was studied. The mutant was 10-to-50-fold impaired in competitive root colonization on tomato, following seedlings inoculation with a 1:1 mixture of parent and mutant strains. The rate of uptake of putrescine by cells of PCL 1206 strain appeared to be increased. Putrescine is an important component of tomato root exudates. It has a bacteriostatic effect on cells of *Pseudomonas* spp. and contains genes involved in putrescine uptake and are highly homologous to the *pot* operon of *Escherichia coli.* A mutation in the promoter region of the *pot* operon resulted in the increased uptake of putrescine which might lead to decreased competitive ability. Hence, the regulated uptake was considered to be an important trait for competition of *Pseudomonas* spp. in the tomato rhizosphere (Kuiper et al. 2001).

The GacS/GacA comprising a two-component regulating system controls the expression of genes required for the synthesis of secondary metabolites with antimicrobial activity in many plant-associated *Pseudomonas* species. Secondary metabolites contributing to biological control that are regulated either directly or indirectly by GacS/GacA system include 2,4-DAPG, phenazines, pyoluteorin, pyrrolnitrin as well as HCN, chitinase and exoproteases. High mutation frequencies of *gacS* and *gacA* in *Pseudomonas* spp. have been observed in liquid culture. In *P. aureofaciens* 30–84, GacS/GacA system controlled the expression of phenazine antibiotics that are inhibitory to pathogenic fungi and enhanced the competitive survival of the BCA. In the natural soil, final rhizosphere populations of wild-type strain 30–84 from mixtures were at least 1.5 times larger than would be predicted from their inoculation ratio and generally were greater than or equal to the population of wild-type alone, despite lower inoculation rates. The results indicated that although *gacS*/*gacA* mutants survive in natural rhizosphere populations, they did not displace wild-type populations (Chancey et al. 2002). *Pseudomonas fluorescens* strain F113 effectively protected beet root plants against *Pythium ultimum*. During colonization of alfalfa rhizosphere, strain F113 produced variants that were characterized by the translucent and diffused colony morphology. The phenotypic variation in this strain appeared to be mediated by the activity of two site-specific recombinases Sss and XerD, since the mutants with disruption in either of the genes involved in the biosynthesis of Sss or XerD showed severe reduction in rhizosphere colonization. Motility is one of the most important traits for competitive rhizosphere colonization and mutants incapable of chemotactic motility were among the most defective colonization mutants. By over-expressing the genes *sss* or *xerD*, a large number of variants (mutants) was generated. By disrupting these genes and complementation analysis, regulation by Gac system of swimming motility by a repression pathway was observed. All isolated variants were more motile than the wild-type strain and

appeared to contain mutations in the *gacA* and/or *gacS* gene. Variants isolated after selection by prolonged cultivation formed a single population with swimming motility that was equal to the motility of *gac* mutants, being 150 % more motile than the wild type. Variants isolated after rhizosphere selection belonged to two different populations: one identical to the population isolated after prolonged cultivation and the other compromising variants that besides a *gac* mutation harbored additional mutations conferring higher motility. The highly motile variants were more competitive than the wild type strain, displacing it from the root tip within 2 weeks (Martinez-Granero et al. 2006).

Colonization of plant roots by introduced PGPR is a complex process involving interactions among the introduced strain, pathogen and native rhizosphere microflora. *Pseudomonas fluorescens* Q8r1-96 is an aggressive colonizer and maintains large populations on the roots of several host plants including wheat, pea, and sugar beet. The role of three genes an *sss* recombinase, *ptsP*, and *orfT* which are important in the interaction of *Pseudomonas* spp. with various hosts was investigated to determine their contribution to the unique colonization ability of the strain Q8r1-96. The *sss* recombinase and *ptsP* genes influenced the global processes, including phenotypic plasticity and organic nitrogen utilization respectively. The *orfT* contributed to the pathogenicity of *P. aeruginosa* in plants and animals and it is conserved among saprophytic rhizosphere pseudomonads. Clones containing the three genes were identified in the genomic library of the strain Q8r1-96. Mutants deficient in these genes were characterized to determine their 2,4-DAPG production, motility, fluorescence, colony morphology, exoprotease and hydrogen cyanide (HCN) production, carbon and nitrogen utilization and ability to colonize rhizosphere of wheat grown in natural soil. The *ptsP* mutant was impaired in wheat root colonization, whereas mutants deficient in the *sss* recombinase and *orfT* were not affected. All the three mutants, however, were less competitive than the parent strain Q8r1-96 in wheat rhizosphere, when they were co-inoculated in pairs with parent strain (Mavrodi et al. 2006).

The mechanism of biocontrol activity of *Pseudomonas fluorescens* isolate 1100–6 effective against *Penicillium expansum* or *P. solitum,* causing apple blue mold was investigated. The wild-type isolate 1100–6 and genetically modified derivative labeled with the gene encoding the green fluorescent protein (GFP) were compared. Both the wild-type and GFP-labeled strain produced large zones of inhibition in dual culture plate tests. Cell-free metabolites of both isolates reduced the colony growth of *Penicillium* isolates to different degrees ranging from 17.3 to 78.5 %. The use of iron chelate did not have a major impact on the antagonistic activity of *P. fluorescens.* The wild-type and GFP-labeled isolates significantly reduced the incidence and severity of apple at 11 days after inoculation at 20 °C and by *P. expansum* and *P. solitum* after 25 days at 5 °C, when the BCA isolates were applied in wounds 24 or 48 h before challenge inoculation with the pathogens. The population of labeled isolate increased in the wound from log 6.95 at the time of inoculation to log 9.12 CFU/ml at 25 days after inoculation at 5 °C. The results indicated that the BCA suppressed the blue mold pathogen through antagonism by producing toxic metabolites and it did not penetrate deep into the wounds made on apples (Etebarian et al. 2005).

Application of genetically modified microorganisms (GMMs) is suggested as an approach to overcome the problem of inconsistency in the performance of biocontrol agents against crop pathogens. The effects of introduced wild-type strains and GMMs on the soil ecosystem have been studied in certain microcosms. The impact of genetically modified, antibiotic-producing *Pseudomonas putida* WCS58r and its two transgenic derivatives was investigated by introducing them as a seed coating into the rhizosphere of wheat in two consecutive years (1999 and 2000) in the same field plots. The transgenic derivatives WCS 358r :: phz and WCS358r :: phl constitutively produced phenazine-1-carboxylic acid (PCA) and 2,4-diacetylphloroglucinol (DAPGs) respectively. The *phz* and *phl* genes stably persisted in the chromosomes of the respective derivatives. The amount of PCA produced in the wheat rhizosphere by the transformant was about 40 ng/g of roots after the first application in 1999. The DAPG-producing derivatives caused a transient shift in the indigenous bacterial and fungal flora in the first year, as determined by amplified ribosomal DNA restriction analysis (ARDRA). However, after the second application of the transformant in 2000, the bacterial or fungal flora was not altered. All bacterial treatments showed positive effect on plant growth, possibly due to suppression of microbial plant pathogens. The results suggested that the effects of GMMs on microflora might be within the range of natural variability as induced by agricultural practices like crop rotation (Viebahn et al. 2003).

5.1.1.3 Prevention of Pathogen Colonization by Bacterial Biocontrol Agents

Suppression of the pathogen and disease development may be achieved, when a biocontrol agent is able to prevent colonization of specific host tissues by the pathogen. *Rhizobium (Agrobacterium) vitis* causes crown gall disease in grapevines. *Pseudomonas fluorescens* (*Pf*) isolate 1100–6 showed potential for suppressing the formation of galls. *Nicotiana glauca* plants were used to study the impact of the BCA, because large uniform gall developed, following inoculation with *R. vitis.* Prophylactic treatment of *N. glauca* with the *Pf* isolate 1100–6 reduced drastically the gall size produced by *R. vitis* and disease suppressive effect was seen at least 86 days after the BCA was applied. The results indicated that the isolate 1100–6 interfered with gall initiation, even when the plants were challenged with *R. vitis* only 20 min after treatment with BCA isolate. The kinetics of inhibition of gall formation by Pf isolate 1100–6 could be the result of direct physical association of the BCA with *R. vitis* or to the plant cells at the wound sites, providing a physical barrier and direct interference with the adhesion process which is an essential requirement for plant cell transformation. Visualization of *Pf* isolate 1100–6 *gfp* (tagged with green fluorescent protein) by epifluorescent microscopy confirmed the ability of this isolate to propagate in the internal and external surfaces of grapevine. Colonization of xylem vessels and pith tissues of grapevines by *Pf* isolate 1100–6 provided evidence for the suitability of this isolate to be applied on grapevines. In addition, the *Pf* isolate 1100–6 was able to survive on the rhizosplane of grapevines for 6 months (Eastwell et al. 2006).

Application of *Pseudomonas fluorescens* CH31 to cucumber significantly reduced root colonization by *Pythium aphanidermatum* and consequently root rot disease development was significantly suppressed (Moulin et al. 1996). Treatment of sugar beet seeds with *P. putida* 40 RNF as pellets significantly reduced the incidence of sugar beet damping-off disease caused by *Pythium ultimum*. The bacterial BCA reduced seed pericarp colonization by *P. ultimum* by 43 % at 48 h after planting and caused a reduction in the number of sporangia produced by 68 % in the soil around the plants. Treatment with *P. putida* was equally effective as the fungicide hymexazole in reducing disease incidence in infested soils (Shah-Smith and Burns 1996). A competitive root tip colonization assay was developed to select the efficient root colonizers of tomato root tips from the bacterial isolates obtained from tomato rhizosphere soil samples. Based on this trait 24 isolates that were equally or better colonizing ability relative to the well known efficient root colonizer *Pseudomonas fluorescens* WCS365. This procedure facilitated the isolation of seven new biocontrol strains including *Pseudomonas rhodesia* effective against tomato foot and root rot (TFRR) disease caused by *Fusarium oxysporum* f.sp. *radicis-lycopersici* (Validov et al. 2007).

The stigma of rosaceous plants being apparently nonselective supports the growth of microorganisms that are pathogenic as well as commensal epiphytes, some of which are antagonistic to *Erwinia amylovora,* causative agent of fire blight disease. Avirulent *hrp* regulatory mutants were found to partially protect greenhouse-grown seedlings from shoot and blossom infection by *E. amylovora* (Tharaud et al. 1997; Faize et al. 2006). The antagonistic bacteria, after establishing themselves on stigmas, utilize the mechanism of competitive exclusion and/or antibiosis to suppress pathogen development. When virulent *E. amylovora* strain Ea153N was inoculated individually on stigmas of pear, apple and black berry, it reached the highest population levels which were double that of the avirulent *hrpL* mutant of Ea153N or bacterial antagonists *P. fluorescens* and *Pantoea agglomerans*. In competition experiments, growth of the avirulent derivative was suppressed by the antagonist mixture to a greater extent than the virulent strain. Addition of a small amount of virulent Ea153N strain to the inoculum of an avirulent *hrpL* mutant significantly increased the population of the avirulent strain. In the orchard trials (9), the avirulent *hrpL* strain significantly suppressed the incidence of fire blight in four trials, whereas the antagonist mixture reduced the disease incidence significantly in six trials (Johnson et al. 2009).

Rhizosphere colonization by biocontrol agents depends on several factors, of which motility is an important trait. In *Pseudomonas fluorescens* F113 rif (F113), motility is a polygenic trait that is repressed by at least three independent pathways. The *kinB* gene encoding a signal transduction protein that together with *AlgB* was implicated in alginate production, participated in swimming motility repression through the Gac pathway, acting downstream of the GacAS two-component system. The Gac mutants were found to be impaired in the production of secondary metabolites, resulting in the loss of biocontrol activity of the mutants. But the *kinB* mutant and a triple mutant impaired in kinB, *sadB* and *wspR* (KSW) had a wild-type phenotype for secondary metabolism. The strain KSW was found to be hypermotile and more

competitive for rhizosphere colonization than the wild-type strain. The biocontrol potential of the mutants was compared with the wild-type strain. The strain KSW possessed improved biocontrol efficiency, compared with wild-type strain, indicating biocontrol potential of the strain could be enhanced by increasing its competitive colonization ability (Barahona et al. 2011).

5.1.1.4 Targeting Pathogenic Potential

Plant pathogens produce different kinds of toxins which may be host-specific or host-nonspecific (general). The host-specific toxins (pathotoxins) are able to induce the major symptoms of the disease as the pathogen itself, whereas host non-specific toxins do not have a major role in symptom development. Some fungal pathogens like *Fusarium culmorum* produce mycotoxins that are harmful, to animals and humans, when contaminated food and feed are consumed. *F. culmorum* causes seedling blight of wheat and barley. This pathogen produces trichothecene and synthesis of this mycotoxin is governed by the gene encoding the trichodiene synthesis. The BCAs *Pseudomonas fluorescens* strains MKB 100 and MKB249, *P. frederiksbergensis* strain 202 and *Pseudomonas* sp. MKB 158 were able to reduce significantly the extent of coleoptile growth retardation and seedling blight disease incidence. These bacterial strains were effective in ameliorating the negative effects of *F. culmorum* on seedling germination. The *Tri5* gene encodes trichodiene synthase that catalyzes the first step in the trichothecene mycotoxin biosynthetic pathway in *Fusarium* spp. Expression of *Tri5* gene was reduced by 33 % in stem base tissue coinoculated with *Pseudomonas* sp. strain MKB 158. As coinoculation of *Pseudomonas* sp. strain MKB 158 induced the expression of a wheat class III plant peroxidase gene, the biocontrol activity of the bacterial strain might be due to induction of systemic resistance to seedling blight disease in wheat (Khan et al. 2006).

5.1.1.5 Induction of Resistance to Crop Diseases

Resistance to crop diseases may be induced in plants by applying various biotic and abiotic agents. Induced resistance may be broadly differentiated into two forms as systemic acquired resistance (SAR) and induced systemic resistance (ISR). In response to pathogen infection or chemical application, “SAR” process is initiated, whereas colonization of plant roots by PGPR leads to ISR. SAR is considered to be activated more commonly by the pathogens, causing cell death reactions, ranging from single-cell hypersensitive reaction [(HR), a plant-specific type of programmed cell death (PCD)] to necrotic disease lesions. SAR is mediated by a salicylic acid (SA)-dependent process (Gaffney et al. 1993). On the other hand, ISR is mediated by jasmonate-or ethylene-sensitive pathway (Pieterse et al. 1998; Walters et al. 2005). SAR is characterized by an early increase in endogenously synthesized SA, coupled with the concomitantly activation of a set of SAR genes (Ryals et al. 1996; Sticher et al. 1997). ISR elicited by PGPR is capable of suppressing disease

development in aerial plant organs which are far separated from the roots that are treated with BCA in various plant species (van Loon et al. 1998; Kloepper et al. 2004).

When a plant is appropriately stimulated, a state of enhanced defensive ability known as induced resistance is observed in the plant. Induced systemic resistance (ISR), as the mode of action of disease suppression by nonpathogenic bacteria present in the rhizosphere of plants, was suggested by two groups of researchers working independently (Van Peer et al. 1991; Wei et al. 1991). SAR requires accumulation of salicylic acid (SA) in the model plant *Arabidopsis thaliana*. ISR is dependent on intact responses to ethylene and jasmonic acid. When these two different signal transduction pathways were triggered simultaneously in *A. thaliana,* the effectiveness of disease suppression was significantly enhanced (van Wees et al. 2000). The bacterial determinants involved in induction of systemic resistance in different host plants by bacterial biological control agents are presented in Table 5.5. The bacterial metabolite SA itself was suggested to trigger the SA-dependent signal transduction pathway. *Pseudomonas fluorescens* strain WCS 374 produced relatively large quantities of SA under iron-limited conditions, compared with conditions of sufficient iron availability. However, no indication for activation of SA-dependent signaling in radish after WCS374 treatment could be noted. WCS 374 strain was more effective in radish against Fusarium wilt under iron-limited conditions (Leeman et al. 1995). The iron- regulated ISR was considered to be the mechanism of biocontrol activity by WCS 374 in radish through mediation by the SA-containing siderophore pseudomine produced by the strain WCS37 (Mercado-Blanco et al. 2001). In the case of *P. aeruginosa* strain 7NSK2 with ability to produce SA, evidence suggested that SA was not the agent inducing resistance, but the compounds pyochelin and pyocyanin produced by this strain were the prerequisite for ISR induced by 7NSK2 strain. Thus SA produced by bacterial strains did not appear to be involved directly in the ISR against plant diseases (Audenaert et al. 2002).

Salicylic acid (SA)-induced defense expression via nonexpressor of pathogenesis related protein (PR)-genes-1 (*NPR1*), a key mediator present in plants. *NPR1* encodes a novel protein with a bipartite nuclear localization sequence and two potential protein-protein interaction domains (Cao et al. 1997; Dong 2004). Activity of NPR1 is dependent on the cellular redox. In *Arabidopsis*, AtNPR1 is predominantly in a monomeric form that can translocate into the nucleus, where it activates defense gene expression through interaction with transcription factors (Subramaniam and Desveaux 2001; Zhang et al. 2003). *Pseudomonas fluorescens* induces ISR which is also mediated by NPR1 as SAR (Spoel et al. 2003). Some strains of *Bacillus* spp. induced ISR dependent on SA and independent of jasmonic acid (JA) and *NPR1*. Furthermore, ISR induced by *Pseudomonas* spp. did not result in accumulation of PR1 in plants. On the other hand, ISR induced by *Bacillus* spp. in some plants, led to accumulation of PR1 in treated plants (Kloepper et al. 2004). *Pseudomonas aeruginosa* 7NSK2 produced SA, a siderophore, that was found to be primarily responsible for induction of ISR to *Botrytis cinerea* causing gray mold disease of bean and this resistance was shown to be iron-regulated (Meyer and Höfte 1997). Root inoculation of *Arabidopsis thaliana* with *P. fluorescens* CHA0 resulted in partial protection

Table 5.5 Bacterial determinants of eliciting systemic resistance in plants against microbial plant pathogens

Bacterial species/strains	Plant host/nature of bacterial determinant	References
Bacillus spp.	2-aminobenzoic acid (2-AB)	Yang et al. (2010)
B. cereus	Tomato/cell dialysates containing macromolecules	Romeiro et al. (2005)
Bacillus mycoides Bac J	Sugar beet/PO, chitinase and β-1,3-glucanase	Bargabus et al. (2002)
B. pumilus	Sugar beet/PO, chitinase, β-1,3-glucanase	Bargabus et al. (2004)
Bacillus subtilis S499	Potato/surfactin	Ongena et al. (2007)
B. subtilis GBO3 and IN 937a	*Arabidopsis* – 2,3 butanediol	Ryu et al. (2004)
Chrysobacterium balustinum AUR 9	*A. thaliana* colo lipopolysaccharides	Solano et al. (2008)
Pseudomonas aeruginosa 7NSK2	Pyochelin and pyocyanin Cotton/ siderophore – pseudobactin	Audenaert et al. (2002), Meyer and Höfte (1997), and Fallahzadeh – Mamaghani et al. (2009)
P. chlororaphis 06	Tobacco/2R, 3R- butanediol	Han et al. (2006)
P. fluorescens CHA0	Tobacco/siderophore	Maurhofer et al. (1994)
P. fluorescens WCS 374	*Arabidopsis thaliana*/salicylic acid-containing siderophore	Leeman et al. (1995)
	Radish/lipopolysaccharide/ siderophore	Mercado-Blanco et al. (2001)
P. fluorescens WCS417	Carnation/lipopolysaccharide	Van Peer and Schippers (1992)
P. putida BTP1	Bean/N-alkylated benzylamine derivative (NABD)	Ongena et al. (2004)
Paenibacillus spp. B2	*Medicago truncatula*/Paenimyxin (lipopolypeptide)	Selin et al. (2010)
Serratia marcescens 90-166	Siderophore – catechol	Press et al. (2001)

of leaves against *Peronospora parasitica* causing downy mildew disease. Induction of ISR to *P. parasitica* required the synthesis of the antibiotic 2,4-diacetylphloroglucinol (2,4-DAPG) by *P. fluorescens*, as application of 2,4-DAPG at 10–100 μM mimicked the ISR effect, indicating the possibility of similar mechanism operating in other pathosystems also (Iavicoli et al. 2003). Another investigation using several strains of *Pseudomonas* spp. indicated that elicitation of ISR was typically dependent on SA and did not result in activation of *PR-1a* gene that encodes PR-1a protein (van Loon and Glick 2004). *P. putida* BTP1 applied on the roots of bean reduced the symptoms of gray mold disease caused by *Botrytis cinerea* on leaves. The molecular determinant of *P. putida* involved in ISR was isolated from cell-culture fluid after growth. The N-alkylated benzylamine derivative (NABD) purified from culture

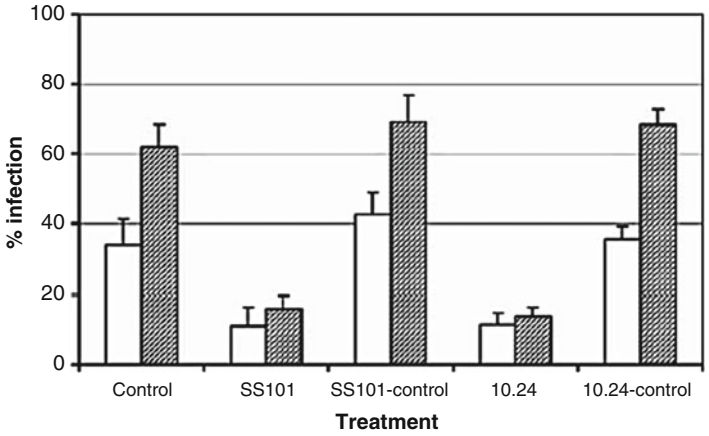

Fig. 5.4 Effect of treatment of wheat or apple with *Pseudomonas fluorescens* SS101 or *massA* mutant 10.24 on root infection by *Pythium* spp. assessed by split-root assays. *White bars*: infection of wheat; *gray bars*: infection of apple. Infection rates for SS101 and 10.24 treatments were significantly lower than the control treatments ($P < 0.001$) (Courtesy of Mazzola et al. 2007 and with kind permission of The American Phytopathological Society, MN, USA)

fluid mimicked the protective effect as induced by crude samples. The mutants impaired in NABD biosynthesis lost the elicitor activity, indicating the requirement of NABD for ISR induction (Ongena et al. 2004).

A split-root plant assay was applied to determine, whether suppression by *Pseudomonas fluorescens* SS101 of *Pythium* spp. pathogenic to wheat or apple, was due to direct or indirect effects. The assays were conducted in orchard soils to explore the possible role of induced resistance. Strain SS101 or the *massA* mutant 10.24 significantly reduced infection by *Pythium* spp. on the component of the apple or wheat root system cultivated in soil treated with the respective bacteria. Wheat root infection was reduced from ≈34 % for plants grown in non-treated soil to ≈11 % for the component of plant root systems grown in SS101- or mutant 10.24-treated soils. Infection of 'Gala' apple seedling roots was reduced from 60 to 70 % in the control to ≈15 % for the portion of the root mass cultivated in SS101- or 10.24-treated soils. For the SS101 and 10.24 treatments, no difference in frequency of *Pythium* root infection was observed for the component of the root system physically separated from bacterially treated soil, relative to that observed for control treatment (Fig. 5.4) (Mazzola et al. 2007).

The capacity of strains of *Pseudomonas* spp. to induce systemic resistance in *Ecalyptus urophylla* against bacterial wilt disease caused by *Ralstonia solanacearum* was assessed. Two strains *P. putida* WCS 358r and *P. fluorescens* WCS374r could trigger ISR, when infiltrated into two lower leaves at 3–7 days before challenge inoculation, but not when these strains were applied to the soil. A mutant of strain WCS 358r defective in the biosynthesis of the siderophore pseudobactin did not induce ISR. The purified siderophore from WCS358r did induce ISR, suggesting

that pseudobactin 358 was the ISR determinant of WCS358r strain. A siderophore-minus mutant of WCS374r could induce disease resistance to the same level as the wild-type strain. The purified siderophore from this strain could induce ISR, indicating that both the siderophore and other uncharacterized ISR determinant of WCS 374r could trigger ISR in *Eucalyptus.* Although soil drench with salicylic acid (SA) induced ISR in *Eucalyptus,* transformation of a siderophore-minus mutant of WCS358 with SA biosynthetic gene cluster from WCS 374 did not restore the ability to cause ISR in *E. urophylla* (Ran et al. 2005). Many determinants of ISR such as iron-regulated metabolites such as pseudobactin siderophore have been demonstrated to have an important role in ISR (Meziane et al. 2005). Production of the siderophores relies on the concentration of iron in the environment and under conditions of low iron availability. Purified siderophores have disease suppressive effect similar to that of the strain producing the siderophore (Neilands and Leong 1986). King's medium B supplemented with 8-hydroxyquinoline (8-HQ) (120 ppm), a chelator, was used for screening of *Pseudomonas* spp. from the rhizosphere of cotton, in order to select the isolates with capacity to produce high concentrations of siderophores that might be equal to or greater than the reference strain *P. aeruginosa* 7NSK2. The strains 35Q and 16Q produced significantly greater quantities of siderophore, the strain 35Q producing a maximum of 381.7 μM. In hydroponic experiments with cotton, the bacterized plants with low iron availability showed high levels of resistance to cotton bacterial blight disease caused by *Xanthomonas campestris* pv. *malvacearum* (*Xcm*). At 48 h after inoculation of plants with *Xcm*, the levels of defense-related enzymes peroxidase (PO) and phenylalanine ammonia lyase (PAL) were significantly higher in plants treated with the strain 35Q. The strain 35Q was found to be a powerful producer of pseudobactin and this siderophore might be a determinant of induction of systemic resistance in cotton under iron-limited conditions (Fallahzadeh-Mamaghani et al. 2009).

The biocontrol potential of grapevine-associated bacterial species *Pseudomonas fluorescens* PTA-CT2, *Acinobacter lwoffii* PTA-113 and *Pantoea agglomerans* PTA-AF1 was assessed against *Botrytis cinerea*, causative agent of gray mold disease in two vineyards with susceptible cv. Chardonnay. The severity of the disease on grapevine leaves and berries was reduced to different levels, depending on the bacterial strain and inoculation method. Systemic resistance was stimulated in grapevine plants drenched with these bacterial BCAs, with a single application. The ISR was associated with a stimulation of plant defense responses such as chitinase and β-1,3-glucanase reaching its maximum activity at ripening stage. The enzyme activities reached their maximum in leaves of grapevine plants treated with *P. fluorescens* and *A. lwoffii*, whereas the peaks of enzyme activities in the berries of plants treated with *A. lwoffi* and *P. agglomerans* were detected. *A. lwoffii* was able to stimulate the activities of chitinase and β-1,3-glucanase more efficiently in both leaves and berries which are vulnerable to the infection by *B. cinerea* (Magnin-Robert et al. 2007). The biotic inducers, *Pseudomonas fluorescens* and *P. putida* were evaluated for their efficacy in inducing systemic resistance in lupine against Fusarium wilt disease caused by *F. oxysporum* f.sp. *lupine* (FOL), when applied as seed treatment. Both biotic inducers significantly reduced the disease incidence under greenhouse

and field conditions. *P. fluorescens* and the abiotic inducer potassium chloride were more effective than *P. putida*, copper sulfate and indole butyric acid. A time-course of defense-related enzymes showed substantial increases in enzymatic activities in induced infected seedlings, compared with untreated unhealthy plants or infected controls. The extent of increase in enzymatic activities varied among treatments. Maximum increases in chitinase and β-glucanase activities were recorded at 12 and 18 days after inoculation with the pathogen respectively. In addition, phenylalanine ammonia lyase (PAL) increased substantially at 8 days after inoculation. Phenolic compounds and specific flavonoids accumulated markedly following infection by FOL in induced and/or infected seedlings compared with healthy plants. The inducers increased the crop parameters and seed yield, compared with untreated control plants (Abd El-Rahman et al. 2012).

Pseudomonas chlororaphis induced ISR in tobacco and cucumber plants against two bacterial pathogens *P. syringae* pv. *tabaci* and *Erwinia carotovora* sub sp. *carotovora* (Spencer et al. 2003) as well as against the fungal pathogen *Corynespora cassiicola* causing leaf spot disease (Kim et al. 2004a, b). Investigations were carried out to monitor the transcriptional response of over 8,000 *Arabidopsis* genes in order to identify ISR-related genes. *P. chlororaphis* 06-mediated ISR was associated with rapid induction of several genes after a challenge inoculation with *C. cassiicola* in cucumber leaves relative to the controls treated with water (Kim et al. 2004a, b). After challenge inoculation with *P. syringae* pv. *tomato* on the *P. fluorescens* WCS 417r-induced plants, 81 genes were evidenced augmented expression patterns within the leaves. These genes were primed to respond faster or more strongly, when exposed to invasion by pathogens. The capacity for augmented defense expression is designated "priming". This process of priming has been observed in several plant species protected by ISR induced by bacterial biocontrol agents (Verhagen et al. 2004).

Induced systemic resistance (ISR) elicited by PGPR has been demonstrated in several crops including bean, carnation, cucumber, radish, tobacco, tomato and *Arabidopsis thaliana* (Van Loon et al. 1998). Plant defensive systemic responses induced by three PGPRs *Azospirillum brasilense* Sp7, *Chrysobacterium balustinum* AUR9, and *Pseudomonas fluorescens* AUR6 on *Arabidopsis thaliana* Col O against *P. syringae* pv. *tomato* (Pst) DC 3000 were studied at the biochemical and transcriptional levels. All three bacterial strains reduced disease severity, when applied prior to challenge inoculation with *Pst.* The maximum protection against the disease was provided by *C. balustinum*. Plants treated with each of the three strains were also reduced in salicylic acid (SA) production after pathogen challenge, compared to untreated controls. *C. balustinum* AUR induced maximum production of SA. The expression level of pathogenesis-related protein PR-1, a transcriptional marker of SA-dependent pathway in *C. balustinum* AUR9-treated plants, was four-fold that of controls, whereas the expression of PDF1.2, a transcriptional marker for the SA-independent pathway was not induced. SA production by PGPR strains could be attributed to the effect of PGPR strains identified as avirulent pathogens. The protection conferred was inversely related to SA production and coincided with reduction of SA accumulation in PGPR-treated plants. *C. balustinum* cell wall lipopolysaccharides being putative bacterial elicitor molecules, were able to

reproduce the systemic induction effect at low doses. It is possible that some PGPR strains may stimulate different systemic responses in host plants. With *C. balustinum* AUR9, the SA-dependent pathway was stimulated first, as indicated by increases in SA levels and PR1 expression, followed by induction of the SA-independent pathway, as indicated by the increases in ethylene (ET) concentrations. The role of ET on ISR-mediated response was detected by its sensitivity (priming response). The PGPR strains giving higher levels of protection against the pathogen caused the release of greater amounts of ET in plants after incubation with its precursor 1-amino-cyclopropane-1-carboxylic acid (ACC). These strains also blocked the SA peak upon pathogen challenge. The induction of both pathways appeared to result in additive effect on disease suppression (Solano et al. 2008).

The impact of strains of *Pseudomonas fluorescens* capable of inhibiting soilborne fungal pathogens during colonization of the wheat rhizosphere was studied. Based on the assumption that *P. fluorescens* induced defense genes in wheat roots, a microarray 192 oligonucleotides representing 84 wheat root expressed sequence tags (ESTs) homologous to defense stress genes were constructed. The ESTs were selected from the wheat EST libraries. Four days after seed inoculation with wheat take-all suppressive strain *P. fluorescens* Q8r1-096, the arrays were integrated with labeled transcript (cDNA) populations from roots or coleoptiles of the cv. Finley. The transcripts encoding jasmonic acid pathways and proteins associated with the hypersensitive response, in addition to stress associated proteins, were induced or repressed in wheat roots during *P. fluorescens* interactions. Transcripts encoding PR-protein Pr-10a and hypersensitive response protein HR in 1 h were also induced in coleoptiles. Real-time PCR assay showed that 11 transcripts were induced in root tissues between 2 and 6 h and remained at higher levels at 24 h post-inoculation. The results suggested that defense/stress gene expression might be modulated by *P. fluorescens* in wheat root tissues (Okubara et al. 2010). The biocontrol mechanism of *Pseudomonas chlororaphis* MA342 and/or *Serratia plymuthica* HRO-C489 applied as seed treatment against *Verticillium longisporum* infecting oilseed rape was investigated. Soil was infested with microsclerotia and mycelium of *V. longisporum* followed by planting seeds treated with rifampicin-resistant bacterial strains at the rate of 10^{6-7} cells/seed. A significant reduction in disease intensity, as reflected by values of area under disease progress curve (AUDPC) was recorded for both bacterial strains. Significant differences in the percentage of healthy plants were noted between the cultivars ranging from 46.5 to 72.6 %. No additional benefit was evident due to combined application of the BCA strains. Growth promotion effects due to the application of BCAs were not related to the extent of disease control. As the possibility of containing this disease through cultivar resistance and/or chemical application is remote, the use of the PGPR that can raise the level of resistance to the disease has to be comprehensively examined (Abuamsha et al. 2011).

Various strains of rhizobacteria have been reported to suppress the development of several plant diseases through induction of resistance in the host plant species. Induced systemic resistance is mediated mainly by fluorescent *Pseudomonas* spp. and also by endophytic bacteria such as *Bacillus* spp. ISR induced against *Tobacco necrosis virus* by *P. fluorescens* CHA0 coupled with stimulation of PR

protein synthesis in tobacco was demonstrated by Maurhofer et al. (1994). Stimulation of phytoalexin synthesis following PGPR treatment was observed in carnation treated with *Pseudomonas* sp. (Van Peer et al. 1991). In a later study, cucumber plants were protected against *Pythium aphanidermatum* causing root rot disease by application of *P. putida* isolate BTP1 and its sid^- mutant M3. The protection was primarily associated with accumulation of antifungal compounds (phenolics) in treated roots. The phenolics were the phytoalexins produced systemically. Analyses of leaf samples revealed increased concentrations of fungitoxic molecules in PGPR-treated plants, although the nature of these molecules appeared to be different from those detected in roots. The results suggested that the PGPR might elicit phytoalexins systemically in cucumber and the overall defense response was not based on a single phytoalexin, but it is chemically complex and organ-specific (Ongena et al. 2000). Watermelon in Mekong Delta of Vietnam was affected seriously by *Didymella bryoniae,* the causative agent of gummy stem blight disease. *Pseudomonas aeruginosa* strain $23_{1\text{-}1}$ suppressed the development of *D. bryoniae* directly by the production of antibiotics locally and/or indirectly by stimulating the defense systems systemically. Foliar infection by *D. bryoniae* was significantly reduced by treating the seeds with *P. aeruginosa,* indicating the ability of the bacteria to induce ISR in watermelon under field conditions. *P. aeruginosa* colonized the watermelon plants endophytically, more actively in infected plants than in healthy plants. Treatment of watermelon seeds with BCA inhibited pathogen penetration which was associated with accumulation of H_2O_2, followed by enhanced peroxidase activity and occurrence of new peroxidase isoforms. The mechanisms of biocontrol activity of *P. aeruginosa* against *D. bryoniae* were antibiosis and ISR under greenhouse and field conditions. The PGPRs present in the native soils have to be screened for their biocontrol potential to select and apply the most efficient strains capable of surviving under field conditions (Nga et al. 2010).

The potential of *Pseudomonas fluorescens* 89B61 to induce ISR in tomato against the late blight pathogen *Phytophthora infestans* was evaluated. This strain elicited systemic protection against the disease and reduced disease severity by a level equivalent to SAR induced by the chemical β-amino butyric acid (BABA) in greenhouse experiments. The results suggested that protection induced by *P. fluorescens* was SA-independent, but ethylene- and jasmonic acid-dependent, whereas SAR elicited by BABA was SA-dependent. Further, the lack of colonization of tomato leaves by strain 89B61 suggested that the ISR was due to systemic protection by this strain and not attributable to a direct interaction between the pathogen and the BCA (Yan et al. 2002). The bacterial strains with multiple mechanisms for biocontrol activity against fungal pathogens are likely to be more efficient. *P. chlororaphis* strain PA-23 produced phenazine-1-carboxylic acid (PCA) and acetamidoanthranilic phenol (AAP) which inhibited the mycelial growth of *Sclerotinia sclerotiorum,* causing stem rot disease of canola. The germination of ascospores of *S. sclerotiorum* in canola petals was inhibited by the strain PA-23, as revealed by microscopic observations. Two applications of PA-23 induced resistance against infection by *S. sclerotiorum*. Enhanced accumulation of PR proteins and oxidative enzymes including chitinase and β-1,3-glucanase by PA-23 in canola leaf tissues

might account for reduction in pathogen infection. The combination of antibiotic production with induction of ISR by *P. chlororaphis* PA-23 could act synergistically in restricting the pathogen growth and colonization, resulting in significant suppression of disease incidence and intensity (Fernando et al. 2007).

Systemic resistance against plant pathogens induced in plants by biocontrol agents and chemicals has been investigated to have an insight into the variations in the biochemical and molecular genetic bases between normal healthy and treated plants, following challenge inoculation with the microbial pathogens. Ultra-structural changes in treated plants resulting in resistance to invading pathogens have been studied only in a few cases. Infection behavior of *Colletotrichum orbiculare,* causing cucumber anthracnose disease, was monitored in the leaves of cucumber preinoculated with *Pseudomonas fluorescens* 89B61, *Serratia marcescens* 90–166, using transmission electron microscope (TEM). Active defense responses such as sheath formation at penetration sites and accumulation of endoplasmic reticula or numerous vesicles around intracellular hyphae were observed in the leaves of plants preinoculated with both bacterial strains. In addition, the electron densities of most intracellular and intercellular hyphae were greater than those of untreated control plants which did not exhibit any of the defense responses detected in BCA-treated leaves as indicated above. In plants treated with chemical inducers of disease resistance like DL-3-amino butyric acid or aminosalicylic acid, no active defense responses were observed and the pathogen hyphae were rarely seen at 5 days after challenge inoculation. The results suggested that the mechanisms of ISR were differentially expressed in plants treated with bacterial BCAs and chemical inducers (Jeun et al. 2007).

The antagonistic activity of *Pseudomonas syringae* against fungal pathogen, causing citrus postharvest diseases was correlated with in vitro production of lipodepsipeptides and induction of resistance in citrus tissues as well, due to a broad spectrum of metabolic modifications. The expression of syringomycin (*syrB1*) and syringopeptin (*sypA*) synthetase genes from *P. syringae* pv. *syringae* (*Pss*) biocontrol strains was assessed on different culture media and in vivo on citrus fruits during interaction with *Penicillium digitatum* by quantitative RT-PCR assay. The *syrB1* and *sypA* genes were more actively expressed, when *Pss* strains were grown on orange peel broth, as compared to nutrient broth and potato dextrose broth. Infection by *P. digitatum* was strongly stimulatory only to *syrB1* expression, suggesting that *syrB1* gene could be involved in the biocontrol activity. Results of QRT-PCR indicated that both *Pss* and *P. digitatum* could enhance transcription of CHI1 in inoculated flavado tissues, compared with untreated control. Further, coinoculation of *Pss* and the pathogen strongly induced the transcription of CHI1. The CHI1 gene was considered to be the most possible part of molecular mechanisms involved in the pathogen defense responses in citrus fruit (Scuderi et al. 2011).

The mechanism of biocontrol activity of endophytic *Pseudomonas putida* MGY2 isolated from papaya fruit against the anthracnose disease of papaya caused by *Colletotrichum gloeosporioides* was investigated. Treatment with MGY2 significantly reduced disease incidence and lesion diameter in papaya fruit inoculated with *C. gloeosporioides*. The strain MGY2 was found to inhibit ethylene production and

reduced the decline of firmness of harvested papaya fruits stored at 25 °C, resulting in maintenance of natural resistance in papaya fruits. MGY2-treated papaya fruit maintained a significantly higher level of PAL activity from day 3 to day 9 of incubation compared to the control fruit. The BCA treatment also enhanced the total phenol contents of the fruits. During the storage, the levels of phenolics in MGY2-treated fruits were higher than the untreated controls. The bacterial treatment induced catalase (CAT) and peroxidase (PO) activities to reach levels significantly higher than in untreated fruits. CAT and PO are important detoxifying enzymes which are known to function together with other enzymes in the ascorbate glutathione cycle for promoting the scavenging of reactive oxygen species (ROS). The expressions of PAL1, CAT1 and PO were analyzed by northern blotting. The expression of PAL1 in MGY2-treated fruit appeared to be stronger compared to controls, as reflected by the mRNA levels which were elevated in MGY2-treated fruits. The reduction of decay in papaya fruit treated with MGY2 might be, due to a combined action of the defense-related enzymes which were stimulated significantly following application of the bacterial strain (Shi et al. 2011) (Appendix 5.4).

Pseudomonas chlororaphis 06, has been reported to induce resistance in tobacco against soft rot pathogen *Erwinia carotovora* subsp. *carotovora* (*Ecc*) and *P. syringae* pv. *tabaci* (*Pst*) causing wild fire disease. The extracellular compounds produced by *P. chlororaphis* were purified to identify the bacterial determinants involved in induction of disease resistance. Based on the results of high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) mass spectrophotometry, the active compound was identified as 2R, 3R-butanediol. This compound induced systemic resistance to *Ecc* SCC1, but not to *Pst*. Treatment of tobacco with volatile 2R, 3R butanediol enhanced aerial growth which was also observed during colonization by *P. chlororaphis*. The global sensor kinase GacS of *P. chlororaphis* 06 was found to be a key regulator for ISR against *Ecc* through regulation of 2R, 3R-butanediol production. The investigation seems to be the first in linking the ISR to 2R, 3R-butanediol, a fermentation product as well as to the sensor kinase GacS for its production (Han et al. 2006). The mechanism of biocontrol potential of *Pseudomonas fluorescens* BK3 against the apple fire blight pathogen *Erwinia amylovora* was studied. The susceptible cultivar Holsteiner Cox was pretreated with 1×10^6 cells of the strain BK3 at 2 days prior to challenge inoculation with *E. amylovora.* The intracellular washing fluid (IWF) was collected from the plants before and after treatment with the BK3. The IWF from treated plants contained chitinase, β-1,3-glucanase, thaumatin-like protein and ribonuclease which belonged to the group of pathogenesis-related (PR) proteins. Investigations on transcript level revealed the up-regulation of 113 EST clones which also belonged to the class of PR-proteins, oxidative stress and transcripts that code for proteins which play important role at different stages of pathogen recognition and signaling pathways (Kürkcüoglu et al. 2007). A noninvasive method of determining populations of BCA and pathogens on the leaf surface or inside plant tissues based on bioluminescence was developed. The pathogen *E. amylovora* and *P. fluorescens* BK3 were transformed with *lux* CDABE gene cluster that codes for two structural genes of the luciferase as well as

for the genes of the substrate biosynthesis. The measurement of bioluminescence revealed that the strain BK3 protected the apple plants, when treated at 2 days prior to inoculation with *E. amylovora.* Application of the strain BK3 did not cause any visible morphological change in treated plants. The results indicated that the strain BK3 protected the apple plants by inducing resistance to fire blight disease (Schmoock et al. 2008).

Pseudomonas fluorescens strains have been reported to induce systemic resistance to some virus diseases affecting different crops. *P. fluorescens* strain 89B-27 induced systemic resistance to *Cucumber mosaic virus* (CMV) in cucumber cv. Straight 8 leading to consistent reduction in the mean numbers of symptomatic plants coupled with delay in symptom expression. No viral antigen could be detected in the asymptomatic plants throughout the experimental period (Raupach et al. 1996). The strains Pf1 and CHA0 of *P. fluorescens* were able to induce systemic resistance in rice against tungro disease, when these BCA strains were applied as seed treatment, root dipping or foliar spray (Narayanasamy 1995). Enhancement of the activities of defense-related enzymes such as peroxidase (PO) and phenylalanine ammonia lyase (PAL) was observed in many crop plants treated with the PGPR strains (Narayanasamy 2005). The ability of three strains CoP-1, CoT-1 and CHA0 of *P. fluorescens* to induce ISR in tomato against *Tomato spotted wilt virus* (TSWV). The BCA strains were used to treat the seed, soil, roots of seedlings and foliage of transplanted tomato plants. Treatment with BCA strains resulted in significant reduction in TSWV infection as well as enhancement of growth of tomato plants both in the glasshouse and field conditions. Increased activity of polyphenoloxidase (PPO), β-1,3-glucanase and chitinase in BCA-treated tomato plants was observed. The presence of a new protein was detected only in BCA-treated plants. Enzyme-linked immunosorbent assay (ELISA) indicated that the viral antigen concentration was reduced in parallel to disease intensity (Kandan et al. 2005).

Pseudomonas chlororaphis strain 06 suppressed the effects of *Cucumber mosaic virus* (CMV) infection in tobacco. The role of global regulator GacS in the strain 06 in stimulating growth promotion and ISR in tobacco was investigated. Root colonization of cv. Samsum with wild-type 06 and the *gacS*-complemented mutant elicited resistance and reduced the intensity of symptoms and titer of CMV. In tobacco cv. GX3, disease intensity was not altered, following root colonization by the wild-type, *gacS* mutant or complemented mutant. But the viral titer was reduced in plants colonized by wild type and the *gacS* mutant strains. The plant growth was not adversely affected by CMV infection in plants treated with wild-type and *gacS* mutant. The bacterial BCA induced systemic resistance in tobacco against CMV without a negative impact on growth (Ryu et al. 2003). *Banana bunchy top virus* (BBTV) causes the destructive bunchy top disease in banana. Strains of *P. fluorescens* CHA0 and Pf1 were able to reduce the incidence of the disease and promote the growth of the plants as well. The bioformulation of these bacterial strains either alone or in combination with chitin were evaluated for their potential to induce systemic resistance against BBTV in greenhouse and field conditions. The bioformulation containing CHA0 amended with chitin stimulated the defense-related enzymes in treated plants challenged with BBTV under greenhouse conditions.

The viral antigen concentration was reduced in BCA-treated plants in proportion to the disease severity. The enhanced growth of BCA-treated plants was reflected in higher bunch yield (Kavino et al. 2008). The effects of treatment of banana with bioformulations containing *Pseudomonas fluorescens* (Pf1) and *Bacillus* sp. (EPB22) on the development of *Banana bunchy top virus* (BBTV) infection were assessed. The disease incidence was found to be reduced by 80 and 52 % respectively in the greenhouse and field evaluations. The virus titer was significantly reduced in plants treated with the bacterial mixture as indicated by enzyme-linked immunosorbent assay (ELISA). Biochemical investigations showed that PR-protein synthesis and activities of peroxidase, polyphenol oxidase and phenylalanine ammonia lyase were activated. In addition, accumulation of phenolic compounds was observed in bacterized banana plants, indicating that the reduction in banana bunchy top disease was likely to be due to in induction of systemic resistance by the BCA mixture. Enhanced plant growth and increase in yield were additional benefits due to the application of bacterial strains (Harish et al. 2009).

5.1.1.6 Factors Influencing Mechanisms of Biocontrol

Application of biocontrol agents has been favored as an alternative crop disease management strategy in place of chemical application. However, the lack of consistency in the performance of bacterial biocontrol agents under field conditions has limited their practical use in commercial agriculture. The inconsistency has been attributed to a greater extent to variability in physical and chemical properties within niches occupied by the biocontrol agents that influence both colonization and expression of various mechanisms of biocontrol activities against microbial pathogens leading to ineffective suppression of disease development.

The method employed to apply bacterial BCAs for treating the seeds may affect the distribution and pattern of colonization of bacteria and subsequently the efficacy of the BCA against seedborne pathogens. It is essential to have the information on parts of the seeds to be colonized for achieving effective biocontrol. Molecular techniques like GFP tagging have been used to monitor the pattern of colonization of bacteria. The *gfp* gene encoding the green fluorescent protein (GFP) was employed to tag *Pseudomonas chlororaphis* MA342 effective against *Drechslera teres,* causing seedborne netblotch disease of barley. The *gfp*-tagged strain MA342 G2 had the same biocontrol potential as the wild-type strain, when it was applied at high cell concentrations to seeds, but was less effective at lower concentrations. The number of culturable cells was significantly less than the total number of bacterial cells on seeds which were dried for 20 h after inoculation. Confocal microscopy and epifluorescence stereomicroscopy were used to determine the pattern of MA342G2 colonization and cell aggregation of barley seeds. Immediately after bacterization of seeds, the BCA cells were seen mainly under the seed glume and no specific pattern could be observed. After the seeds were sown, aggregation of bacteria towards and near the embryo, but not in the embryo was recognizable. Aggregates of bacteria were commonly present in the groove of each seed formed by the base of the

coleoptile and the scutellum. The results suggested that the strain MA342 might colocalize with the pathogen, facilitating the antifungal compound (2,3-deepoxy-2,3-didehydrorhizoxin) produced by the BCA to reach the target pathogen propagules (Tombolini et al. 1999).

Many abiotic factors existing in soil such as pH, temperature, moisture, texture and inorganic and organic constituents can significantly influence the activities of biocontrol agents. Temperature is one of the important factors, influencing both colonization of roots by rhizobacterium, as well as the expression of their biocontrol mechanisms. In the case of *Fusarium oxysporum* f.sp. *ciceris* race 5, incubation temperature and inoculum density of the pathogen strongly interacted in modulating the expression of Fusarium wilt in chickpea by four rhizobacteria viz., *Pseudomonas fluorescens* RGAF19, *P. fluorescens* RG26, *Bacillus megaterium* RGAF51 and *Paenibacillus marcesans* RGAF101. When the conditions became favorable for wilt disease development, the effectiveness of biocontrol by the rhizobacteria decreased (Landa et al. 2001). In a later study, seed and soil treatment with *P. fluorescens* isolates RGAF 19 and RG26, significantly increased chickpea shoot dry weight at 20 °C and root dry weight at 25 and 30 °C. They colonized the chickpea rhizosphere and internal stem tissues at 20, 25 and 30 °C and there was a positive linear trend between bacterial population size in the rhizosphere and temperature increase. The maximum inhibition of mycelial growth and conidial germination of *F. oxysporum* f.sp. *ciceris* race 5 in vitro occurred at a temperature range optimal for bacterial growth and production of toxic metabolites like pyoverdin (Landa et al. 2004).

The knowledge of interactions between crop cultivars, pseudomonads and soil types may be useful to optimize cultivar-soil combinations for the promotion of growth through beneficial BCAs. Three Swiss winter wheat (*Triticum aestivum*) cultivars Arina, Zinal and cimetta were characterized for their ability to accumulate naturally-occurring plant-beneficial pseudomonads in the rhizosphere. The ability to select for specific genotypes of 2,4-DAPG producers in two different soils was used as the basis to determine the cultivar performance cultivar, specific differences were strongly influenced by the soil-type. The *phlD* diversity among the *Pseudomonas* spp. substantially varied between the two soils, as indicated by the denaturing gradient gel electrophoresis (DGGE) analysis of fragments of the DAPG biosynthetic gene *ph1D* amplified from natural *Pseudomonas* rhizosphere populations. Further, there was a cultivar-specific accumulation of certain *phlD* genotypes in only one soil. Among the three cultivars tested, Arina was protected most effectively against *Pythium ultimum* infection by treatment with *P. fluorescens* CHA0. However, in terms of growth promotion, this cultivar derived the least benefit from the interaction with the BCA, in the absence of pathogen infection. The results suggested that it might be possible to improve the plant-beneficial effects of root colonizing pseudomonads by breeding wheat genotypes with greater potential to sustain interactions with the PGPR (Meyer et al. 2010).

Bacterial species/strains have been reported to promote fungal spore germination and/or root colonization by arbuscular mycorrhizal (AM) fungi. The bacteria promoting the establishment of mycorrhizal symbiosis (ecto-and endo-mycorrhizas) by increasing root-fungus contacts and colonization are designated mycorrhizae

helper bacteria (MHB). *Pseudomonas fluorescens* C7R12 and BEG12, the cell organization of C7R12 was found earlier to promote colonization of *Medicago truncatula* roots by *Glomus mosseae* BEG12. To understand the underlying mechanism of interaction between C7R12 and BEG12, the cell organization of C7R12 was characterized on adventitious roots mycorrhized or not with BEG12 and extraradical hyphae by employing the immunofluorescence technique and confocal laser scanning microscope. Bacterial cells more frequently remained single on mycorrhizal than on non-mycorrhizal roots and in microcolonies and strings on mycorrhizal roots. In addition, the root area covered by bacterial cells, as revealed by image analysis, appeared to be significantly lower on mycorrhizal than on non-mycorrhizal roots. Cells of C7R12 were abundant on extraradical hyphae and organized both as single cells and microcolonies. The results suggested that *P. fluorescens* C7R12 cells became less active and were found to be less abundant on mycorrhizal than on nonmycorrhizal roots (Pivato et al. 2008).

The effectiveness of the biocontrol by introduced bacteria relies primarily on their ability to maintain stable populations and to remain metabolically active in the rhizosphere. It is known that the sizes of introduced pseudomonad populations may decline appreciably within a few weeks and consequently the beneficial effects of the introduced bacteria tend to be variable. Among the biological factors affecting the biocontrol activity, bacteriophages which are ubiquitous in the soil environment are potentially important. They are considered to be important as an ecologically important factor with negative impact on bacterial populations. The practice designated phage therapy involves the use of bacterial viruses that can only infect specific bacteria by lysing the susceptible cells of the bacterial species/strains/pathogens. A lytic bacteriophage ØGP100 that could specifically infect *Pseudomonas fluorescens* CHA0 and some closely related *Pseudomonas* strains were isolated from soil. The influence of phage ØGP 100 on the biocontrol potential strain CHA0 and its rifampicin resistant derivative CHA0-Rif was assessed. The phage ØG100 has double-stranded DNA as the genome with an icosahedral head and stubby tail. In the presence of ØGP100, the population size of the strain CHA0-Rif in soil and on cucumber roots was reduced by more than 100-folds. As a result, the biocontrol potential to protect cucumber against *Pythium ultimum* was entirely abolished. However, the phage did not affect either root colonization and/or the disease suppression by a ØG100-resistant variant of the strain CHA0-Rif. The results suggested that the emergence of a phage resistant subpopulation strain CHA0-Rif might occur too slowly to allow for the build up bacterial population that might effectively suppress *P. ultimum* that infects the plant roots at very early stages of growth (Keel et al. 2002). Introduction of *lux* genes into bacterial strains and measurement of the resulting bioluminescence can provide a sensitive marker to track introduced rhizobacteria in nonsterile soil environments and to estimate their physiological activity in situ (White et al. 1996). Root colonization and in situ bioluminescence are considered to be indicators of biocontrol activity in the rhizosphere of plants. Bioluminescence was strongly correlated with dehydrogenase of activity of *Pseudomonas fluorescens* in in vitro cultures. In situ bioluminescence indicated the physiological activity of *Pseudomonas fluorescens* B5. Colonization of the roots at ≥4 cm below the seed

decreased at very low soil water matric potential (-330×10^3 Pa). Total population size of the strain B5 per seedling was significantly increased at -140×10^3 Pa. However, matric potential had no significant effect on the bacterial population density per g of root fresh weight and did not affect the distribution of the population down the root. Total population size per seedling and downward colonization by the strain B5 were significantly reduced at high temperatures (25–35 °C). Antagonistic activity of *P. fluorescens* B5 against *Pythium ultimum* causing beet root damping-off disease decreased with increasing soil temperature and decreasing matric potential (Schmidt et al. 2004).

5.1.2 Bacillus *spp.*

The use of Gram-positive *Bacillus* spp. as biocontrol agents has been relatively less frequent compared with the Gram-negative *Pseudomonas* spp., despite the fact that the strains of *Bacillus* spp. are widely distributed, have high thermal tolerance and grow very rapidly in liquid cultures. The genus *Bacillus* includes several bacterial species such as *B. subtilis, B. cereus, B. amyloliquefaciens, B. pumilus, B. mycoides, B. pastueri* and *B. sphaericus* which suppress development of diseases affecting a wide range of crop plants. The antagonistic activity of *Bacillus* spp. against crop pathogens may be due to antibiosis, competition for nutrients and space, hyperparasitism and induced systemic resistance to diseases. Antibiosis is the mechanism of biocontrol activity of most *Bacillus* spp. and in many cases the precise mechanism of biocontrol activity of bacterial biocontrol agents remains unclear. *Bacillus* spp. produce dormant spores that are resistant to desiccation, heat, UV irradiation and organic solvents. The ability to produce the endospores and various antibiotics makes the *Bacillus* spp. to be attractive candidates suitable for formulation and commercialization. Many commercial products containing *Bacillus* spp. as the active ingredient have been launched.

5.1.2.1 Metabolites-Mediated Antagonism

Antibiotics

Most of the spore-forming members of *Bacillus* spp. produce antibiotics that are low molecular weight peptides produced via the non-ribosomal biosynthetic pathway which involves specific enzymes known as peptide synthetases. The peptides exhibit a broad-spectrum of biological activities including antifungal, antibacterial, antiviral and antitumoral activities (Emmert et al. 2004). The metabolites with antimicrobial properties may be produced via ribosomal biosynthetic pathway also (Zuber et al. 1993). *B. subtilis* was reported to synthesize about 60 different types of antibiotics, many of which have antifungal properties of the compounds belonging to the iturin family (Phae and Shoda 1991). Detection of antibiotic

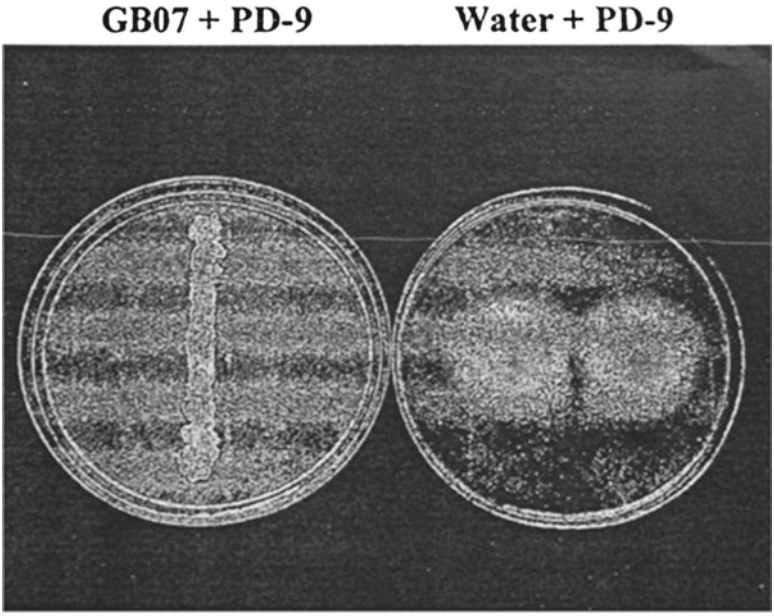

Fig. 5.5 In vitro assessment of antibiotic activity of *Bacillus subtilis* GB07 against *Penicillium digitatum*, causative agent of green mold disease of orange (Courtesy of Zhang and Dou 2002 and with kind permission of Florida State Horticulture Society, Florida, USA)

production by the bacterial isolates is important in determining their biocontrol potential in suppressing the plant pathogens and the diseases caused by them. Various biochemical and molecular methods have been employed to detect and characterize the secondary metabolites produced by *Bacillus* spp.

Cultural Methods

The antibiotic activity of *Bacillus subtilis* strain GB07 against the post- harvest pathogen *Penicillium digitatum,* causing green mold of oranges was assessed in vitro. The strain GB07 was streaked on potato dextrose agar (PDA) medium at the center of the plate. After 2 days of incubation, aliquots of 10 µl of the pathogen conidial suspension (10^6 spores/ml) were dispensed on either side of the bacterial streak at a distance of 2 cm. Suitable control plates streaked with sterile water and inoculated with pathogen were maintained. The antibiotics secreted by the strain GB07 strongly suppressed the growth of *P. digitatum,* as there was no visual mycelial growth (Fig. 5.5) (Zhang and Dou 2002). The antagonistic activity of *Bacillus* spp., filtered culture filtrates and selected secondary metabolites was assessed in vitro against *Xanthomonas campestris* pv. *campestris* (*Xcc*) causal agent of cabbage black rot disease. All three species *B. amyloliquefaciens, B. subtilis* and *B. pumilus* were found to be efficient metabolic producers, when growing in half-strength trypticase soy broth (TSB) or in cabbage broth (CB). Generally TSB stimulated

Table 5.6 Production of secondary metabolites by three species of *Bacillus* in tryptic soy broth and cabbage broth at 30 °C (Wulff et al. 2002)

	Bacillus spp.		
Secondary metabolite matches	*B. amyloliquefaciens*	*B. subtilis*	*B. pumilus*
Surfactin	12/18[a]	17/19	13/14
Iturin	11/18	2/19	–
Bacillomycin	9/18	1/19	–
Azalomycin F	15/18	–	–
Amphomycin	1/18	1/19	10/14
Acivicin	3/18	5/19	2/14
Anthrobactin	8/18	14/19	8/14
Rhodotorola acid	7/18	5/19	–
Valinomycin	3/18	1/19	12/14
Stenothricin	1/18	2/19	5/14
Colistin	–	–	3/14
Enterochelin	1/18	–	1/14
Nocardamin	2/18	–	–

[a]Number of isolates producing the designated antibiotic/total number of isolates tested
–Absence of detectable amounts of the designated antibiotic

greater production of metabolites compared with control. *B. amyloliquefaciens* isolates produced surfactin, iturin, bacillomycin and/or azalomycin F, while *B. subtilis* isolates were mostly able to synthesize surfactin and arthrobactin. The culture filtrates of *B. pumilus* isolates contained surfactin, amphomycin, arthrobactin and valinomycin. All three species produced surfactin in the cultures (Table 5.6) (Wulff et al. 2002). *B. subtilis* strains GB-017 and GB-0356 isolated from the soil were able to inhibit the mycelial growth of the fungal pathogens *Botrytis cinerea*, *Fusarium* sp., *Pythium* sp. and *Rhizoctonia solani.* The disc diffusion method was employed to assess the antifungal activity of the bacterial strains. The antagonistic activity was maintained up to pH 9.0 and remained stable at 80 °C for 1 h. The antifungal compounds were purified using ion-exchange and adsorption columns and the exhibited characteristics corresponded to polyenes and lactones (Kim et al. 2003).

The potential of antibiotic production by *Bacillus subtilis* effective against soybean seed pathogenic fungi was assessed. *B. subtilis* strain PRBS-1 (from soybean rhizosphere) and AP-3 (reference strain from rice rhizosphere) were compared for their efficacy in their antifungal and growth promotion activities. Both strains inhibited the mycelial growth of the fungal pathogens *Rhizoctonia solani*, *Colletotrichum truncatum, Sclerotinia sclerotiorum, Macrophomina phaseolina* and *Phomopsis* sp. pathogenic to soybean. The antibiotic effect of compounds purified from the culture filtrates appeared to be similar to the compounds of iturin group. The growth promotion effect of the strain PRBS-1 was at least partially related to the production of indole-acetic acid. The results indicated that the biocontrol potential and growth-promoting activity of *B. subtilis* strain PRBS-1 may be useful for effective management of soybean seed pathogenic fungi (Araujo et al. 2005). *B. amyloliquefaciens* strain Bg-C31 produced an antimicrobial substance which was identified as a protein with resistance to heat and protease K activity. The antagonistic gene was located

in the chromosome by plasmid curing. A 29-kDa protein encoded by the *LC1* gene was expressed. The antimicrobial activity of the fusion protein to *Ralstonia solanacearum* was detected. The antibacterial protein showed the potential for suppressing the Capsicum bacterial wilt disease and the pathogen causing the disease (Hu et al. 2010).

The ability of *Bacillus subtilis* to inhibit the mycelial growth of *Fusarium verticillioides* causing Fusarium head blight disease of wheat and to inhibit accumulation of fumonisin B1 was assessed in vitro. All strains (10) of *B. subtilis* significantly inhibited the pathogen growth, the strain CE1 exhibiting greatest antifungal activity. The strains CE1 and 86 only were able to reduce the mycotoxin production by 50 and 29 % respectively. The strain CE1 was the only strain capable of inhibiting the growth of and toxin production by *F. verticillioides*. The antagonistic activity of the strain CE1 was assessed in the greenhouse to find a link with the results obtained in vitro. There was significant antibiotic activity (60 %) and reduction of *F. verticillioides* population (40–50 % in CFU) in the in vitro assays. A similar biocontrol efficacy was observed also in the greenhouse experiments which showed inhibition of colonization reaching high values (98.55–99.86 %) (Cavaglieri et al. 2005). *Bacillus subtilis* strain EIR-j, an endophyte isolated from wheat roots showed high antifungal activity against *Gaeumannomyces graminis* var. *tritici* (*Ggt*), causative agent of wheat take-all disease. Soil drenches with bacterial cell densities of 10^6, 10^9 and 10^{12} CFU/ml reduced the infection by *Ggt* in wheat seedlings by 62.6, 68.6 and 70.7 % respectively at 4 weeks after sowing. Treatment with the strain EIR-j increased the growth and yield parameters, compared to untreated wheat plants and *Ggt*-inoculated plants. EIR-j treatment alleviated the deleterious effects of take-all on grain parameters to an extent similar to that of fungicide Triadimefon. Observations with scanning electron microscope (SEM) showed that in the presence of the bacterial strain, hyphae of *Ggt* showed leakage, appeared ruptured, swollen and shriveled. Examination under transmission electron microscope (TEM) revealed that cells of EIR-j were present in the root tissues of wheat seedlings and effectively retarded infection and colonization of *Ggt* in root tissue. Disintegration of pathogen hyphal cytoplasm occurred, following the suppressive activity of the BCA strain. In addition, formation of wall appositions of papillae, representing morphological defense reactions triggered by the bacterial strain could also be observed in the treated root tissues of wheat inoculated with the pathogen (Liu et al. 2009a, b).

The biocontrol potential of two bacterial BCAs *Bacillus subtilis* (MAB) and *Paenibacillus polymyxa* (MAP) obtained as two commercial products was assessed against *Phytophthora capsici*, causal organism of pepper (chilli) Phytophthora blight disease. *B. subtilis* inhibited the mycelial growth of *P. capsici.* On the other hand, *P. polymyxa* inhibited formation of zoosporangium by *P. capsici.* Release of zoospores from zoosporangium was significantly inhibited by both BCAs. The results indicated that the secondary metabolites of these BCAs target the pathogen at different stages of its life cycle (Kim et al. 2010). *Bacillus subtilis* CMB32 isolated from soil, was able to inhibit the mycelial growth of *Colletotrichum gloeosporioides*, causative agent of anthracnose diseases. In addition, this strain was antagonistic also to other fungal pathogens such as *Fusarium solani, Botrytis*

cinerea, F. oxysporum, Rhizoctonia solani and *Phytophthora capsici*. The strain CMB2 produced antifungal biosurfactant lipopeptides iturin A, fengycin and surfactin A which were detected by MALD1-TOF mass spectrophotometry. Iturins and fengycins exhibited powerful antifungal activity and growth inhibition of several fungal pathogens. Surfactins were not toxic by themselves, but sustain some synergistic effect on the antifungal activity of iturin A (Pyoung II et al. 2010).

Strains of *Bacillus subtilis* produce cyclic lipopeptides which constitute the iturin family which are powerful antifungal agents. *B. subtilis* strain KS1 was isolated from grape berry skin and it was identified as a new strain based on the morphological, biochemical and genetic analyses. The strain KS1 suppressed the mycelial growth of the gray mold pathogen *Botrytis cinerea* and grape ripe rot pathogen *Colletotrichum gloeosporioides.* Under field conditions KS1 reduced the grape downy mildew caused by *Plasmopara viticola* on berry skins and leaves. The KS1 genome had *ituD* and *Ipa-14* genes which were shown to have a role in the biosynthesis of iturin A. The mutants lacking both the genes lost the antagonistic activity of the parent strain KS1 against *B. cinerea* and *C. gloeosporioides* and lacked the ability to produce iturin A. The results suggested that the antagonistic activity of KS1 against grapevine fungal pathogens might depend on the ability to produce iturin A. The strain KS1 was found to be tolerant to various pesticides. Hence, the application of chemicals, either prior to or after treatment with KS1, can be included as a component of integrated disease management system for grapevine diseases (Furuya et al. 2011). The isolate SB10 of *Bacillus* spp. reduced the incidence of Phytophthora blight disease of pepper caused by *P. capsici* by 72.2 %. The antifungal compounds produced by SB10 were identified as lipopeptide complex, namely surfactins, iturins and fengycins, by employing Matrix-assisted Laser Desorption Ionization-Time of Flight (MASDI-TF) mass spectrometry technique (Oh et al. 2011).

Bacillus amyloliquefaciens strain RC-2 was able to effectively suppress the development of mulberry anthracnose disease caused by *Colletotrichum dematium*. The culture filtrate (CF) of the strain RC-2 suppressed the appearance of anthracnose lesions significantly, only when applied prior to inoculation of cabbage with *C. dematium,* but not after pathogen inoculation. This suggested that the antifungal compounds secreted by the strain RC-2 had only a preventive effect on the disease. Addition of peptone significantly enhanced the production of antifungal compounds by the bacterial strain. Observations under the scanning electron microscope (SEM) showed that the conidial germination was entirely inhibited on mulberry leaves pretreated with undiluted CF of RC-2. The CF could also inhibit the growth of other plant pathogens such as *Pyricularia oryzae. Xanthomonas campestris* pv. *campestris* and *Agrobacterium tumefaciens.* Different kinds of antifungal compounds were isolated by high performance liquid chromatography (HPLC) analysis. Iturin A2, a cyclic peptide was one among the antifungal compounds identified by nuclear magnetic resonance and fast atom bombardment mass analysis (Yoshida et al. 2001). *Bacillus amyloliquefaciens* strain MET0908 isolated from soil was found to be effective suppressing the development of *Colletotrichum lagenarium,* causing anthracnose disease infecting leaves, stems and fruits of watermelon. The strain

MET0908 produced antifungal compounds in cocultures, but not when grown in pure cultures. The supernatant of the coculture with *C. lagenarium* inhibited the mycelial growth of the pathogen. An antifungal protein was purified by 30 % ammonium sulfate saturation and it was concentrated using Centricon 10, DEAE- Sepharose™ Fast Flow column and Sephacryl S-100 gel filtration chromatography. The purified protein with a MW 40-kDa inhibited the growth of several other plant pathogens like *Fusarium graminearum, Colletotrichum gloeosporioides, Pythium ultimum, Phytophthora capsici, Didymella bryoniae* and *Monosporascus cannonballus*. The BCA strain secreted an extracellular β-1,3-glucanase that acted on fungal cell walls. Confocal image analysis microscopy showed that the antifungal protein was embedded in the septa of the hyphal wall of *C. lagenarium.* Scanning electron microscopy revealed abnormal swelling, degradation and burst by the excretion of lytic enzymes of the BCA. The mode of action of the antifungal protein was by the disruption of the pathogen cell wall. The antifungal proteins secreted by *B. amyloliquefaciens* strain MET 0908 showed no significant homology with any known proteins (Kim and Chung 2004).

Bacillus amyloliquefaciens strain DGA 14 isolated from the surface of banana fruits was able to inhibit the mycelial growth of the fungal pathogens *Thielaviopsis paradoxa, Colletotrichum musae*, *Fusarium verticillioides,* and *Lasiodiplodia theobromae* which collectively cause the postharvest crown rot disease of banana. The strain DGA 14 produced a diffusible metabolite that inhibited all fungal pathogens tested in culture. The bacterial strain moved and attached to fungal pathogens significantly suppressing mycelial growth and inhibited conidial germination in liquid medium. In addition, the bacterial strain parasitized the fungal pathogens effectively. DGA14 survived and colonized banana fruits after 2 days. The incidence of crown rot disease of banana was significantly reduced by the application of the strain DGA 14 which was more effective than the fungicide used to treat the banana fruits in the packing house (Alvindia and Natsuaki 2009). The endophytic bacteria *Bacillus amyloliquefaciens* isolated from poplar (*Poplus* spp.) trees showed antimicrobial activity against fungal and bacterial pathogens with variable efficacy. The strain PEBA 20 effectively reduced infection by *Botryosphaeria dothidea,* causing canker disease in poplar. Aberrant hyphae were observed after the treatment of *B. dothidea* with bacterial suspension or fermentation filtrates. The hyphal aberrations increased with treatment duration, exhibiting bead-like appearance and cluster-like structures at the hyphal tips. Cut shoots dipped in bacterial suspension (10 CFU/ml) for 30 min had reduced incidence of canker (60 %), compared to controls (100 %). Delay in canker development and reduction in lesion size were also recorded in treated shoots. As *B. amyloliquefaciens* is known to produce a range of antifungal dipeptides or cyclic peptides, the antagonistic activity of the strain PEBA 20 could be due to its ability to secrete antifungal metabolites capable of inducing abnormalities of pathogen hyphae, resulting in reduction in canker incidence in cut shoots, following treatment with bacterial suspension/culture filtrates (Yin et al. 2011).

Bacillus licheniformis strain P40 inhibited the development of *Erwinia carotovora* causing soft rot disease in stored potatoes. The strain P40 produced a novel

bacteriocin-like substance (BLS) which exhibited bactericidal effect on *E. carotovora* cells at 30 µg/ml. The effect of treatment of pathogen cells with BLS was assessed using transmission electron microscope (TEM). The BLS-treated cells showed wrinkled bacterial surfaces and shrinkage of whole bacterial cell, indicating plasmolysis. Treatment of potato tubers with BLS, at a concentration of 240 µg/ml or higher, substantially reduced the symptoms of soft rot and the symptom development was completely arrested at a concentration of 3.7 mg/ml of BLS. The results showed the effectiveness of the strain P40 and its metabolite for control of potato soft rot disease (Cladera-Olivera et al. 2006). The endophytic *Bacillus vallismortis* ZZ185 isolated from healthy stems of Broadleaf Holly (*Ilex latifolia*) showed strong antagonistic activity against *Fusarium graminearum, Alternaria alternata, Rhizoctonia solani, Cryphonectria parasitica* and *Phytophthora capsici* in vitro, when exposed to the culture filtrate and n-butanol extract of this strain. The antifungal activity of the culture filtrate (CF) was significantly correlated with cell growth of the BCA strain. The secondary metabolite of the BCA was relatively heat stable with more than 50 % of the antifungal activity of the CF being retained after exposure to a temperature of 121 °C for 30 min. The antifungal activity of the CF against the mycelial growth of *A. alternata* and *F. graminearum* remained almost unaltered, even after exposure to a range of pH from 1 to 8. The antifungal compounds were purified from the n-butanol extract of the CF and they were identified as a mixture of bacillomycin D (*n*-C14) and bacillomycin D (iso-15). This bacterial strain ZZ185 of *B. vallismortis* appears to have potential for the management of fungal diseases of several crops (Zhao et al. 2010).

Biochemical and Physiological Methods

The antibiotic producers of *Bacillus* isolates were identified and grouped by applying fatty acid methyl ester (FAME) analysis. The fatty acid methyl esters were extracted from each isolate using standard procedures for gas chromatographic (GC) FAME analysis. The isolates *B. amyloliquefaciens* were identified by FAME analysis. These isolates produced surfactin, iturin, bacillomycin and/or azalomycin. Their ability to inhibit *Xanthomonas campestris* pv. *campestris* (*Xcc)* causal agent of cabbage black rot disease, varied considerably from weak to a very strong effect. However, no general relationship was observed between in vitro inhibition and biocontrol effect. Isolates identified by FAME analysis as *B. pumilus* were molecularly very heterogeneous. The isolates varied substantially in their ability to inhibit *Xcc* in vitro and their biocontrol activity in vivo was not related to the in vitro growth inhibition effect on the pathogen (Wulff et al. 2002). *B. luciferensis* strain KJ2 C12 isolated from pepper roots, was subjected to FAME analysis. The FAME profiles of this strain were composed mainly of anteiso 15:0 and iso IS:0 which were similar to that of *B. marinus* (similarity = 0.535 %). The identity of this BCA was, however, established based on the similarity of 16S rDNA sequences. *B. luciferensis* effectively protected pepper plants against Phytophthora blight disease caused by *Phytophthora capsici* (Kim et al. 2009).

Molecular Techniques

Cultural methods are time-consuming and labor-intensive in providing results. On the other hand, molecular methods may be useful for rapid identification of antibiotic-producing strains of bacteria. The strains of *Bacillus* obtained from soils naturally suppressive to plant diseases and other substrates could be screened using polymerase chain reaction (PCR)-based methods. *Bacillus subtilis* and four strains of *Bacillus* sp. were screened by PCR amplification, using degenerate primers corresponding to peptide synthetase genes. Four isolates of *Bacillus* sp. gave positive amplification signal and three isolates exhibited inhibitory activity against the mycelial growth of *Sclerotinia sclerotiorum*, indicating that the PCR- based techniques could be employed for rapid selection of antibiotic-producing strains of *Bacillus* spp. (Giacomodonato et al. 2001). Suppressive substrative hybridization (SSH) was employed to identify genetic markers associated with biological control activity of by *B. subtilis* against microbial plant pathogens. Sixteen subtracted fragments with high degree of similarity to sequences present in several strains of *B. subtilis* with known biocontrol potential were selected. Oligonucleotide primers specific to nine of these genes were designed. The targeted genes included five genes involved in antibiotic synthesis (*bmyB, fenD, ituC, srfAA* and *srfAB*) and four additional genes. All nine markers were amplified from three commercialized *B. subtilis* strains with the exception of *ituC*. The strains positive for amplifiable markers generally were more effective in inhibiting the growth of *Rhizoctonia solani* and *Pythium ultimum.* The presence of amplifiable markers in the isolates of *Bacillus* spp. may be used as a basis of selecting the isolates rapidly (Joshi and McSpadden Gardener 2006).

Screening of potential strains of *Bacillus* spp. with antibiotic and biocontrol activities for the presence of specific antibiotic-encoding sequences by employing molecular methods forms a rapid approach in comparison with traditional method of selection. The presence of antibiotic biosynthetic genes for the antibiotics surfactin, iturin A, bacillomycin D, fengycin, mycosubtilin, and zwittermicin A by specific PCR using 21 *Bacillus* spp. which showed >50 % mycelial inhibition or leaf/stem infection reduction. MALDI-TOF-MS method is an easy and less time-consuming method for confirming the production of antibiotics, compared with other available procedures. The PCR products amplified from all strains using the primer pair SUR3F/3R showed a 441-bp band corresponding to the surfactin antibiotic biosynthetic gene. The antibiotic biosynthetic gene for iturin A was detected in all strains except the strain 3039, using the primer pair ITUDIF/IR. The presence of bacillomycin D biosynthetic genes could be detected only in strains BS6, 3059 and 4079. Two primer pairs had to be employed for the detection of zwittermycin A in the strains BS6 and BS8. The strains possessing the multiple genes BS6, BS8, H-08-02, S-07-01, 3057 and 4079 along with other positive strains were subjected to MALDI-TOF-MS analysis. The antibiotics produced by four strains are presented in Table 5.7. Production of antibiotics by some strains could not be recognized by MALDI-TOF-MS, even in the presence of relevant genes were detected by PCR assay. All isolates of *B. subtilis, B. mycoides, B. amyloliquefaciens, B. licheniformis* and *B. cereus/thuringiensis* were positive for

Table 5.7 Production of antibiotics by *Bacillus* spp. as determined by MALDI-TOF-MS analysis (Athukorala et al. 2009)

Bacterial BCA/strain	Antibiotics produced
Bacillus amyloliquefaciens BS6	Surfactins, bacillomycin D, fengycins
B. mycoides 4079	Surfactins, bacillomycin D, iturin A,, fengycin
B. subtilis 3057	Surfactins, bacillomycin D, fengycin
B. subtilis H-08-02	Surfactins, iturin A, fengycin

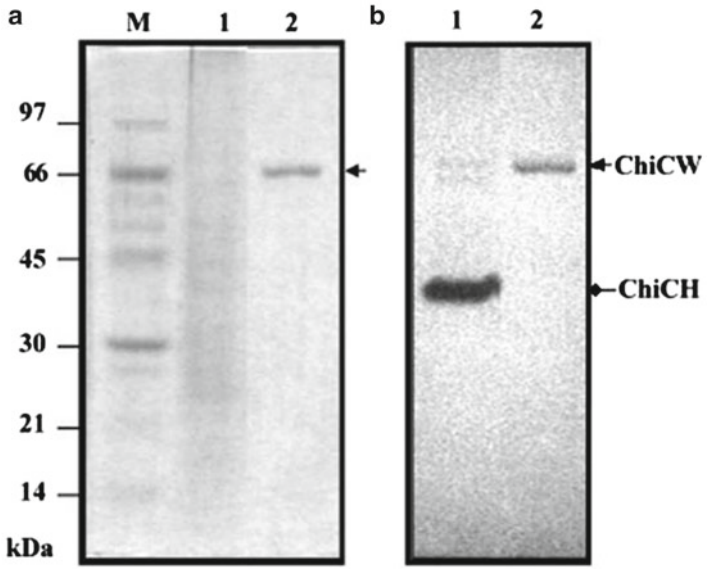

Fig. 5.6 Separation of two chitinase ChiCW and ChiCH secreted by *Bacillus cereus* 28–9 using SDS-PAGE technique. (**a**): gel stained with Coomassie Brilliant Blue; (**b**): gel stained with Calcoflour White M2R; *M* low molecular weight protein standard (Courtesy of Huang et al. 2005 and with kind permission of Journal of Biochemistry and Molecular Biology)

surfactin and iturin A. The presence of biosynthetic genes for and production of fengycin and surfactin by *B. mycoides* has been reported for the first time (Athukorala et al. 2009).

Hydrolytic Enzymes

Various species and strains of *Bacillus* have been demonstrated to produce antibiotics and/or enzymes in the cultures either naturally or stimulated in the presence of required substrates. *B. cereus* has been shown to be a reliable BCA of soybean Phytophthora damping-off and root rot disease. The cotton root rot disease caused by *Rhizoctonia solani* was suppressed by an endophytic strain of *B. cereus* (Pleban et al. 1997; Emmert et al. 2004). *B. cereus* 28–9 exhibited high biocontrol activity against Botrytis leaf blight of lily. This bacterial strain produced at least two chitinases ChiCW and ChiCH which could be extracted from the culture filtrates (Fig. 5.6).

Table 5.8 Effect of purified preparation of ChiCW on the conidial germination of *Botrytis elliptica* (Huang et al. 2005)

Activity of ChiCW (μU)[a]	Inhibition rate of conidial germination (%)
28.0[a]	84 ± 1
14.0	78 ± 3
7.0	29 ± 8
3.5	20 ± 2
0.0	0

[a]A mixture of 4 μl of purified ChiCW at different concentrations and 4 μl of conidial suspension of *B. elliptica* (10^5 conidia/ml) incubated for 12 h prior to microscopic determination of conidial germination

The purified enzymes inhibited the conidial germination of *Botrytis elliptica.* The conidia of *B. elliptica* incubated with ChiCW became enlarged and formation of germ tubes was retarded (Huang et al. 2005). In a later study, a chitinase-secreting *B. cereus* strain CH2 was isolated from the rhizosphere of eggplant. Based on the activity and purification, using SDS-PAGE technique, the enzyme was identified as a 15.0-kDa chitinase. On glass slides, germination of the fungal spores was effectively suppressed by the bacterial suspension, supernatant from the suspension, and 0.005 % solution of chitinase extracted from the strain CH2. The chitinase required pH 7.1 and 40 °C for its optimal activity. In greenhouse assays, the severity of Verticillium wilt disease of eggplant was reduced by 69.69, 54.04 and 53.13 % respectively by cell suspension, supernatant and diluted enzyme preparation (0.01 %). This strain CH2 appeared to have good commercial potential for the control of eggplant Verticillium wilt (Table 5.8) (Li et al. 2008a, b).

Various kinds of ion-exchange and adsorption chromatography were employed for estimating antifungal activity of *B. subtilis* against *Botrytis cinerea* and other fungal pathogens. Thin layer chromatography (TLC) procedure was used to separate the antifungal compounds which had properties corresponding to polyene and lactone (Kim et al. 2003). *B. amyloliquefaciens* effective against *Colletotrichum lagenarium* causing anthracnose disease of watermelon produced an antifungal compound in the cultures grown in potato dextrose broth. The cell-free filtrates were concentrated by centrifugation. Solid ammonium sulfate was used to precipitate proteinaceous substances. DEAE-Sepharose™ Fast Flow Column was used for further purification by employing sodium dodecylsulpahte (SDS)- polyacrylamide gel electrophoresis (PAGE) method. A single band with a molecular weight of about 40-kDa showed antifungal activity against *C. lagenarium* and other plant pathogens such as *F. graminearum, Pythium ultimum* and *Phytophthora capsici*. The purified antifungal protein was identified as β-1-3-glucanase. *B. amyloliquefaciens* strains MET 0908 exhibited strong activity against *C. lagenarium* (Kim and Chung 2004). *Bacillus subtilis* NSRS 89–24 has been shown to be effective against two important fungal pathogens, causing rice blast disease (*Magnaporthe grisea*) and rice sheath blight disease (*Rhizoctonia solani*). The

bacterial strain inhibited the growth of *M. grisea* and *R. solani* in vitro. NSRS 89–24 produced a heat stable antibiotic and labile enzyme β-1,3-glucanase. The glucanase activity in the culture medium of NSRS 89–24 strain was inducible in the presence of chitin (0.3 %), reaching the maximum activity at 5 days after incubation. The targets for the antifungal compounds were in the cell wall of fungi at each hyphal apex which is composed of chitin, β-glucans and other oligosaccharide compounds. The β-glucanase activity of *B. subtilis* NSRS 89–24 might have an important role in the degradation of pathogen cell wall. The extracellular β-1,3-glucanase produced by NSRS 89–24 was purified and the molecular mass was estimated. Both purified enzyme and the antibiotic could separately inhibit the growth of *M. grisea* and *R. solani*. The results indicated that glucanase and antibiotic could act in concert, exhibiting, strong synergistic effect on both rice pathogens. Production of these bioactive compounds in vivo has to be demonstrated (Leelasuphakul et al. 2006).

Bacillus cereus strain UW85 produced two antibiotics, zwittermicin A and kanosamine in its culture supernatant. Zwittermicin A is a water soluble, acid-stable, linear aminopolyol molecule with broad-spectrum activity against numerous fungal and bacterial pathogens (He et al. 1994). Later *Bacillus* spp. have been shown to produce lytic enzymes such as chitinases, proteases or glucanases that may have a role in their biocontrol activities against plant pathogens. *B. cereus* strain AU 004 isolated from soil samples was evaluated for its ability to produce hydrolytic enzymes. This bacterial strain secreted complex of hydrolytic enzymes such as chitinase, chitosanase and protease, when grown in a medium containing chitosan flakes of marine waste. The culture supernatant significantly inhibited the growth of *Fusarium oxysporum*, *F. solani* and *P. ultimum*. The protease from the culture supernatant was purified by sequential chromatography and characterized as a neutral protease with MW 28.8-kDa, and optimal pH and temperature for protease activity at 7 and 50 °C respectively. The purified protease inhibited both spore formation and the hyphal development of *P. ultimum* in vitro, indicating the role of the enzyme in the biocontrol activity of *B. cereus* AV004 against the fungal pathogen. Isolation of a protease from *Bacillus* spp. appears to have been achieved for the first time in this investigation (Chang et al. 2009).

Bacillus subtilis strain CHU26 isolated from potato field exhibited strong extracellular chitinase activity on the colloidal chitin-containing agar plate. The strain CHU 26 strongly inhibited the mycelial growth of *Rhizoctonia solani*. The gene encoding chitinase (*chi18*) was cloned from the constructed *B. subtilis* CHU26 genomic DNA library. The *chi18* gene consisted of an ORF of 1,791 nucleotides and encoded 595 amino acids with a MW of 64-kDa. The amino acid sequence of the chitinase gene showed 62 and 81 % similarity to those from *B. circulans* WL-12 and *B. licheniformis* respectively. *Escherichia coli* was transformed with *chi18* gene and the transformant exhibited chitinase activity on colloidal chitin agar plate as the CHU26 strain. The transformant reduced infection of radish seedlings by *R. solani* by more than 90 %, indicating the role of chitinase as an important mechanism in the biocontrol activity of *B. subtilis* strain CHU26 (Yang et al. 2009). *Bacillus pumilus*

strain SG2 effective against the fungal pathogens *Fusarium graminearum* and *Bipolaris sorokiniana*, produced two different chitinases in the presence of colloidal chitin. The chitinases inhibited the mycelial growth of *F. graminearum* and caused abortion of hyphal elongation of *B. sorokiniana.* In contrast, expression of chitinases by the strain SG2 was repressed, when glucose was used as the carbon source and the antifungal activity of *B. pumilus* strain SG2 was consequently abolished. The hyphal inhibition of *F. graminearum* and *B. sorokiniana* by the bacterial strain remained stable for a minimum period of 14 days. These results confirmed that expression of the *B. pumilus* SG2 chitinases was under the control of two types of regulation, special regulation of chitin and global regulation by glucose. The results revealed that activity of chitinases was the principal mechanism of the biocontrol activity of *B. pumilus* SG2 against the wheat pathogens (Shali et al. 2010).

5.1.2.2 Prevention of Colonization of Plant Tissues by Pathogens

The principal mechanism of biocontrol activity of *Bacillus* spp. against plant pathogens may be through production of antibiotics and/or hydrolytic enzymes that act adversely on the pathogens directly. Rhizosphere colonization by *Bacillus* spp. was reported to suppress root disease in cotton (Mahaffe and Backman 1993). Root infection by *Fusarium verticillioides* results in systemic invasion of maize plants, leading to the accumulation of the mycotoxin, fumonisins in maize grains and products. The influence of *B. subtilis* CE1 on native *F. verticillioides* colonization at different inoculum concentrations and maize root levels was investigated. Bacterization of maize seeds with CE1 strain at 10^6–10^8 cells/ml inoculum concentration inhibited *F. verticillioides* counts at the rhizosphere levels, whereas all bacterial treatments reduced the fungal CFUs at the endorhizosphere level. The highest bacterial concentration provided the maximum level of inhibition percentages of *F. verticillioides* infection of maize roots. The results indicated the ability of *B. subtilis* CE1 to reduce rhizosphere and endorhizosphere colonization by the pathogen *F. verticillioides,* when the strain CE1 was applied as seed inoculants (Cavaglieri et al. 2005). The ability of four *Bacillus* spp. isolated from vegetable crops such as sugar beet, tomato and potato was assessed for colonizing cocoa (*Theobroma cacao*) seedlings and reducing the severity of black pod rot disease caused by *Phytophthora capsici*. The bacterial strains exhibited differential ability to colonize cocoa leaves. The strains BT8 and BP24 of *B. cereus* were able to establish long-term colonization, whereas the strains BacJ (of *B. mycoides)* and 203–7 (of *B. pumilus*) could colonize the cocoa leaves for short periods, their populations declining at different rates. Total foliar colonization in plants treated with BT8 and BP24 was relatively at high levels. In the leaf disk bioassay, only the strain BT8 was able to suppress the expansion of lesions induced by *P. capsici*, although the strain BP24 could colonize the disk tissues. Foliar colonization of leaves by these strains was primarily epiphytic with limited endophytic invasion. Suppression of *Phytophthora* by BT8 occurred in non-colonized plants. The results suggested that

systemic resistance might be induced in other plant parts by colonization of leaves of cocoa (Melnick et al. 2008).

Bacillus subtilis strain EXWB1 isolated from healthy melon (*Cucumis melo* L.) fruits has been demonstrated to be a unique mechanism of biocontrol activity against *Alternaria alternata,* infecting melon fruits during transport and storage. The droplet of the strain EXWB1, when placed on melon skin, spread as a thin film on the hydrophobic fruit surface, indicating that the bacterial strain established effective attachment to the fruit surface. Further the rate of EXWB1 colony extension on melon skin was 125 μm/h, suggesting that the bacterial strain could rapidly occupy the same niche as the fungal pathogen. Production of biosurfactant was found to be useful for attachment of the bacterial cell to the fruit surface. The hyphae of *A. alternata* could not grow on melon skin or wounds inoculated with EXWB1 cell suspension and no rotting of the fruit flesh in fruits treated with bacterial cell suspension could be seen. Ethylene is known to accelerate fruit senescence. Treatment of melon fruits with EXWB1 cell suspension delayed ethylene production by 2 days and the amount of ethylene produced was also reduced. In addition, the respiration of melon fruits treated with EXWB1 cells suspension was suppressed, when challenged with the pathogen inoculation. EXWB1 inoculation was beneficial in maintaining the fruit firmness which was drastically affected by *A. alternata* in untreated control fruit. As this bacterial strain existed on healthy fruit surfaces, the potential of EXWB1 could be advantageously exploited for protecting melon fruits under storage conditions (Wang et al. 2010).

5.1.2.3 Induction of Resistance to Crop Diseases

Elicitation of induced systemic resistance (ISR) to diseases infecting various crops by strains of *Bacillus* has been demonstrated in greenhouse or field trials on cucumber, muskmelon, watermelon, tomato, bell pepper, sugar beet, and tobacco. Different species of *Bacillus, B. subtilis*, *B. amyloliquefaciens*, *B. cereus, B. pumilus, B. mycoides, B. pastuerii* and *B. sphaericus* have been evaluated for their ability to elicit ISR resulting in significant reduction in the incidence and/or severity of diseases on a wide range of host plant species. Elicitation of ISR by *Bacillus* spp. was associated with ultrastructural changes in plants and cytochemical alterations during pathogen attack. Activation of some of the same pathways as in *Pseudomonas* spp. and some additional pathways has been demonstrated in different pathosystems. ISR elicited by several strains of *Bacillus* spp. was found to be independent of salicylic acid (SA), but dependent on jasmonic acid (JA), ethylene (ET) and the regulatory gene *NPR1*. Nevertheless, other strains induced ISR dependent on SA and independent of JA and *NPR1*. Further, ISR induced by *Pseudomonas* spp. does not result in accumulation of *PR1* gene in plants. On the other hand, ISR induced by *Bacillus* spp. in some cases led to accumulation of *PR1* gene in treated plants. Promotion of plant growth by treatment with *Bacillus* spp., in addition to disease suppression, has been found to be an added advantage (Kloepper et al. 2004).

In the roots of pea bacterized with *Bacillus pumilus* strain SE34, challenged with *Fusarium oxysporum* f.sp. *pisi* (*Fop*), colonization of *Fop* was restricted to endodermis and paratracheal parenchyma cells and radiated towards the vascular stele. The restriction of pathogen in SE34-treated plants was due to strengthening of the epidermal and cortical cell walls. In addition, cell wall appositions with large amounts of callose and infiltrated with phenolic compounds were also present in the root tissues of bacterized pea plants (Benhamou et al. 1996). This bacterial strain induced systemic resistance in tomato plants against *F. oxysporum* f.sp. *lycopersici* (*Fol*) resulting in reduction in severity of typical symptoms and number of brown lesions formed on lateral roots of treated tomato plants. By using gold-complexed β-1,3-glucanase assay, higher amounts of β-1,3-glucans in the root tissue were detected, when plants were treated with strain SE34 and chtiosan, compared with individual treatments and untreated control plants (Benhamou et al. 1998). In tobacco-blue mold (*Peronospora tabacina*) infected tobacco plants treated with *Bacillus pumilus* strain SE34, significantly increase in the levels of salicylic acid (SA) at 1 day post-inoculation with *P. tabacina* was observed (Zhang et al. 2000).

A detached leaf and microtiter plate bioassays were developed to assess elicitation of ISR by bacterial BCAs strains. Application of *Bacillus pasteuri* C-9 and *B. pumilus* SE34 and T4 as soil drenches to three tobacco cultivars reduced significantly the mean percentage of leaf area with lesions induced by *Peronospora tabacina*, in addition to reduction in the sporulation of the pathogen. Disease severity was substantially reduced by strains SE34 and T4, but not by strain C-9, as determined by detached leaf and microplate assays (Zhang et al. 2002). In a later study, the efficacy of modes of BCA application, seed treatment and/or soil drench in inducing ISR was determined. The strains SE34 and C-9 significantly enhanced the growth of tobacco as seed treatment. ISR was elicited only by C-9, but not by SE34 or T4. As seed treatment the T-4 had no effect either on growth enhancement or ISR induction in plants. However, when the bacterial strains were applied as seed treatment followed by soil drenches, all strains promoted the plant growth. When the interval between the last application of bacteria and challenge inoculation with *P. tabacina* was 6 weeks, all bacterial strains could induce ISR. The results suggested that plant growth promotion and elicitation of ISR by tested bacterial strains may be linked with each other (Zhang et al. 2004).

Induction of systemic resistance can be demonstrated through challenge assays in which distal, untreated leaves are challenged with a pathogen, following a short priming period with an inducing on a primary, spatially separated leaf or root system (Conrath et al. 2002). *Bacillus mycoides* strain Bac J reduced the severity of Cercospora leaf spot disease of sugar beet caused by *Cercospora beticola.* The leaf was sprayed with BacJ strain was bagged. The plant was challenge-inoculated by spraying the conidial suspension of *C. beticola* (10^8 CFU/ml) after bacterial treatment of bagged leaf. The disease severity was significantly reduced on both highly susceptible and moderately resistant sugar beet varieties (Bargabus et al. 2002). The ability of *Bacillus mycoides* isolate BmJ and *B. mojavensis* isolate 203–7 in suppressing the development of cucumber anthracnose disease caused by *Glomerella*

cingulata var. *orbiculare* by inducing systemic acquired resistance (SAR) in treated plants. The isolates BmJ and 203–7 delayed disease incidence and reduced total and live conidial production per mm^2 of lesion area significantly relative to the untreated control plants. Cucumber apoplastic proteins were assayed at 6 days after induction. The isolates BmJ and 203–7 enhanced β-1,3-glucanase activities by 135 and 72 % respectively. The isolate 203–7 also increased peroxidase activity by 79 % over control treatment. In the field trials, the isolate BmJ applied 1 week before inoculation reduced significantly the AUDPC (=0.05) in cucumber, compared to water control in 1 year (2004) and on cantaloupe for both years (2004 and 2005). The disease severity was reduced by 41 % in cucumber and >20 % in cantaloupe levels equal to that obtained by using fungicides azoxystrobin and chlorothalonil (Neher et al. 2009).

In a later study, treatment of sugar beet leaves with live BacJ strain or avirulent strain of *Erwinia carotovora* pv. *betavasculorum* (*Ecb*) resulted in significant control of leaf spot disease. In addition, this treatment resulted in a two-fold increase in chitinase- and β-glucanase-specific activity, indicative of systemic resistance induction. Hypersensitive cell death was induced by avirulent *Ecb*, but not by BacJ strain. An oxidative burst elicited by spray application of BacJ strain under both light and green light conditions was not dependent on the stomata for entry into the sugar beet tissues. The BacJ strain elicited an oxidative burst in sugar beet similar in timing, but not in intensity to that elicited during incompatible interaction. The oxidative response was observed, only following live BacJ strain cell treatment, a requirement for effective disease control as well (Bargabus et al. 2003). *Bacillus cereus* selected from 500 rhizobacteria isolated from soil, rhizosphere and rhizoplane of healthy tomato plants, was found to be effective against foliar fungal and bacterial pathogens infecting tomato. After the removal of bacterial cells by centrifugation, the supernatant was dialyzed repeatedly. The dialysates, when applied to the roots, protected the tomato plants against diseases caused by fungi and bacteria. The results indicated that the macromolecules secreted by *B. cereus* could act as elicitors of systemic resistance in treated tomato plants against diseases (Romeiro et al. 2005).

The efficacy of *Bacillus amyloliquefaciens* IN937a, *B. subtilis* GB03 and a mixture of these two strains in reducing the severity of cucumber angular leaf spot disease due to *Pseudomonas syringae* pv. *lachrymans* was assessed. Seed treatment with bacterial BCAs reduced severity of disease and it increased plant growth also significantly by inducing systemic resistance (Raupach and Kloepper 2000). *Bacillus pumilus* strain SE34 incorporated into the potting medium provided systemic protection to tomato against the late blight disease caused by *Phytophthora infestans*. The chemical inducer of disease resistance DL-β-amino-*n*-butyric acid (BABA) was applied as a foliar spray to one half of the nonbacterized plants as a positive control at 4 weeks after seeding. The plants were challenge-inoculated with the pathogen after 1 week. The disease severity was expressed as the percentage of leaves covered with late blight lesions. Treatment with strain SE34, significantly reduced the severity of late blight disease. In addition, the plant height and overall plant weight were increased in plants treated with the strain SE34 compared with untreated control plants (Yan et al. 2002, 2003). *Bacillus amyloliquefaciens* strain

BS6 and *Pseudomonas chlororaphis* strain PA-23 were evaluated for their potential in reducing the incidence of stem rot disease of canola under greenhouse and field conditions caused by *Sclerotinia sclerotiorum*. The effectiveness of these BCA strains in controlling the canola stem rot disease was in equivalence to the fungicide Rovral Flo ® (iprodione). The suppression of stem rot disease development by the BCA strains was achieved by reducing canola flower petal infection by *S. sclerotiorum* through both direct antimicrobial action and/or induction of plant defense enzymes (Fernando et al. 2007).

The mechanism of biocontrol activity of *Bacillus pumilus* 7 km isolated from wheat rhizosphere against *Gaeumannomyces graminis* var. *tritici* causing take-all disease, was studied. The soil was drenched with bacterial cell suspension and changes in the defense-related enzymes and total phenolics contents were determined. Disease severity was significantly reduced in the bacterized roots of wheat plants and plant growth was promoted markedly due to treatment. The activities of soluble peroxidase (SPOX), ionically cell wash bound peroxidase (CWPOX), β-1,3-glucanase, β-1,4-glucanase, were increased in plants treated with *B. pumilus.* In addition, the phenolic contents of treated plants also showed higher levels, compared with that of untreated control plants. The enzyme activities reached the peak at 4–8 days after application of BCA to the wheat roots. The disease suppressive activity of *B. pumilus* might be linked to its ability to stimulate the plant's defense systems operating in the root system locally or systemically (Sari et al. 2007). The ability of *Bacillus subtilis* strains S2BC-1 and GIBC-Jamog was assessed as biocontrol agents against tomato wilt disease caused by *Fusarium oxysporum* f.sp. *lycopersici*. Seed bacterization and soil application of the mixture of these two strains reduced the wilt disease incidence significantly as determined by localized and split-root-experiments. Enhanced activities of chitinase and β-1,3-glucanase was observed in root samples from plants treated with the bacterial strains. High intensity peroxidase isoforms could be detected in gels for samples from localized and ISR experiments. The results indicated the possibility of mechanisms of both direct antibiosis and induction of ISR operating in concert in tomato plants protected by the BCA strains (Shanmugam and Kanoujia 2011).

The secondary metabolites secreted by *Bacillus* spp. were evaluated for their ability to induce ISR in treated plants/organs. *Bacillus* spp. produce different lipopeptide antibiotics. Treatment of potato tuber cells with purified fengycins resulted in the accumulation of phenolic compounds involved in or derived from phenylpropanoid metabolism (Ongena et al. 2005b). The purified surfactin of *B. subtilis* S499 was able to induce systemic resistance against *Botrytis cinerea* in bean and tomato plants. In tomato cells, activation of key enzymes of lipoxygenase pathway appeared to be activated in resistant plants, following induction by surfactin-overproducing isolates (Ongena et al. 2007). Similar reaction was observed in canola plants receiving double application of *Pseudomonas chlororaphis* strain PA23 on canola petals. The activity of chitinase and β-1,3-glucanse was enhanced resulting in the suppression of *Sclerotinia sclerotiorum* (Fernando et al. 2007). Blackleg disease caused by *Leptosphaeria maculans* (anamorph: *Phoma lingam*) is another economically important disease of canola. *Bacillus cereus* strain DFE4 and

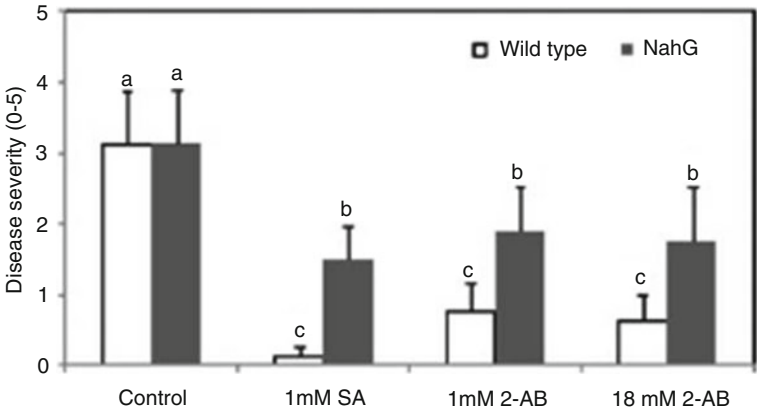

Fig. 5.7 Induction of systemic resistance in wild type and NahG transgenic tobacco in wild type and NahG transgenic tobacco by 2-AB from *Bacillus* sp. BS107 against *Pectobacterium carotovorum* subsp. *carotovorum* SSC1 2-AB was applied at 1.0 and 18.0 mM on 3-week old Xanthi nc tobacco and NahG transgenic tobacco seedlings. *Different letters on bars* indicate significant differences between treatments according to Fisher's protected LSD test at P = 0.05 (Courtesy of Yang et al. 2010 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

B. amyloliquefaciens strain DFE16 produced the lipopeptide antibiotics iturin A, bacillomycin D and surfactin. The results indicated that the direct antifungal activity of the antibiotics was the most dominant of black leg disease control by the bacterial BCAs. However, low but significant disease suppression from induced resistance also occurred (Ramarathnam et al. 2011).

Bacillus sp. strain BS107 was able to elicit ISR against soft rot disease of tobacco caused by *Pectobacterium carotovorum* subsp. *carotovorum* (*Pcc*). A determinant of ISR secreted by the strain BS107 was isolated from the cell-free culture supernatant and identified by mass spectrometry and NMR analyses as 2-aminobenzoic acid (2-AB) as a principal ISA determinant. 2-AB displayed effective ISR activity against soft rot disease development on the tobacco leaves. Treatment of tobacco roots with the 2-AB exhibited protective effects against *Pcc*. No inhibition of the pathogen occurred at the concentration of 2-AB that induced resistance. Reverse transcription (RT)-PCR assays of tobacco leaves of plants treated with 2-AB on the roots, showed up-regulation of the induced resistance marker genes such as *PR1a PR1c, PR2* and *PR4*. 2-AB is biosynthesized from chorismic acid which is a precursor for salicylic acid (SA). It is well known that SA has a vital role in mediating plant defenses. Salicylic acid treatment at 1.0 mM significantly reduced tobacco soft rot disease severity as 0.75. In contrast, it lost the capacity, when NahG plants were treated. Application of 2-AB at 1.0 and 18.0 mM into NahG plants resulted in significant increase in the symptom severity to 2.5- and 2.8-fold respectively than that was observed in wild type plants (Fig. 5.7). The results indicated that 2-AB protected systemically NahG tobacco against *Pcc* and 2-AB-elicited ISR required SA signaling. Colonization of BS107 on plant root system could play a role in

promoting plant defenses, as it secreted bacterial determinants including 2-AB for protecting plants against challenge by diverse pathogens (Yang et al. 2010).

Demonstration of induction of systemic resistance by root-colonizing bacteria to diseases caused by soilborne pathogens may be difficult, since the requirement of spatial separation of ISR-eliciting bacterium and the pathogen may not be satisfied in some pathosystems. Treatment of cucumber with *B. pumilus* strain INR7 was reported to induce ISR against cucurbit wilt disease caused by *Erwinia tracheiphila*. The number of wilted leaves per plant was reduced in INR7-treated cucumber plants challenged with inoculation of the pathogen by natural insect vectors, striped cucumber beetle (*Acalymma vittatum*) and spotted cucumber beetle (*Diabrotica undecimpunctata*). Elicitation of systemic resistance by the INR strain was observed under field conditions, where the bacterial strain was applied as seed treatment and soil drench (Zehnder et al. 1997). In a later study, bacterial strain INR7 was applied as seed treatment and drenches, while preparing transplants. The mean percentage of wilted vines was significantly reduced in plants treated with strain INR7 and insecticide, compared with untreated control plants. The bacterial treatment was not significantly different from the level of protection obtained from insecticide treatment (Zehnder et al. 2001).

Antibiotic production by bacterial BCAs has been shown earlier to be primarily responsible for suppression of several crop pathogens. But several strains of *Bacillus* spp. and *Pseudomonas* spp. have been shown to be capable of inducing systemic resistance to crop pathogens. *B. subtilis* strain BSCBE4 and *P. chlororaphis* PA23 were able to induce systemic resistance against *Pythium aphanidermatum* in hot pepper (chilli) and also to promote the growth of treated plants. Defense-related enzymes phenylalanine ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO) were stimulated, in addition to accumulation phelolic compounds. Incidence of damping-off was significantly reduced due to treatment with the bacterial BCA strains (Nakkeeran et al. 2006). *B. subtilis* M4 was able to stimulate a systemic response in cucumber and tomato leading to protection against *Colletotrichum lagenarium*, causing anthracnose disease of cucumber and *Pythium aphanidermatum*, inducing damping-off disease of tomato seedlings. The pathogen and M4 strain were spatially separated from each other, thus excluding the possibility of antibiotic secreted by the bacteria having a role in disease control. Treatment of plant roots with M4 strain was found to induce systemic molecular modifications. The RNA expression profiles in control and treated plant leaves were compared using the cDNA-AFLP technique. Several AFLP fragments corresponded to genes, not expressed in control plants treated with M4 strain. The differential accumulation of mRNA indicated the plant reaction following perception of M4 strain. Evidence for the specific modulation of gene expression by *B. subtilis* strain in tomato and cucumber triggering plant defense machinery was established in this investigation (Ongena et al. 2005a).

The biocontrol potential of strains (20) of *Bacillus* spp. for suppression of development of *Penicillium digitatum*, causing the citrus green mold disease was assessed. Volatile compounds of 9 of 20 strains tested, inhibited the mycelial growth of the pathogen by more than 80 %. The ethanol extract from *B. subtilis* 155 cell-free

supernatant containing secondary metabolites (SMs) exhibited maximum inhibitory effect on mycelial growth and spore germination of *P. digitatum* with EC_{50} values of 77.26 and 82.10 µg/l respectively. The protein separated from the SMs by ammonium sulfate precipitation had an EC_{50} of 288 µg/l. The antifungal protein activity was associated with lowest band obtained after polyacrylamide gel electrophoresis (PAGE) procedure. Inoculation with 20 µl of a 10^8 CFU/ml of *B. subtilis* endospore suspension at 24 h, prior to fungal spore inoculation, decreased infection of *P. digitatum* by 86.7 % and production of disease symptoms was delayed by 6 days and decay symptoms by 9 days. Addition of SMs solution (10 µg/ml) simultaneously with the pathogen, decreased disease incidence by 72.5 % and symptom appearance was delayed by 5 days after inoculation. No sign of decay could be seen up to 9 days. The results indicated that antifungal compounds produced by *B. subtilis* 155 might have an important role in suppressing the growth of the green mold pathogen infecting citrus fruits (Leelasuphakul et al. 2008).

The ability of *Bacillus cereus* strain CIL to induce systemic resistance in *Lilium formosanum,* when applied as soil drench against leaf blight disease caused by *Botrytis elliptica*, was assessed. Protection provided by the bacterial strain was for about 10 days against the leaf blight disease. *B. cereus* strain CIL was assayed for ISR-eliciting activity and root colonization trait. A 40 % reduction in disease severity could be achieved, when the bacterial strain suspension was applied to the rhizosphere. The bacterial strain could effectively colonize the roots of lily, maintaining a population of over 10^4 CFU/cm at 10 days after application. The expression of *LfGRP1* and *LsGRP1* genes encoding glycine-rich protein associated with *L. formosanum* and oriental lily cv. Stargazer respectively was analyzed. *LsGRP1* was found to be an SA-dependent pathogen-inducible gene. *LfGRP1* expression showed that it could be induced by the pathogen. Both *LfGRP1* and *LsGRP1* transcripts decreased after the application of the BCA strain. The expression of *LfGRP1* and *LsGRP1* were extensively suppressed, when *B. elliptica* was inoculated on CIL-treated lily plants. The negative regulation was considered to be controlled by a signal transduction pathway induced by *B. cereus* strain CIL that differed from the pathogen-induced SA-directed pathway (Liu et al. 2008a, b).

The physiological responses of lily cv. Star Gazer with induced systemic resistance, triggered by *Bacillus cereus* CIL against *Botrytis elliptica* were studied by using histological and biochemical analyses. Leaves inoculated with *B. elliptica* displayed cell death, H_2O_2 accumulation and lignin deposition. In plants treated with *B. cereus,* cell death and H_2O_2 accumulation and lignin deposition in leaves caused by *B. elliptica* infection were suppressed, indicating that suppression of oxidative burst might be associated with *B. cereus* C12-induced systemic resistance. In reactive oxygen species inhibitors assays, *B. elliptica*-induced lesion numbers and H_2O_2 accumulation in lily leaves were significantly reduced in leaves pretreated with catalase or diphenylene iodonium. In addition, the expression of *LsGRP1* and *LsPsbR* in leaves, elicited with *B. cereus* CIL and inoculated with *B. elliptica,* was decreased. Similar expression pattern was also observed in leaves pretreated with catalase or diphenylene iodonium and inoculated with *B. elliptica.* The results suggested that *B. cereus*

CIL-induced systemic resistance might be related to suppression or alleviation of oxidative stress and cell death of lily caused by *B. elliptica* (Huang et al. 2012).

Bacillus vallismortis strain EXTN-1 isolated from red pepper was able to effectively suppress tomato bacterial wilt disease caused by *Ralstonia solanacearum*. *Bacillus subtilis* strain 816–6, *B. pumilus* strain 228–7, *Bacillus* sp. strain 113–3 and *Paenibacillus polymyxa* strain H32-5 were also evaluated along with strain EXTN-1 for their efficacy against *R. solanacearum*. The tomato seedlings roots were bacterized with the bacterial strains and the tomato plants were grown in perlite-hydroponic system. Upon challenge inoculation with the pathogen, all bacterial strains suppressed the development of bacterial wilt disease. *B. vallismortis* strain EXTN-1 was the most effective, in reducing the infection to 65 % as against 95 % infection in untreated control plants. The movement of the pathogen from the site of inoculation was hampered in plants treated with the strain EXTN-1. As the strain EXTN-1 did not have any direct antagonistic activity against *R. solanacearum*, the suppression of disease development was probably achieved by a mechanism other than antibiosis. The strain EXTN-1 produced an efficient elicitor for inducing systemic resistance in many crops (Park et al. 2007). The bacterial BCA mixture of strains containing *Bacillus atrophnius* S2BC-2 and *Burkholderia cepacia* TEPF-Sungal protected the gladiolus plants against *Fusarium oxysporum* f.sp. *gladioli* causing vascular wilt and corm rot disease to the maximum extent. The protection by these strains was attributed to induction of systemic resistance as deduced from the stimulation of PR-proteins and activities of PO, PPO and PAL in addition to phytoalexins and/or chalcone synthase (Shanmugam et al. 2011). *Bacillus pumilus* strain INR-7 was able to reduce the incidence of pearl millet downy mildew disease caused by *Sclerospora graminicola* by inducing systemic resistance in treated seedlings. Resistance induced by the strain INR-7 was associated with the expression of hypersensitive response (HR), enhanced lignifications, callose deposition and hydrogen peroxide as well as increase in expression of defense-related enzymes such as β,1,3-glucanase, chitinase, phenylalnine ammonia lyase (PAL), peroxidase (PO) and polyphenol oxidase (PPO). Microscopical examination of inoculated pearl millet tissues revealed accumulation of lignin, callose and hydrogen peroxide earlier and to higher levels in resistant and induced resistant seedlings. Tissue print analysis revealed localization of defense- related enzymes in the vascular bundles and difference in the expression pattern of β-1,3-glucanase, chitinase, PAL, PO and PPO in genetically resistant, INR-7-treated and susceptible pearl millet seedlings (Raj et al. 2012).

The ability of *Bacillus cereus* AR156 to provide systemic protection to tomato against bacterial speck disease caused by *Pseudomonas syringae* pv. *tomato* DC 3000 and root knot caused by *Meloidogyne incognita* and to promote plant growth was assessed. The strain AR156 population reached 10^5–10^6 CFU/g in the rhizosphere of tomato plants for more than 2 months. The bacterial BCA increased the average biomass of tomato plants by 47.7 %. AR156 elicited induced systemic resistance against DC3000, resulting in reduction in disease severity by 1.6 fold and inhibition of pathogen proliferation by approximately 15-fold. DC 3000 strain

triggered accumulation of defence-related genes (*PR1* and *PIN2*) in tomato leaves and primed the leaves for accelerated defense-related gene expression upon challenge with DC 3000. The results suggested that simultaneous activation of salicylic acid (SA) and jasmonic acid dependent signaling pathways by AR156 against DC 3000. The bacterial strain AR 156 was able to form robust colonies in the roots of tomato and exerted same beneficial effects, including suppression of development of bacterial speak disease via ISR and plant growth promotion (Niu et al. 2012).

Plant growth-promoting rhizobacteria (PGPR) have been demonstrated to enhance the levels of resistance to several fungal and bacterial diseases infecting plants. The ability of PGPR to induce resistance to virus diseases of plants has been assessed in some pathosystems. PGPR-mediated induced resistance against *Tobacco necrosis virus* (TNV) and *Cucumber mosaic virus* (CMV) was demonstrated earlier (Maurhofer et al. 1994; Raupach et al. 1996). The potential of *Bacillus* spp. strains to induce systemic resistance to *Cucumber mosaic virus* (CMV) was tested by treating the seeds of cucumber and tomato with single strains of bacteria under greenhouse conditions. Treatment with bacterial strains significantly reduced the area under disease progress curve, when the cotyledons of plants were inoculated mechanically with CMV. There was delayed symptom development in some of the treated plants (Zehnder et al. 2000). The ability of *Bacillus* spp. to reduce the incidence and severity of disease caused by *Tomato mottle virus* (ToMV) transmitted by whiteflies was assessed, by employing combination of bacterial strains under field conditions, when high populations of whitefly vectors were available. The presence and titre of ToMV was determined by quantitative estimation of ToMV DNA. Treatment with bacterial strains resulted, in lower percentage of infected plants and larger yields, compared with non-bacterized control plants (Murphy et al. 2000). The efficacy of combination of *Bacillus subtilis* GB03 plus any one of the strain of *Bacillus pumilus* (SE34, INR7, T4), *B. amyloliquefaciens* (IN 937a) and *B. subtilis* (IN937b) formulated with the carrier chtiosan was assessed in enhancing resistance to CMV infection and growth of tomato. Tomato plants treated with the biopreparations, each of which contained two bacterial strains, appearing similar to the untreated controls which were 10 days older, were challenged with CMV. The CMV disease severity ratings were significantly lower and treated plants had better growth than the control plants of same age at 14 and 28 days post-inoculation (dpi). Accumulation of CMV in young noninocualted leaves was much less in all biopreparation-treated plants except the plants treated with the combination of T4 strain, as revealed by the virus concentration assessment by enzyme-linked immunosorbent assay (ELISA). The protection provided by biopreparation appeared to have resulted from the enhanced growth of tomato plants, thereby allowing them to respond to inoculation with CMV in a manner similar to that of more mature plants exhibiting adult plant resistance, a phenomenon frequently observed in different virus-host combinations (Murphy et al. 2003).

Several isolates of bacteria obtained from plant samples were screened for their antiviral activity against *Tobacco mosaic virus* (TMV). The isolate ZH14 identified as *Bacillus cereus* secreted antiviral compounds which inhibited virus infection by 94.2 %. The isolate ZH14 had the ability to degrade the ribonucleic acid. The antiviral product of ZH14 has an extracellular protein with high molecular mass with

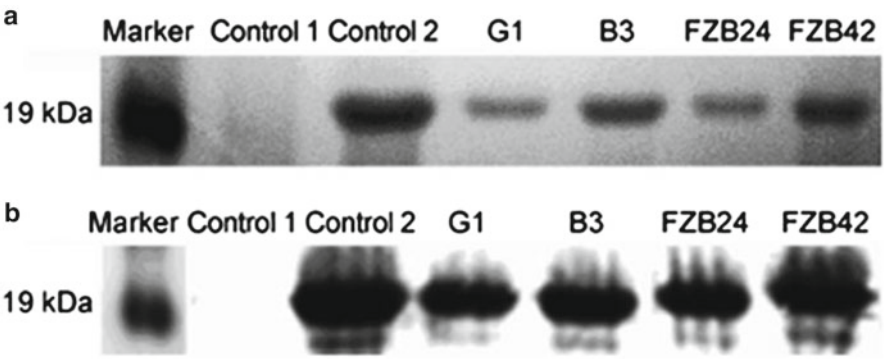

Fig. 5.8 Detection of *Tobacco mosaic virus (TMV)* coat protein (*CP*) in tobacco treated with different PGPR strains at 28 days post-inoculation (*dpi*). (**a**): SDS-PAGE analysis; (**b**): Western blot analysis. *Control 1*: tobacco without TMV inoculation; *Control 2*: tobacco inoculated with TMV and treated with water G1, B3, FZB24 and FZB42 represent tobacco treated with bacterial strains and inoculated with TMV (Courtesy of Wang et al. 2009 and with kind permission of Korean Society for Microbiology and Biotechnology, South Korea)

temperature optima between 15 and 60 °C and pH optimum of 6–10. The results indicated that the BCA strain had the potential to remain antagonistic to TMV at a wide range of temperature and pH conditions (Zhou et al. 2008). *Bacillus subtilis* strain G1 and B3 and *B. amyloliquefaciens* strains FZB24 and FZB42 were evaluated for their potential for inducing systemic resistance to *Tobacco mosaic virus* (TMV) and improving plant growth in tobacco plants under greenhouse conditions through application of the bacterial strains as seed treatment. The bacterial strains significantly reduced disease severity induced by TMV, as indicated by visible symptoms and reduced virus accumulation as revealed by ELISA tests. The amount of coat protein (CP) (20-kDA) was significantly lower in plants treated with *Bacillus* strains (Fig. 5.8). The plants treated with G1 and F2B24 strains contained lower levels of TMV-CP than that was present in B3- and FZB42-treated plants. The *NPR1* gene is a regulator in the expression of various sets of defense genes involved in the SAR and ISR resistance pathways. The gene *Coi1* positively regulates the JA-dependent ISR pathway. An RT-PCR analysis of the signaling regulatory genes *Coi1* and *NPR1* and defense genes *PR-1a,* and *PR-1b* in treated tobacco leaves revealed an association with enhancement of systemic resistance of tobacco to TMV. The results of this investigation provided evidence indicating the disease protection to tobacco by *Bacillus* strain G1 was based on plant-mediated ISR. In addition, *Bacillus* strains stimulated the growth of tobacco plants substantially that could be recognized both in the roots and leaves of tobacco. Thus treatment of tobacco with *Bacillus* strains was able to enhance the systemic resistance to TMV and to promote the growth of tobacco as well (Wang et al. 2009).

The efficacy of *Bacillus pumilus* strain EN16 and *B. subtilis* strain SW1 in inducing systemic resistance in tobacco against *Tobacco mosaic virus* (TMV) was assessed under greenhouse conditions. Treatment with strain EN16 and SW1 significantly reduced the intensity of mosaic symptoms and disease severity, resulting in 52 and 71 % protection at 14 days after inoculation respectively. Virus titer in tobacco was

determined by employing the enzyme-linked immunosorbent assay (ELISA). The bacterial BCA-treated plants showed decrease in TMV concentration. A period of 5 and 7 days interval between inducer treatment and challenge inoculation with TMV was required for induction of resistance respectively by EN16 and SW1. The activities of phenylalanine ammonia lyase (PAL), peroxidase, polyphenol oxidase and pathogenesis-related (PR) proteins were assessed in tobacco plants treated with BCA strains and inoculated with TMV. The amounts of defense-related enzymes and PR-proteins significantly increased in *Bacillus*-treated plants challenged with pathogen, when compared with control (Lian et al. 2011). *Bacillus subtilis* strains are able to induce resistance to virus diseases of crops and to promote growth of the treated plants. The gene encoding the $HPaG_{xooc}$ protein from the rice pathogenic bacterium *Xanthomonas oryzae* pv. *oryzicola* which elicited hypersensitive cell death in non-host plants, thereby inducing resistance to diseases and pests. The strain OKB105 of *B. subtilis* was transformed with the gene encoding $HpaG_{xooc}$ protein to know, if there would an additive effect due to the presence of the bacterial gene. The greenhouse experiments showed that the transformant OKBHF was more effective in enhancing plant growth of tomato plants and in lowering the disease severity of *Cucumber mosaic virus* at 28 days post-inoculation (dpi). Plant height, fresh weight and flower and fruit number were greatly increased. The reverse transcription (RT)-PCR analysis revealed the molecular mechanisms of $HPaG_{xooc}$ and *B. subtilis* in tomato plants. The results suggested their synergistic roles in inducing enhanced expression of three expansin genes LeEXP2, LeEXP5 and LeEXP18 which could regulate plant cell growth and two defense-related genes *Pti4* and *Pti6* which could activate expression of a wide array of PR genes and one defense gene PR-1a (Wang et al. 2011).

5.1.2.4 Factors Influencing the Biocontrol Activity

Environmental factors have definite influence on the host plant, bacterial pathogen and the biocontrol agent expected to suppress the development of the pathogen and the disease caused. *Bacillus* spp. are known to produce endospores that are resistant to adverse environmental conditions and these spores remain alive for several years facilitating perpetuation and persistence of these bacteria in the soil and other substrates. The biocontrol agents, in most cases, are confined to the plants/soils on which they are applied. Rarely they are able to spread to other plants in the same field. Natural agencies like wind and insects may aid the biocontrol agents to spread to other plants or locations. Honey bees are important natural pollinators of commercial blueberries in the United States. But they are also the vector of the fungal pathogen *Monilinia vaccinii-corymbosi,* causing mummy berry disease. *Bacillus subtilis* was effective in suppressing flower infection by *M. vaccinii-corymbosi*. Individual honey bees could carry $5.1 \times 6.4 \times 10^5$ CFU of *B. subtilis*, when exiting hive-mounted dispensers with Serenade. On caged rabbiteye blue berry bushes in the field, population densities of *B. subtilis* vectored by honey bees reached a carrying capacity of $<10^3$ CFU per flower stigma within 2 days of exposure. There was a

highly significant non-linear relationship between *B. subtilis* populations per stigma. The bee activity was expressed as number of legitimate flower visits per time interval per cage. The results suggested that the use of a hive-dispersed biocontrol product as a supplement during pollination might reduce the risk of mummy berry disease (Dedej et al. 2004).

The ability of two pollinators *Apis mellifera* and mason bee *Osmia cornuata* was assessed as carriers of the BCAs from flower to flower (secondary colonization on apple cv. Golden Delicious). *Bacillus subtilis* strain BD170 (commercial product Biopro®) was developed for the control of fire blight disease caused by *Erwinia amylovora.* The behavior and capacity of the insects to deposit the BCA in the most receptive flower parts were compared both by washing, diluting and plating the flower organs on a recovery medium and also by applying PCR assays, based on a molecular marker. *O. cornuata* was efficient than *A. mellifera.* Under field conditions, the efficacy of pollinators in carrying the BCA from sprayed flowers to the stigmas of newly opened ones at different intervals after the spray application of the BCA product was determined, by detecting the BCA using PCR assays. The percentages of positive PCR flower samples were higher in the internal treated areas of the fields, compared with external untreated ones. However, the high colonization level found in the external untreated areas and in the flowers opened in both areas at several days after the treatment, indicated that the pollinators could play an important role as secondary carriers (Maccagnani et al. 2009).

Strains of *Bacillus* spp. have been shown to exhibit a desirable trait of eliciting ISR in different plant species against several microbial plant pathogens (Jetiyanon and Kloepper 2002). The individual *B. amyloliquefaciens* strain IN937a, and compatible mixtures of strains of *B. pumilus* strains 937b, SE34, SE49, T4 and INR7 were evaluated for their ability to elicit ISR against Southern blight disease *Sclerotium rolfsii* in tomato, anthracanose disease (*Colletotrichum gloeosporioides*) in long Cayenne pepper (*Capsicum annuum* pv. *acuminatum*) and cucumber mosaic disease (*Cucumber mosaic virus*) in cucumber. The strain mixtures could suppress more consistently both disease incidence and/or severity in two seasons, when compared to individual strain IN937a. Mixture IN937a+IN937b elicited ISR effectively against all diseases under field conditions. Cumulative marketable yields were also increased by some mixtures of BCA strains (Jetiyanon et al. 2003).

5.1.3 Paenibacillus *spp.*

The genus *Paenibacillus*, earlier included under the genus *Bacillus* comprises about 80 species differentiated on the basis of 16S rRNA analysis. They are facultatively aerobic, endospore-forming and low G+C Gram-positive bacilli (Ash et al. 1994; Raza et al. 2008). Several strains of *P. polymyxa* have been reported to effectively suppress the development of plant diseases such as seedling blight, wilt and root rot diseases of cucumber and water-melon caused by *Fusarium oxysporum* and *Pythium* spp. (Dijksterhuis et al. 1999; Yang et al. 2004) and sesame damping-off caused by

Pythium spp. (Ryu et al. 2006). The strains of *Paenibacillus* may have different mechanisms of biocontrol activity against fungal pathogens, causing different economically important plant diseases. In addition to the role as biological control agents of plant diseases, *Paenibacillus* spp. are able to fix atmospheric nitrogen in plant rhizosphere, improving the soil fertility (von der Weid et al. 2002).

5.1.3.1 Antibiosis

Paenibacillus lentimorbus isolate CBCA-2 isolated from pistachio leaves showed significant biocontrol potential against *Botryosphaeria dothidea,* causing pistachio panicle and shoot blight disease. The isolate CBCA-2 inhibited the germination of pycnidiospore entirely after 24 h of incubation at 25 °C. Malformation of pycnidiospores and hyphae, in addition to lysis of the pycnidiospores, was observed. Nutrient yeast dextrose broth induced the secretion of antifungal compounds to the maximum extent, among the five media tested. Development of the lesions on detached leaves was suppressed by application of culture filtrates (CF) of CBCA-2, while the washed bacterial cells did not show any inhibitory effect on lesion development. Application of CF on excised dormant stems, prior to challenge inoculation by the pathogen, was effective in arresting lesion development. Pruning wounds could be effectively protected by spraying a suspension of CBCA-2, prior to inoculation with the pycnidiospore suspension of *B. dothidea* (Chen et al. 2003). Foliar application of *P. lentimorbus* B-30488 reduced the incidence of tomato early blight disease caused by *Alternaria solani* by 45.3 %, compared with control. The bacterial strain reduced the radial growth of *A. solani* in dual culture. Observations under scanning electron microscope (SEM) revealed complete degradation of pathogen hyphae on co-culture with the strain B-30488, indicating direct inhibitory effects of the bacterial BCA and *A. solani*. Gene expression studies, using RT-PCR analyses, indicated that the defense and growth-related genes were up-regulated significantly in tomato plants treated with B-30488 foliar spray and *A. solani*-inoculated plants treated with B-30488 strain. Tomato plants inoculated with *A. solani* affected microbial community structure and population of rhizosphere, as compared with B-30488. The results indicated the multiple modes of action of *P. lentimorbus* B-30488 for its biocontrol activity against *A. solani* through degradation of pathogen cell wall, induction of resistance and possibly by competing for similar sources of nutrients, since the BCA and pathogen exhibited high similarity in nutrient utilization of carbon sources present in tomato tissues (Khan et al. 2012).

Production of fusaricidin-type antibiotics was observed in *Paenibacillus polymyxa* by Beaty and Jensen (2002). *P. polymyxa* 1460 secreted lectins that could enhance cellulose β-glucosidase activity in wheat root cell wall (Karpunia et al. 2003). *Paenibacillus polymyxa* has been reported to produce peptide antibiotics more commonly. *P. polymyxa* E681 produced polymyxin, fusaric acid and polyketides (Ryu et al. 2006). Polymyxin is the main type of peptide antibiotics produced by some strains of *P. polymyxa*, while other strains secreted different peptides including

polypeptins. Polymyxins A, B, C and D have been distinguished, based on the differences in amino acid or fatty acid composition (Orwa et al. 2002). Polymyxins have bactericidal effect on Gram-negative bacilli, especially *Pseudomonas*. Polymyxins alter cytoplasmic membrane permeability by binding to a negatively charged site in the lipopolysaccharide layer (Wiese et al. 1998).

The antagonistic activity of *Paenibacillus brasilensis* strain PB177 against *Fusarium moniliforme* and *Diplodia macrospora* infecting maize was investigated. The antifungal compounds produced by *P. brasilensis* inhibited the mycelial growth of these fungal pathogens and also *Rhizoctonia solani* and *Verticillium dahliae* capable of infecting a wide range of crops. The inhibitory effect of PB177 was stronger on *D. macrospora* than on *F. moniliforme*. Conidial structures of the fungal pathogens were not formed in the presence of PB177, indicating that cellular differentiation of both *D. macrospora* and *F. moniliforme* was inhibited. In the control without bacteria, conidia were produced abundantly (von der Weid et al. 2005). The mechanism of biocontrol activity of *Paenibacillus polymyxa* strains B2, B5 and B6 against *Pythium aphanidermatum* and *Phytophthora palmivora* was investigated using dual culture method on plates containing V8 medium (20 % v/v), consisting of 0.3 %, $CaCO_3$, 1.5 % nutrient agar and 0.003 % cholesterol (w/v, at 28 °C for 48 h in the dark and an additional 48 h under illumination). None of the strains of *P. polymyxa* inhibited the radial growth of *P. aphanidermatum*. In contrast, all strains drastically inhibited the radial growth of *P. palmivora.* However, *P. polymyxa* strains B2 and B5 were able to antagonize the zoospores of both *P. aphanidermatum* and *P. palmivora* around the roots of *Arabidopsis.* The strains B5 and B6 produced similar quantities of antagonistic substances, but the strain B5 was superior in the production of mycoidal substance and biofilm (Timmusk et al. 2009).

Strains of *Paenibacillus polymyxa*, in addition to antibiotics, are able to produce hydrolytic enzymes that play an important role in the biocontrol of plant diseases. *P. polymyxa* 72 produced an amylase (MW 48-kDa) composed of 1,161 amino acids. The gene encoding the biosynthesis of the amylase appeared to be divided into two segments by a direct-repeat sequence located almost at the center of the gene (Sakurai et al. 1989). Two *P. polymyxa* strains could produce cellulase and mananase independent of the presence of monosaccharide products in growth media. Such media-independent expression of enzymes for degradation of cellulose containing cell wall components indicated that these strains could be promising candidates for application as biocontrol agents against fungal pathogens (Nielson and Sørensen 1997). *Paenibacillus polymyxa* strain BRF-1 isolated from soybean rhizosphere was able to suppress the soybean root rot disease caused by a complex of *Fusarium oxysporum* var. *redolens, F. avenaceum, F. solani, Pythium ultimum, Rhizoctonia solani* and *Phytophthora* f.sp. *glycinea*. In a dual culture, the strain BRF-1 inhibited the growth of *R. solani* and *F. oxysporum* as well as other fungal pathogens. The strain BRF-1 produced antifungal peptide. The antifungal compound was separated by ammonium sulfate precipitation and purified by Sephadex G-50. The peptide had a MW of about 35.4-kDa with antifungal activity against *R. solani* (Chen et al. 2010).

5.1.3.2 Colonization of Plant Surfaces

Colonization of plant surfaces by bacterial biocontrol agents prior to initiation of infection by the plant pathogens in specific sites on plant organs is important for successful and effective prevention/inhibition of the development diseases. The importance of bacterial biofilm established on plant roots as a biocontrol mechanism has been emphasized by some investigations. *Paenibacillus polymyxa* could excrete its biofilm formation at the sites of infection by oomycetes as a niche exclusion approach. Microbial biofilms are constituted by both bacterial cells and extracellular matrix which may form upto 98 % of the biofilm (Sutherland 2001; Kolter 2005). Plant root exudates and root electrical signals selectively influence bacterial colonization and biofilm formation. Colonization rarely occurs as individual cells (Van Loon 2007). Efficient root colonization by certain PGPRs is linked to their ability to secrete a site-specific recombinase. Genetically unmodified *P. polymyxa* strains colonize plant root tips as a biofilm. The pattern of biofilm formation has been characterized. The biofilm contains almost infinite range of macromolecules produced by the bacteria.

Paenibacillus brasilensis strain PB177 was tagged with the *gfp* gene encoding the green fluorescent protein (GFP) in order to monitor interactions between PB177 and *Diplodia macrospora* and *Fusarium moniliforme* infecting maize. Bacterial colonization of plant surfaces such as roots and leaves occurs commonly in the form of aggregates or microcolonies. The seeds were treated with GFP-tagged bacteria prior to challenging them with the fungal pathogens on agar plates. Both GFP-tagged PB177 strain and fungal hyphae were detected along with maize root surface in most treated plants. The results indicated that the bacterial cells were mobilized to the maize roots in the presence of the fungal pathogens *D. macrospora* and *F. moniliforme*. The GFP-tagged *P. brasilensis* introduced into the soil could be detected as aggregates attached to plant roots from second day after planting, but colonization patterns were heterogenous and patchy. GFP-tagged PG177 cells introduced into the soil were not commonly observed inside the maize root tissue indicating that *P. brasilensis* could not be considered as an endophyte of maize plants (von der Weid et al. 2005). *Paenibacillus polymyxa* strains B2 and B5 formed biofilms that enlarged throughout 25 h of experimental duration. *P. polymyxa* was found to be effective in protecting the *Arabidopsis thaliana* model system against root colonization by zoospores of the oomycete pathogens *Pythium aphanidermatum* and *Phytophthora palmivora*. The zoospores of *P. palmivora* colonized plant roots in liquid assays and the presence of *P. polymyxa* prevented colonization prior to encystment and infection. The strains of *P. polymyxa* were able to antagonize the zoospores of *P. aphanidermatum* and *P. palmivora* around the *Arabidopsis* root. Most of plants treated with *P. polymyxa* survived the *P. aphanidermatum* inoculations in soil assays. The results indicated that biofilm formation and niche exclusion mechanisms do have a role in the biocontrol activity of bacterial biocontrol agents against plant pathogens (Timmusk et al. 2009).

5.1.3.3 Induction of Resistance to Crop Diseases

The plant growth promoting rhizobacteria (PGPR) *Paenibacillus alvei* strain K-165 was found to be effective against *Verticillium dahliae* causing wilt diseases of several solanaceous crops, including the model plant *Arabidopsis thaliana* (Tjamos et al. 2004). Strain K-165 significantly reduced the development of vascular symptoms in *A. thaliana.* The BCA and the pathogen were spatially separated and hence, antagonism by direct interaction could be ruled out. The strain K-165 induced systemic resistance in *A. thaliana* wild-type (wt) plants against *V. dahliae.* ISR triggered by strain K-165 differed from ISR induced by *P. fluorescens* WCS417r or CHA0. ISR induced by strain K165 was salicylic acid (SA)-dependent, while the resistance induced by *Pseudomonas* strains was entirely independent of SA. ISR induced by strain K-165 against *V. dahliae* was blocked in *Arabidopsis* mutants *sid1/eds 5, sid2* and *npr1-1,* indicating that components of pathway from isochorismate, most likely SA, as well as a functional NPR1 play a crucial role in the ISR induced by the strain K-165. In addition, the concomitant activation and increased transient accumulation of the PR-1, PR-2 and PR5 genes were discernible in the treatment in which both the BCA strain and the challenging pathogen were present in the rhizosphere of *A. thaliana* plants (Tjamos et al. 2005). The biocontrol potential of chitinase-producing *Paenibacillus illinoisensis* strain KJA-424 was assessed against *Phytophthora capsici* infecting pepper (chilli) plants. The growth response of pepper plants and kinetics of pathogenesis-related (PR) protein production were determined after challenge inoculation of *P. capsici* and combined inoculation with the pathogen and the bacterial strain. Root mortality of plants treated with the pathogen and BCA was significantly reduced, compared with pathogen inoculated plants. The activities of β-1,3-glucanase, cellulase and chitinase were significantly increased in plants inoculated with the pathogen and bacterial strain, compared with plants inoculated with the pathogen alone. The activities of these defense-related enzymes were negatively correlated with root mortality. The fresh weight of plants in this treatment was also increased indicating the growth promotion effect of the treatment with *P. illinoisensis* on pepper plants (Jung et al. 2005).

The mechanisms underlying the biocontrol activity of strains of *Paenibacillus polymyxa* MB02-1007 and chitosan against *Ralstonia solanacearum*, causative agent of bacterial wilt diseases of tomato, potato and a large number of plant species, were studied. Most of the strains (14 of 16) did not show any direct inhibitory effect on *R. solanacearum* in dual cultures. On the other hand, chitosan exhibited strong antibacterial activity in paper disk and agar well tests. In the greenhouse assays, chitosan was more effective as soil drench. The effects of treatments with the bacteria and/or chitosan on the enzymatic activities involved in the defense mechanisms operating in the plants were assessed. The chitinase activity was enhanced by both chitosan and *P. polymyxa,* irrespective of method of application (seed treatment/soil drenching) in the presence or absence of the pathogen. *P. polymyxa* increased the chitinase activity as a soil drench or seed treatment by 68 and 78 % respectively, while chitosan increased chitinase activity to a greater extent

(96 %) as soil drench. Changes in the β-1,3-glucanase activity induced by the bacteria and chitosan were less marked, compared with those of chitinase activity. In addition, *P. polymyxa* and chitosan improved the growth of treated tomato plants significantly, demonstrating the beneficial effects of the bacteria and organic compound (Algam et al. 2010).

The ability of lipopolypeptides (paenimyxin) secreted by *Paenibacillus* sp. strain B2 to elicit production of hydrogen peroxide (H_2O_2) and to activate defense-related genes in the model legume *Medicago truncatula* was assessed. A pathosystem between *M. truncatula* cell suspension and *Fusarium acuminatum* was established to investigate the process of induction of resistance to disease. Induction of H_2O_2 reached the peak at 20 min after elicitation with paenimyxin in plant cell cultures. Higher concentration of paenimyxin (>20 μM) inhibited the induction of H_2O_2. In plant roots, paenimyxin at a low concentration (1 μM) applied prior to inoculation with *F. acuminatum* showed protective effect and suppressed 95 % of necrotic symptoms. Inhibitory effect of paenimycxin was observed at higher concentration on plant growth. A semi-quantitative reverse transcription (RT)-PCR assay was applied to quantify the gene responses in *M. truncatula*. Genes involved in the biosynthesis of phytoalexins (enzymes phenylalanine ammonia lyase, chalcone synthase and chalcone reductase), antifungal activity (PR-proteins and chitinase) or cell wall (invertase) were highly upregulated in roots or cells after paenimyxin treatment (Selin et al. 2010).

5.1.4 Burkholderia *spp.*

Burkholderia cepacia as a plant pathogen was first reported on onion bulbs by Burkholder (1950). The bacterial species was later found to be a ubiquitous soil bacterial species and has emerged as an important biocontrol agent of plant pathogens. *Burkholderia cepacia* complex (*Bcc*) is naturally abundant in soil, water, on plant surfaces and in clinics. The *Bcc* consists of nine discrete genomic species and a genetic scheme based on the *recA* gene facilitated greatly the identification of *B. cepacia* complex species. This molecular approach was useful to differentiate the pathogenic and nonpathogenic human forms and nine current species within the genus *Burkholderia* (Mahenthiralingam et al. 2000). Different strain of *B. cepacia* were shown to be effective against *Pythium*-induced damping-off disease (Parke et al. 1991), *Aphanomyces*-induced root rot disease of pea (King and Parke 1993) and *Rhizoctonia*-induced root rot of poinsettia (Cartwright and Benson 1994). The effectiveness of *B. cepacia* against the bacterial pathogen *Ralstonia solanacearum* causing tomato bacterial wilt disease was also demonstrated (Sfalanga et al. 1999). The use of *B. cepacia* as a biocontrol agent of phytopathogens became a point of great concern, because of the report providing evidence for its ability to cause cystic fibrosis in human beings suffering from respiratory ailments (Isles et al. 1984).

Burkholderia cepacia produces antimicrobial compounds inhibiting the development of plant pathogens. *B. cepacia* AMMD effectively suppressed the development

of Pythium damping-off and Aphanomyces root rot disease of peas, when applied as seed treatment in growth chamber and field experiments (Parke et al. 1991). The antagonistic effects of *B. cepacia* strain AMMDR1 on post-infection stages in the life cycles of *Pythium aphanidermatum* and *Aphanomyces euteiches* infecting peas were assessed. The total numbers of oogonia per root were reduced by approximately 60 % at 12 days after inoculation with zoospores in treatments where *B. cepacia* AMMDR1 strain was applied, independent of whether the bacterial strain was applied to seeds or roots. In contrast, treatment with *B. cepacia* strain 1324 did not exert any adverse effect on oogonial production by the pathogens. Cross-sections of the root tissues in the area of zoospore inoculation showed that the reduction in the number of oogonia formed by the strain AMMDR1 was most dramatic in the cortex and much less in the stele. This situation may suggest that the antibiotics produced by the BCA may diffuse with difficulty across the endodermis. The bacterial strain had no effect on the production of secondary zoospores of *A. euteiches* from infected pea roots. The results indicated that all effects of *B. cepacia* strain AMMDR1 were dependent on the application or in situ production of antibiotics at the site of pathogen activity. *B. cepacia* could restrict the seed infection by *P. aphanidermatum* and reduce the mycelial growth and production of oogonia by *A. euteiches* (Heungens and Parke 2001). *Burkholderia cepacia* strain AMMDR1 was able to reduce effectively the severity of damping-off disease caused by *Pythium aphanidermatum* and root rot disease caused by *Aphanomyces euteiches* in peas. In order to investigate the underlying mechanisms of biocontrol activity of the strain AMMDR1, in addition to antibiosis, an antibiosis deficient strain was employed. *B. cepacia* strain 1324, a seed and root colonizing Tn5 mutant of the strain AMMDR1 was evaluated for its biocontrol potential in suppressing the development of pea damping-off and root rot diseases, along with the wild-type strain. The seedling emergence was substantially increased from 0 to 46 %, when seedlings were treated with strain AMMDR1 at 4 h after inoculation with *P. aphanidermatum.* On the other hand, the mutant 1324 did not show any effect on seedling emergence. The wild-type strain significantly reduced post-infection colonization of pathogen and damping-off of pea seedlings, even when the bacteria were applied at 12 h after zoospore inoculation. The mutant strain had no effect on mycelial colonization of seeds or roots by *A. euteiches* also, suggesting that the primary mechanism of biological control by *B. cepacia* AMMDR1 was through antibiosis (Heungens and Parke 2001).

Two isolates of *Burkholderia cepacia* BC-S and BC-TM were evaluated for their potential as biocontrol agents against *Schizophyllum commune* causing seed rot of oil palm and *Fusarium oxysporum* f.sp. *lycopersici* (*Fol*) causing wilt disease of tomato. Pathogenicity tests of the two isolates of *B. cepacia* on onion bulbs showed that they induced non-soft rot symptoms. Sunken dried lesions appeared on the outside surface of bulbs around the site of inoculation. Dual culture tests showed that both isolates of *B. cepacia* inhibited the mycelial growth of both pathogens to different degrees. The isolate BC-S was more inhibitory to *S. commune,* while BC-TM was able to inhibit *Fol* more effectively. In addition, these two BCA strains could inhibit the mycelial growth of other fungal pathogens *Fusarium solani* and

Colletotrichum dematium (Sijam and Dikin 2005). A novel compound designated CF661 with antifungal activity was isolated from *B. cepacia* strain CF661. This compound was inhibitory to several fungal pathogens such as *Alternaria alternata*, *Bipolaris sorokiniana, Colletotrichum lindemuthianum, Rhizoctonia solani, Fusarium graminearum* with varying minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC). No visible fungal growth could be observed at CF661 concentrate above 20.0 μg/ml. Microscopic observations revealed complete inhibition of conidia with concomitant protoplasm aggregation and broken mycelia in *Fusarium graminearum*, *F. oxysporum* and *Curvularia lunata* treated with CF661 (Li et al. 2007). In the further study, the antifungal effects of CF661 from *B. cepacia* were studied by dual staining with propidium iodide (PI) and fluorescein diacetate (FDA) on *Fusarium solani*. Incubation with 120.0 μg/ml of CF661 showed collapsed hyphae with visualizations of PI and FDA straining, indicating extensive cell death and membrane permeation. High doses of CF661 might kill *F. solani* by acting primarily on cell membrane. At lower concentration (20.0 μg/ml) of CF661, the growth of *F. solani* was entirely inhibited with marked morphological changes in the hyphae which exhibited swelling and numerous balloon-shaped cells. In addition, abnormal chitin deposition in hyphae was also seen (Li et al. 2009).

In cocultures, *B. cepacia* strain Lu 10–1 inhibited the mycelial growth of *Colletotrihum dematium,* causing mulberry anthracnose disease. Hyphae growing close to the bacterial colonies showed excessive branching, irregular swelling, curling of hyphal tips and disruption of apical growth. Coagulation of cytoplasm, degradation of the mycelium and presence of large vesicles inside fungal cell walls were also discernible. Inhibition of conidial germination by cell-free culture filtrate of Lu10-1 indicated the direct antagonistic effects of this bacterial strain (Ji et al. 2010). *B. cepacia* TIA-2B effectively suppressed the mycelial growth of *Rhizoctonia solani* and *Sclerotium rolfsii* infecting tomato. The roots were treated with the bacterial strain. Observations under SEM showed that the isolate TIA-2B could adhere to tomato roots producing biofilm structures at 48 h after application. High colonization density was observed in the apical root zone (De Curtis et al. 2010).

Burkholderia cepacia strain Lu 10–1, was able to suppress the development of the mulberry anthracnose disease caused by *Colletotrichum dematium* through multiple mechanisms of biocontrol activity. In addition to the production of antifungal compound, the strain Lu 10–1 produced siderophores, indole acetic acid (IAA) and solubilized phosphates and exhibited nitrogenase activity, indicating its ability to compete with the pathogen for nutrients and to promote the growth of mulberry plants. The strain Lu10-1 could reduce the disease severity significantly, when applied to inoculated leaves or to the soil, but the extent of disease suppression varied with time interval between pathogen inoculation and bacterial treatment. Presence of green fluorescent protein (GFP)-labeled Lu10-1 cells was monitored in the plants, after application of the labeled bacteria to the soil. The labeled cells were detected mainly in the intercellular spaces of roots and stems. The bacterial cells were found in the xylem vessels and in leaf veins at 11 and 20 days after inoculation respectively, indicating the ability of bacterial cells to migrate from the site of application

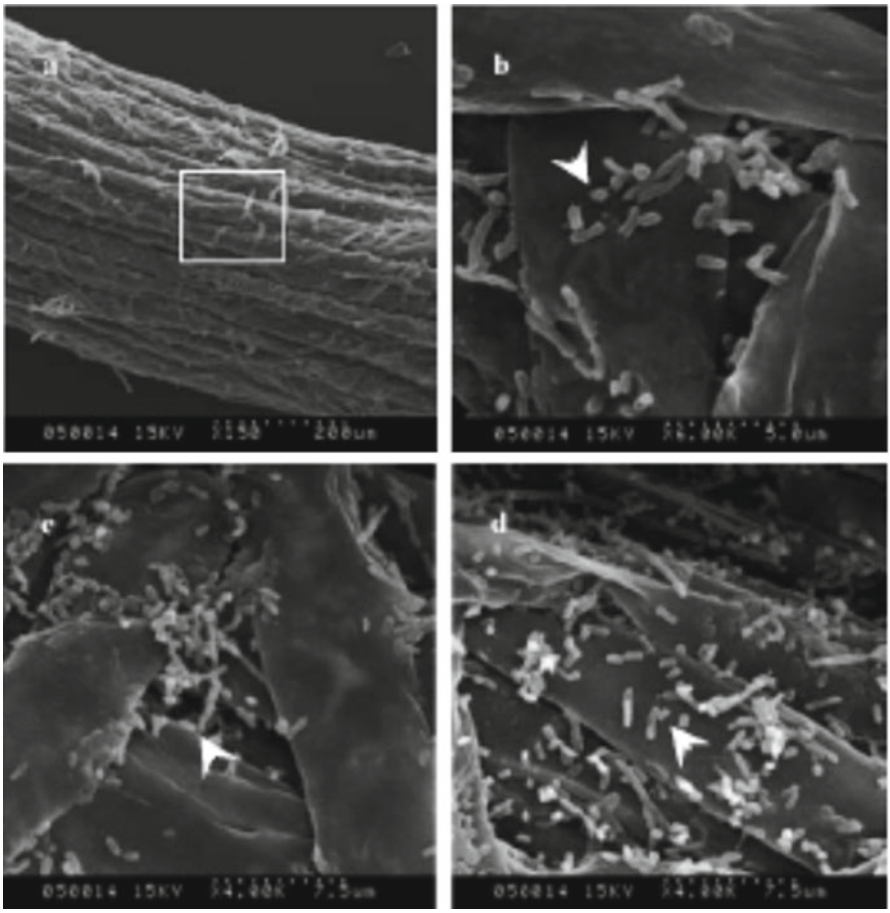

Fig. 5.9 Visualization of colonization of mulberry roots by *Burkholderia cepacia* strain Lu 10–1. (**a**): colonization of root hair zone; (**b**): magnified image of the framed region; (**c**): colonization of sites of root hair emergence; (**d**): colonization of the surface of the root hair (Courtesy of Ji et al. 2010 and with kind permission of BioMed Central, BMC Plant Biology)

(Fig. 5.9). Observations under SEM confirmed that the bacterial cells congregated at many entry sites along the length of the roots. Some bacterial cells were able to enter the cortex directly through the epidermis. The junctions between the primary and secondary roots were heavily colonized, indicating that the bacteria could enter the roots through the fissures or cracks present at the site of emergence of lateral roots and of the radicle. The treated plants showed resistance to anthracnose disease at sites away from the site of Lu10-1 application. The results indicated that the strain Lu10-1 might suppress development of anthracnose disease through direct antagonism, competition for nutrients, as well as by induction of disease resistance (Ji et al. 2010).

5.1.5 Lysobacter

These bacteria formerly placed within the order Myxobacteriales and Cytophagales were transferred to the genus *Lysobacter* that encompases nonfruiting gliding bacteria with a high G+C ratio. *Lysobacter* included *L. antibioticus, L. brunescens, L. enzymogenes* and *L. gummosus* and several strain earlier identified as *Stenotrophmonas* spp. or *Xanthomonas* spp. have been included in the genus *Lysobacter* based on the analysis of 16S rRNA gene sequences and fatty acid composition (Sullivan et al. 2003). Several strains of *Lysobacter* spp. have been reported to be effective biocontrol agents of plant pathogens. The strains of *Lysobacter* spp. produce antibiotics and lytic enzymes that can suppress the development of fungal pathogens. The ability of *L. enzymogenes* strain C3 to induce systemic resistance in treated plants against fungal pathogen has also been demonstrated (Kilic-Ekicio and Yuen 2003). Three strains of *Lysobacter enzymogenes* have been described as biocontrol agents of plant pathogens. Strain 3.IT8 was antagonistic to fungal and oomycetous pathogens, including various species of *Pythium*. Strain N4-7 was found to be effective in suppressing the development of summer patch disease of turfgrass caused by *Magnaporthe poae* (Islam et al. 2005). *Lysobacter enzymogenes* strain C3 was found to be natural antagonist capable of suppressing diseases caused by *Rhizoctonia solani* and *Bipolaris sorokiniana* on turf grass (tall fescue) (Zhang and Yuen 1999; Yuen and Zhang 2001). *L. enzymogenes* isolated from rockwool, effectively suppressed damping-off disease of cucumber caused by *Pythium aphanidermatum*. Another strain *L. capsici* isolated from the rhizosphere of pepper (chilli) plants exhibited antimicrobial activity (Park et al. 2008).

Lysobacter sp. strain SB-K88 suppressed the development of damping-off disease of sugar beet caused by *Pythium* sp. The metabolites inhibiting the mycelial growth were identified as xanthobaccins A, B and C. Seed bacterization and seed treatment with isolated xanthobaccin A suppressed sugarbeet damping-off disease in sugar beet plants grown in *Pythium* sp. infested soil (Nakayama et al. 1999). In a later investigation the modes of biocontrol activity of *Lysobacter* spp. strain SB-K88 against *Aphanomyces cochlioides,* causative agent of damping-off disease of sugar beet were studied. Microscopic observation of *A. cochlioides* hyphae growing close to the bacterial colonies showed excessive branching, irregular swelling, curling of hyphal tips and loss of apical growth. SEM observations revealed dense colonization of the bacteria on the root surfaces and a characteristic perpendicular pattern of *Lysobacter* colonization, possibly generated via development of polar, brush-like fimbrae. The strain SB-K88 was able to colonize also the roots of several plants, including spinach, tomato and amaranthus. Plants growing from the seeds of sugar beet and spinach treated with strain SB-K88 exhibited resistance to the damping-off disease induced by *A. cochlioides.* Exposure of zoospores of *A. cochlioides* to the bacterial cell suspension, cell-free supernatant of SB-K88 or pure xanthobaccin A for a minute, resulted in immobilization of zoospores. In all treatments lysis followed within 30 min in the presence of the inhibiting factors. The results indicated that a combination of antibiosis and characteristic biofilm formation at the rhizoplane

of the host plant might be the mechanism of biocontrol activity of *Lysobacter* sp. strain SB-K88, resulting in effective suppression of damping-off disease of sugar beet (Islam et al. 2005).

Lysobacter enzymogenes strain C3 was shown to be effective against *Bipolaris sorokiniana,* causing Bipolaris leaf spot in turf grasses and *Fusarium graminearum*, causative agent of Fusarium head blight disease of wheat. A heat-stable antifungal factor (HASF) was isolated from the culture supernatant. HSAF was an antibiotic complex consisting of dihydromaltophilin and structurally related to macrocyclic lactams which exhibited suppressive effect on several fungal and oomycete pathogens such as *Bipolaris sorokiniana, Fusarium graminearum, Rhizoctonia solani, Sclerotinia sclerotiorum, Pythium ultimum* var. *ultimum* and *Phytophthora sojae*. When applied to tall fescue leaves as a partially-purified extract, HASF (>25 μg/ml) inhibited germination of *B. sorokiniana* conidia. Two mutants of the strain C3 disrupted in the hybrid polyketide synthase-nonribosomal peptide synthetase gene for HSAF biosynthesis were less efficient in suppressing the development of Bipolaris leaf spot, compared with the wild type strain due to their in ability to synthesize HSAF. In contrast, the biocontrol potential of the mutants in reducing the severity of Fusarium head blight was in equivalence with the wild-type strain. The role of HSAF was not important for the strain C3 as a biocontrol agent against all fungal pathogens, since the HSAF-minus mutants were equally effective as the wild-type against diseases like Fusarium head blight of wheat (Li et al. 2008a, b). *Lysobacter enzymogenes* strain 3-IT8 was effective in suppressing the development of root and crown rot disease caused by *Pythium aphanidermatum* in rockwool hydroponic system. Application of the strain 3-IT8 in combination with chtiosan (the deacetylated derivative of chitin) reduced the number of diseased plants by 50–100 %, compared with control. Application of chitin alone or the bacterial strain alone was ineffective. The chitosan disappeared from the hydroponic system within 24 h after application which could be attributed to the expression of enzymes of the strain 3-IT8 induced by the exposure to chitosan. The results indicated that addition of chitosan enhanced the biocontrol efficacy of *L. enzymogenes* and chitosan might serve as C- and N-source for the bacterial strain, and induce antagonist gene expression or both (Postma et al. 2009).

Lysobacter enzymogenes produces abundantly extracellular enzymes such as chitinases and glucanases that can degrade the major cell wall components of fungi and oomycetes. The role of extracellular enzymes in the biocontrol activity of *L. enzymogenes* strain C3 has been studied. Purified chitinolytic fractions inhibited the spore germination and infection of plants by *Bipolaris sorokiniana* (Zhang and Yuen 2000). *Lysobacter enzymogenes* strain C3 produced chitinases in broth media containing chitin. The conditions for production of chitinases by the strain C3 were studied and the partially purified chitinases were characterized after purification by ammonium sulfate precipitation and chitin affinity chromatography. The partially purified fraction inhibited conidial germination and germ tube elongation of *Bipolaris sorokiniana*. The fraction exhibited strong exochitinase and weak endochitinase activity. The activity was maximum at 45–50 °C and pH 4.5–5.0 and Hg^{2+} and Fe^{3+} ions inhibited the activity. Of the five proteins separated from partially purified preparations, three

proteins showed homology to known bacterial chitinases. At least two chitinases produced by the strain C3 were found to have antifungal properties (Zhang et al. 2001). *L. enzymogenes* strain 34S1 was found to be an effective biocontrol agent against *Magnaporthe poae* causing summer patch disease of Kentucky bluegrass (*Poa pratensis*). A chitinase gene was cloned on a 2.8-kb DNA fragment from the strain 34S1 by heterologous expression in *Burkholderia cepacia*. A 51-kDa protein with chitinolytic activity was purified from the culture filtrates of the BCA strain by hydrophobic interaction chromatography. The mutant strain C5 defective in the function of *chiA* gene lacked chitinolytic activity and it did not secrete the 51-kDa protein in culture filtrates. The strain C5 showed decreased ability to suppress summer patch disease on Kentucky grass, indicating the important role of chitinolytic enzyme in the biocontrol activity of *L. enzymogenes* strain 34S1 (Kobayashi et al. 2002).

Lysobacter enzymogenes strain C3 produced multiple extracellular hydrolytic enzymes which were involved in the biocontrol activity of this strain. The regulation of biosynthesis of these enzymes was investigated by generating mutants of strain C3 disrupted in extracellular enzyme production and evaluating the efficiency of the biocontrol activity of the mutants. The *clp* gene encoding a global regulator was found to govern the lytic enzyme production and a number of other factors also in the strain C3. A single min-Tn5-*lacA*,-cat transposon mutant 5E4 of the strain C3 that was globally affected in a variety of phenotypes was isolated. The activities of several extracellular lytic enzymes, gliding motility and in vitro antimicrobial activity of the mutant 5E4 were reduced. The *clp* gene was chromosomally inserted into the mutant 5E4, resulting in complemented strain P1. All mutant phenotypes were restored in P1. But the gliding motility was found to be excessive, compared with that of the wild-type strain. The *clp* mutants had reduced biocontrol efficiency against sugar beet damping-off and tall fescue Bipolaris leaf spot diseases. The biocontrol efficiency was partially restored in the complement strain P1 (Kobayashi et al. 2005). A new isolate *Lysobacter* sp. was obtained from the rhizosphere of tobacco plant and it was identified as *L. capsici* based on the sequencing and phylogenetic analysis of 16S rRNA and characteristics in the Biolog Identification System. The strain PG4 of *L. capsici* exhibited chitinase and protease activity, capable of digesting yeast cells. No siderophore production could be detected on CAS agar plates. The strain PG4 was highly antagonistic to *Rhizoctonia solani* and *Sclerotinia sclerotiorum* and several other fungal pathogens. Seed treatment with the strain PG4 drastically reduced the plant mortality caused by *F. oxysporum* f.sp. *radicis-lycopersici* in the greenhouse experiments (Puopolo et al. 2010).

Lysobacter enzymogenes strain N4-7 produced three β-1,3-glucanases, and the genes *gluA* and *gluC* encoded the biosynthesis of these enzymes. The molecular and biochemical characterization of three enzymes in strain N4-7 provided the basis to assess the role of β-1,3-glucanases in biocontrol activity of the bacterial strains (Palumbo et al. 2003). The strains C3 and N4-7 are closely related. The strain C3, unlike N4-7, is genetically tractable and it has been studied as a biocontrol agent against many pathogens. *L. enzymogenes* strain C3 produced multiple extracellular β-1,3-glucanases encoded by the genes *gluA*, *gluB* and *gluC*. These glucanases of strain C3 resolved in native gels produced patterns nearly identical to those produced by strain N4-7, sharing >95 % amino acid sequence identity to their

Table 5.9 Effect of treatment of wheat and tall fescue with *L. enzymogenes* strain C3 on incidence of severity of diseases (Kilic-Ekicio and Yuen 2003)

Treatments	Bipolaris leaf spot		Rhizoctonia leaf blight area (%)
	Incidence (lesions/10 cm of leaf)	Severity (% of leaf area infected)	
Live C3 cells	4b	1b	20b
Killed C3 cells	6b	2b	35b
Distilled water	41a	13a	74a

In the same column figures followed by the same letter are not significantly different (P = 0.05)

counterparts. Mutational analysis indicated that the three genes accounted for the total β-1,3-glucanases activity detected in culture. Using gene-specific mutagenesis approach, direct evidence was obtained supporting a role for β-1,3-glucanases in the biocontrol activity of the strain C3 against *Bipolaris sorokiniana* and *Pythium ultimum*. The strain G123, mutated in all three glucanase genes, showed significantly reduced biocontrol efficiency against the Bipolaris leaf spot of tall fescue and Pythium damping-off of sugar beet. The results revealed the important role of the β-1,3-glucanases produced by the strain C3 in its biocontrol activity against fungal pathogens (Palumbo et al. 2005).

Induced systemic resistance (ISR) is one of the important mechanisms of biocontrol activity of rhizobacteria against microbial plant pathogens. The chitinase and glucanase activities of *Lysobacter enzymogenes* strain C3 could not account for all its disease suppressive activity. The spatial distribution of strain C3 on host plant shoots suggested the possibility of induction of resistance to diseases being a possible mechanism of biocontrol activity. Resistance elicited by the strain C3 suppressed germination of *Bipolaris sorokiniana* conidia on the phylloplane of tall fescue, in addition to reducing the severity of leaf spot. Direct pathogen-inhibitory effect could be separated from antibiosis by applying heat inactivated cells of the strain C3 that retained no antifungal activity. Localized resistance occurred, when the leaves were treated with live or heat-killed cells, whereas systemic resistance was induced, when the roots of tall fescue plants were treated. The resistance induced by foliar or root treatments persisted for 15 days. In addition, the dose-relationship typical of antagonism of the BCA was absent, indicating the ability of strain C3 to elicit ISR. The resistance induced by strain C3 was not host or pathogen-specific, since the foliar application of heat-killed cells of C3 suppressed the development of *B. sorokiniana* on wheat and reduced the severity of brown patch caused by *R. solani* in tall fescue (Table 5.9) (Kilic-Ekicio and Yuen 2003). The ability of *Lysobacter enzymogenes* strain C3 to elicit induced resistance in wheat against *Fusarium graminearum* (= *Gibberella zeae*) causing Fusarium head blight (FHB) disease was assessed in the greenhouse. Chitin broth cultures of the strain C3 reduced FHB severity to <10 % infected spikelets, compared to >80 % severity in control treatment. The C3 broth cultures, heated to inactivate cells and lytic enzymes, but retaining the elicitor factor for induced resistance, also effectively reduced FHB

severity, suggesting induced resistance is one mechanism of biocontrol activity. Protection by treatment with C3 was not systemic, since application of the bacterial strain uniformly to cover all susceptible florets was required (Jochum et al. 2006).

5.1.6 Serratia *spp.*

The genus *Serratia* includes *S. liquefaciens, S. marcescens, S. plymuthica* and *S. rubidaea. S. marcescens* and *S. liquefaciens* are frequently found associated with human infections as opportunists. *S. plymuthica* has also been recovered from body, but it has rarely been isolated as the sole bacterial species present. *S. plymuthica* is classified as a member of risk group1. No strong evidence of causing human infections has been obtained (de Vleeschauwer and Höfte 2007). However, *S. plymuthica* has been found in the rhizosphere, as endophyte of plants and it has been demonstrated to be a biocontrol agent that can suppress the development of many soilborne and airborne plant pathogens. Some of the plant pathogens that have been reported to be controlled by strains of *S. plymuthica* include *Rhizocto solani* infecting cotton and bean (Chernin et al. 1995; Ovadis et al. 2004), *Pythium aphanidermatum* infecting cucumber (Ovadis et al. 2004). *Colletotrichum lindemuthianum* and *Botrytis cinerea* infecting bean (Meziane et al. 2006a, b), *Verticillium dahliae* and *Phytophthora cactorum* infecting strawberry (Berg et al. 2001) and *Phytophthora capsici* infecting pepper (chilli) (Park and Shen 2002; Shen et al. 2002). The effectiveness of *S. marcescens* as a biocontrol agent against *Phytophthora parasitica* causing citrus gummosis disease was reported by de Queiroz and de Melo (2006). Strains (16) of *Serratia plymuthica* showed antifungal activity against *Verticillium dahliae, Rhizoctonia solani* and *Sclerotinia sclerotiorum* in in vitro bioassays. Dipping strawberry roots in a suspension of cells of *S. plymuthica* strain HRO-C48 reduced the Verticillium wilt disease incidence by 18.5 % and root rot disease caused by *Phytophthroa cactorum* by 33.5 % (Kurze et al. 2001). *S. plymuthica* strain A21-4 isolated from the rhizosphere soils and onion roots, colonized the roots of pepper, when it was applied to the seeds or roots of pepper. It was inhibitory to *Pythium* sp. and *Phytophthora capsici*, but not to *Fusarium* sp. and *Rhizoctonia solani*. The strain A21-4 population on the rhizosphere remained at high levels (up to 10^6 CFU/g soil) after a period of 21 days (Shen et al. 2002). *Serratia marcescens* strain R-35 was isolated from the roots of healthy citrus plants. In plate confrontation tests, the strain R-35 was able to inhibit the mycelial growth of *Phytophthora parasitica* causing citrus gummosis disease. The bacterial strain lysed the oospores of *P. parasitica*. The antagonistic activity of the strain R-35 was considered to be due to antibiotics as well as siderophores produced by the BCA. Application of the strain R-35 reduced the citrus seedling infection by 50 % (de Queiroz and de Melo 2006).

S. plymuthica strain A153 suppressed the production of apothecia by *Sclerotinia sclerotiorum* and the inhibitory effect was attributed to the production of chlorinated macrolides (Thaning et al. 2001; Levenfors et al. 2004). In addition, production of

antibiotics such as pyrrolnitrin (Prn), prodigiosin and haterumalides by *S. plymuthica* has also been reported (Chernin et al. 1996; Thaning et al. 2001; Levenfors et al. 2004). Isolated haterumalides, purified from the supernatant of *S. plymuthica* strain A153 drastically inhibited the mycelial growth, apothecial formation and ascospore germination of many filamentous fungi and oomycetes in vitro (Strobel et al. 1999; Levenfors et al. 2004). *S. plymuthica* IC1270 was found to be effective against *Penicillium expansum,* causing apple blue mold disease and *Monilinia fructicola* infecting peaches. Pyrrolnitrin was considered to be the key factor in suppressing the postharvest diseases of apples and peaches (Rite et al. 2002). Gene expression and regulation of biosynthetic genes for Prn in *S. plymuthica* strain IC1270 was studied. The Prn-deficient *grrA, grrS* and *rpoS* gene replacement mutants were less efficient in suppressing the development of *R. solani* and *P. aphanidermatum* in greenhouse assays, indicating that the biocontrol activity of this strain was tightly modulated by the GrrA/GrrS global regulatory cascade and the sigma factor RpoS (Ovadis et al. 2004). The soilborne pathogen *Pythium ultimum* causes damping-off disease in greenhouse and field grown cucumber. Live cells and ethanol extracts of cultures of *Serratia marcescens* strain N4-5 significantly suppressed the development of damping-off disease, when applied as seed treatment for cucumber seeds. Culture filtrates of N4-5 exhibited chitinase and protease activities, whereas the ethanol extracts contained the antibiotic prodigiosin, the surfactant serrawettin W1 and possibly other unidentified surfactants. Production of prodigiosin and serrawettin W1 was temperature dependent, since their production occurred at 28 °C, but not at 37 °C. Prodigiosin purified from two consecutive TLC runs, using different solvent systems, inhibited germination of sporangia and mycelial growth of *P. ultimum.* The surfactant serrawettin did not have any inhibitory effect on the pathogen. Disease suppression by *S. marcescens* strain N4-5 was in part due to the production of the antibiotic prodigiosin (Roberts et al. 2007).

Biocontrol agents parasitic on fungal pathogens excrete extracellular cell wall-degrading enzymes such as chitinases, glucanases and proteases that target pathogen cell wall, resulting in lysis of the pathogen cells. The biocontrol potential of *Serratia marcescens, Streptomyces viridodiasticus* and *Micromonospora carbonacea* against *Sclerotinia minor* causing basal drop disease of lettuce was evaluated. These bacteria and actinomycete effectively suppressed the pathogen development and disease incidence. All the three BCAs were efficient producers of chitinase and β-1,3-glucanase and caused extensive plasmolysis and cell wall lysis of *S. minor*. Production of chitinases and β-1,3-glucanases was considered to be the main mechanism of biocontrol activity of these BCAs, since only the production of these enzymes was related to the extent of in vitro pathogen inhibition and disease suppression. The transfer of the plugs containing *S. minor* inhibited by the antifungal enzymes, to a fresh medium and their inability to grow further, indicated a fungicidal effect of the metabolites produced by the BCA isolates. When a mixture of all three antagonists was applied to soil in glasshouse trials, disease incidence was not significantly different from the most effective strain *S. marcescens*. This indicated that there was no additive effect on disease incidence due to the presence of the actinomycetes. In addition, *S.*

marcescens was the most competent rhizosphere colonizer and reduced the incidence of basal drop disease caused by *S. minor* (El-Tarabily et al. 2000). Different types of chitinases were produced by the strains of *S. plymuthica*. A chitinase-encoding gene *chiA* from *S. plymuthica* strain IC1270 was cloned and sequenced. The *chiA* gene had an open reading frame coding for 562 amino acids of a 61-kDa precursor protein with a putative leader peptide at the N-terminus. *Escherichia coli* transformed with *S. plymuthica chiA* gene was able to suppress the development of *R. solani* and inhibit spore germination of *F. oxysporum* f.sp. *melonis* in vitro. In addition, the transformant suppressed root rot disease development in cotton seedlings under greenhouse conditions (Chernin et al. 1997). The role of chitinases in the biocontrol activity of *S. plymuthica* strain IC14 against *S. sclerotiorum* and *B. cinerea* appeared to be less important. Synthesis of proteases and other biocontrol traits were required for the suppression of *B. cinerea* and *S. sclerotiorum* by this strain (Kamensky et al. 2003). Biocontrol activity of the strain IC1270 against *R. solani* and *P. aphanidermatum* was demonstrated to be, not dependent on the production of chitinase by this strain (Gavriel et al. 2004). Biocontrol activity dependent on chitinolytic enzymes was variable and strain-specific.

Competition for nutrients among microorganisms especially for bioavailable iron in soil habitats and on plant surfaces, under iron-limiting conditions, is well known. Strains of *Serratia* may produce a range of low-molecular weight compounds or siderophores to sequester iron compounds, thereby depriving the fungal pathogens of this element required for their growth. *S. plymuthica* strains IC1270, IC14, and HRO-C48 were shown to produce potent siderophores functioning as iron-chelators in vitro under iron-limited conditions (Kamensky et al. 2003; Berg et al. 2005; Ovadis et al. 2004; Faltin et al. 2004). The Prn- and/or – endochitinase mutants of the strain IC1270 exhibited residual biocontrol activity. Despite the loss of ability to secrete the antibiotic and enzyme considered to have a role in the biocontrol activity of these mutants was suggested to be due to their ability to compete for nutrients like iron (Gavriel et al. 2004; Ovadis et al. 2004). The mechanism of biocontrol activity of two strains IC1270 and IC14 of *Serratia plymuthica* against *Penicillium digitatum* (orange green mold) or *P. italicum* (blue mold of orange) was investigated. Two mutants of strain IC1270, one deficient in chitinolytic activity (IC1270-C7) and another deficient in pyrrolnitrin production (IC1270-P1) were generated by gene replacement technique. On orange, the mutants were as effective as wild-type strain, suppressing the development of *P. digitatum.* However, the loss of antifungal activity by IC1270-P1 in the in vitro assays was observed, as no inhibition zone could be recognized, when tested against *P. digitatum* or *P. italicum*. The chitinase-deficient mutant of strain IC14 was found to be equally efficient as the wild-type strain, suggesting that chitianses did not have any role in the suppression of green mold disease caused by *P. digitatum*. Interactions between strains IC1270 or IC14 and *P. digitatum* were investigated, using tissue culture plates with diluted orange peel extract as the nutrient source. Strain IC1270 decreased germination of *P. digitum* conidia, when it was physically separated from the pathogen by a membrane filter, which permitted nutrient and metabolite interchange, while strain IC14 did not inhibit spore

germination. But significant inhibition of conidial germination of *P. digitatum* was recorded, when the pathogen and IC14 were in physical contact with each other. Competition for nutrient appeared to be the principal mechanism of strain IC1270, while a direct cell to-cell interaction between IC14 and the pathogen was required for antagonistic activity (Meziane et al. 2006a, b).

Enhancement of levels of resistance of host plant by rhizobacteria is well known. Some strains of *Serratia plymuthica* have been reported to elicit induced systemic resistance (ISR) against plant pathogens. *S. plymuthica* strain 2–67 induced systemic resistance in cucumber against the anthracnose pathogen *Colletotrichum orbiculare* (Gang et al. 1991). Elicitation of ISR in cucumber against *Pythium ultimum* by *S. plymuthica* strain RICG4 was also observed. The structural and biochemical changes in cucumber treated with the strain RICG4 were studied using electron microscopy. ISR, elicited by the bacterial strain, correlated with the formation of structural barriers that might prevent pathogen ingress towards the vascular stele. This was accompanied by the deposition of a phenolic enriched occluding material. The responses linked to the development of ISR included oxidation and polymerization of pre-existing phenols and synthesis of new phenolic compounds via activation of the phenyl-propanoid pathway. By priming the susceptible cucumber plants using suspension of the strain R1CG4, the development of root rot disease caused by *P. ultimum* could be suppressed (Benhamou et al. 2000). The strain IC1270 could elicit ISR against *Botrytis cinerea* and *Colletotrichum lindemuthianum* in bean and tomato (Meziane et al. 2006a, b). *Serratia plymuthica* HRO-C48 isolated from the rhizosphere of oilseed rape produced *N*-acyl homoserine lactone (AHL) signals which mediated quorum sensing regulation. The strain HRO-C48 produced the broad-spectrum antibiotic pyrrolnitrin. This strain induced systemic resistance against *Botrytis cinerea* causing gray mold disease in bean and tomato plants. Colonization of the rhizosphere depended on AHL signaling. The results indicated that quorum sensing regulation might be generally involved in interactions between plant-associated bacteria, fungal pathogens and host plants (Pang et al. 2009).

The differential effects of the strain IC1270 against rice pathogen have been reported. This bacterial strain induced systemic resistance against the blast disease caused by *Magnaporthe grisea* and bacterial blight disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). In contrast, the infection by the necrotrophic pathogens *Rhizoctonia solani* causing sheath blight disease and *Bipolaris oryzae* causing brown spot disease was significantly promoted. The differential effectiveness of IC1270 may possibly be due to its capacity to modulate oxidative machinery of the rice plant (de Vleeschauwer and Höfte 2007). The root colonization by IC 1270 in rice did not cause a strong constitutive resistance phenotype, but rather primed plants to hyper-respond to subsequently inoculated plants, resulting in excessive defense activation and enhanced resistance to *Magnaporthe oryzae*. This priming effect of IC1270 was revealed by the observation that challenge inoculation of IC1270-colonized plants with *M. grisea* entailed a rapid accumulation of autofluorogenic phenolic compounds in and around epidermal cells displaying dense cytoplasmic granulation (Fig. 5.10). The IC1270-inducible ISR response seemed to get as a double-edged sword within the rice defense

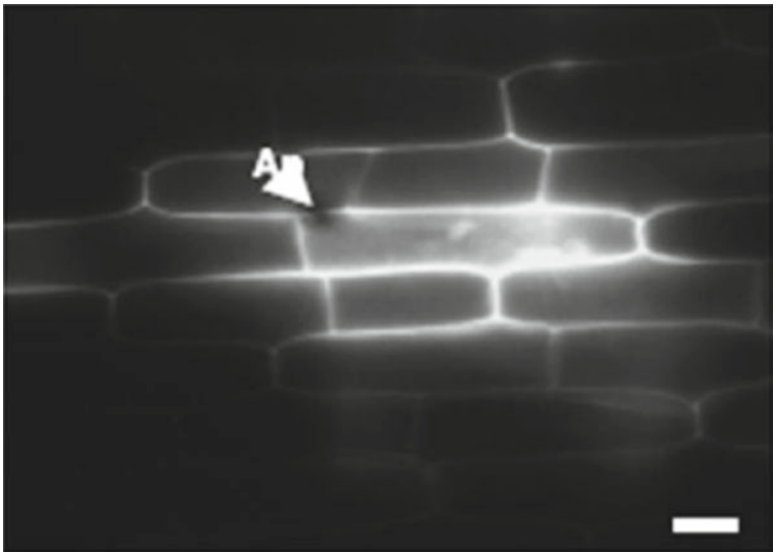

Fig. 5.10 Accumulation of autofluorescent phenolics in *S. plymuthica* IC1270-primed rice leaf sheath cells following challenge inoculation with conidial suspension of rice blast pathogen. Epifluorescence image of IC1270-induced sheath cells at 24 h post-inoculation; *Ap* appressorium; Scale bar: 20 μm (Courtesy of de Vleeschauwer and Höfte 2007 and with kind permission of BioMed Central, BMC Plant Biology)

network, as induced plants displayed an enhanced susceptibility to *R. solani* and *B. oryzae.* Artificial enhancement of reactive oxygen species (ROS) levels in inoculated leaves faithfully mimicked the opposite effects of IC1270 strain on these two pathogens, suggesting a central role for oxidative events in the IC1270-induced resistance mechanism (de Vleeschauwer et al. 2009).

Serratia marcescens is another rhizobacterial species, capable of inducing systemic resistance in plants against fungal and bacterial plant pathogens. The strain 90–166 elicited induced systemic resistance (ISR) in cucumber against *Pseudomonas syringae* pv. *lachrymans, Colletotrichum orbiculare* and *Erwinia tracheiphila* under field conditions. The ISR development was found to be influenced by bioavailability of iron and ISR was reduced, when iron was made available in planting medium. ISR induced by the strain 90–166 was not very much dependent on salicylic acid (SA), since three mutants of 90–166 lacking SA production were able to induce resistance in cucumber against anthracnose caused by *C. orbiculare* (Press et al. 1997). Seed treatment with the strain 90–166 suppressed anthracnose disease of cucumber, when iron concentrations of the planting mix was decreased by the addition of an iron chelator ethylenediamine [di(o-hydroxyphenyl acetic acid)] (EDDHA). Suppression of the disease by the strain 90–166 was significantly improved. A mutagenesis approach was applied to evaluate the role of siderophore production by 90–166 in ISR. A mutant 90-166-2882, lacking catechol siderophore production, had a transposon insertion in an *entA* homolog. The *entA* mutant

90-166-2282 could not induce resistance to anthracnose, supporting the hypothesis that catechol siderophore produced by the wild-type strain 90–166 is necessary for elicitation of ISR. The *entA* mutant still retained the ability to produce SA, but not the ability to elicit ISR, indicated that catechol rather than SA was required for induction of ISR against cucumber anthracnose disease. The lack of capacity to elicit ISR by mutant strain 90-166-2882 was not attributed to inefficient rhizosphere colonization, since the total root population sizes of the mutant and wild-type strains were similar. The results suggested that catechol siderophore biosynthesis genes in *S. marcescens* 90–166 might be associated with ISR (Press et al. 2001). The ability of chitinolytic *S. marcescens* GPS5 with and without chitin supplementation (1 % colloidal chitin) to activate defense-related enzymes in groundnut leaves was assessed, following challenge inoculation with *Phaeoisariopsis personata,* causing late leaf spot disease. Chitin supplementation reduced the lesion frequently by 64 % as against 23 % with chitin supplementation. Chitin-supplemented GPS strain was much more effective in the activation of defense-related enzymes and this enhanced enzyme activities continued up to 13 days after pathogen inoculation (Kishore et al. 2005a, b).

The mechanism of antagonistic activity of three strains of *Serratia marcescens* (CFFSUR-B2, CFFSUR-B3 and CFFSUR B4) against the fruit anthracnose pathogen *Colletotrichum gloeosporioides* was studied. All the three strains showed similar pattern of antagonistic activities with differences in their extent of inhibition of mycelial growth (>40 %) and germination of conidia (81–89 %) of *C. gloeosporioides*. The bacterial strains produced prodigiosin and chitinases in oilseed-based media (peanut, sesame, soybean or castor bean) and Luria-Bertani medium. Production of prodigiosin reached the maximum (40 µg/ml) level in the peanut-based medium, while cultivation in soybean-based medium induced the highest quantity of chitinase (56 units/ml) in all strains of the BCA. Prodigiosin production was not influenced by changes in pH of the medium. The strains of *S. marcescens* used in this investigation showed the ability of adapting to tropical climates (Gutiérrez-Román et al. 2012).

5.1.7 Pantoea *sp.*

Biological suppression of a plant disease with *Pantoea agglomerans* (earlier known as *Erwinia herbicola*) was first attempted in the 1930s (Beer et al. 1984). *P. agglomerans* (*Pa*) is a Gram-negative bacterium included in the family Enterobacteriaceae. *Pa* is primarily a plant epiphyte, but commonly found in diverse niches including aquatic environment, soil and sediments. Some strains are capable of inducing human ailments. Fire blight disease of apple and pear caused by *Erwinia amylovora* is a destructive disease. Many strains of *Pa* have been developed as commercial products for application as biocontrol agents against fire blight and other crops diseases caused by bacterial pathogens (Rezzonico et al. 2009). Biological control focused primarily on suppression of epiphytic growth phase of *E. amylovora* on

blossoms prior to infection and epiphytic growth. Strains of *P. agglomerans* were evaluated as potential biocontrol agents against *E. amylovora*, in part, due to their ability to produce antibiotics inhibitory to the pathogen in synthetic culture media. The antibiotics produced by the strains of *P. agglomerans* were identified as herbicolins (Ishmaru et al. 1988), pantocins (Brady et al. 1999; Wright et al. 2001) or microcins (Vanneste et al. 1998). Some strains of *P. agglomerans* could produce single antibiotics, while some produced multiple compounds that suppressed the pathogen development. The genes encoding for antibiotic biosynthesis were either localized in the chromosomes and/or on plasmids. Pear juice contains several amino acids including arginine and proline which can reverse inhibition of *E. amylovora* by the antibiotics produced by *P. agglomerans.* The laboratory assay has limitations in that strains that suppress fire blight through competitive exclusion of the pathogen from sites and nutrients on stigmas might be lost, because of their inability to inhibit the pathogen growth.

The role of antibiotics produced by strains of *Pantoea agglomerans* in fire blight disease suppression was investigated by employing transformed bacterial species and mutants deficient in the production of antibiotics. *Escherichia coli* was transformed with a 12-kb plasmid isolated from *E. herbicola*. The transformants that were able to produce antibiotics, inhibited the growth of the pathogen in the culture and symptoms development on inoculated hawthorn blossoms. In contrast, the wild-type strain did not inhibit the growth of and induction of symptoms by the pathogen (El-Masry et al. 1997). In the another approach to study the role of antibiotics in disease suppression, mutants of the strain Eh318 deficient in pantocin A, pantocin B or both antibiotics were generated. Mutants of Eh318 deficient in the production of pantocin A or pantocin B were equally effective in reducing infection by *E. amylovora,* as the wild type strain in the greenhouse experiments. On the other hand, the mutant deficient in the production of both antibiotics were less effective in suppressing the development of fire blight disease, indicating that the strain Eh318 might suppress the pathogen development through other mechanism (s) in addition to antibiosis (Wright and Beer 1996; Wright et al. 2001).

The effectiveness of secondary metabolites produced by *Pantoea agglomerans* in suppressing fire blight disease caused by *Erwinia amylovora* was assessed in apple or pear orchards. The population dynamics and disease suppression with *P. agglomerans* Eh252, a strain that could produce a single antibiotic, were compared with its near-isogenic antibiotic deficient derivative, strain 10:12. Water or suspensions of Eh 252 or 10:12 (1×10^8 CFU/ml) were applied at 30 and 70 % bloom to pear or apple trees. Aqueous suspensions of freeze-dried cells of *E. amylovora* (3×10^5 CFU/ml) were applied at full bloom. Population sizes of Eh252 or 10:12 on pear blossoms were estimated by spreading dilutions of blossom washes on culture media. Average population sizes of Eh252 and 10:12 on blossoms ranged from 10^5 to 10^7 CFU. The antibiotic deficient strain 10:12 was generally less effective than Eh 252 in suppressing fire blight. However, it significantly reduced the incidence of fire blight in four of seven experiments, compared with inoculated water-treated controls. Eh 252 significantly decreased the incidence of fire blight in six of seven field trials, compared with the incidence on plants treated with 10:12 mutant and

Table 5.10 Effect of treatment with strain Eh252, mutant 10:12 and commercial antibiotics on incidence of fire blight disease on pear cultivars during 1998–2000 (Stockwell et al. 2002)

Year/cultivars		Treatments				
		Water	Eh 252	10: 12	Oxytetra-cycline	Strepto-mycin
1998	Bartlett	1.00a (188)	0.32c	0.65b	1.65a	0.41c
1999	Bartlett	1.00a (160)	0.36bc	0.48b	0.51b	0.12c
	Bosc	1.00a (29)	0.88a	0.90a	NT	0.54b
	Bartlett (Medford)	1.00a (63)	0.12d	0.31c	0.62b	0.06d
2000	Bartlett	1.00a (166)	0.59b	0.78ab	0.69b	0.17c
	Bosc	1.00a (204)	0.51a	0.98a	NT	0.08c
	Rome beauty	1.00a (50)	0.16c	0.50bc	0.66b	0.32bc
	Pooled data	1.00a	0.45d	0.70c	0.84b	0.25e

Figures for each of the pear cultivars in each year (*horizontal*) with same letter are not statistically significant (P = 0.05) according to Fischer's protected least difference; number in parentheses are the average number of fire blight strikes on water-treated (*control*) trees
NT not tested

water control. Overall the strain Eh 252 reduced the incidence of fire blight by 55 ± 8 %, 10:12 by 30 ± 6 %, streptomycin by 75 ± 4 % and oxytetracycline by 16 ± 14 %. The moderate level of effectiveness of strain 10:12 compared with water control, indicated that other mechanisms such as competitive exclusion or habitat modification might also contribute to suppression of fire blight disease. Disease suppression by Eh 252 exceeded the level that was provided by oxytetracycline, but it was similar to that provided by streptomycin in several field trials (Table 5.10). The results indicated that antibiosis was an important mechanism of biocontrol activity of *P. agglomerans* Eh 252 against *E. amylovora*, causal agent of fire blight disease of pear and apple (Stockwell et al. 2002).

Pantoea agglomerans strain E325 was selected through screening, based on suppression of *Erwinia amylovora* on flower stigmas. In order to have an insight into the underlying mechanism of biocontrol activity of strain E325, bacterial modification of pH was evaluated by analyzing exudates extracted from 'Gala' apple stigmas. Under ideal laboratory conditions for bacterial colonization of flowers, *P. agglomerans* strain E325 was able to reduce the pH on stigmas to levels that could reduce growth of *E. amylovora.* In contrast, an increase in pH occurred on flowers inoculated with the pathogen alone. Similar trend of enhancement of pH in pathogen-inoculated flowers and decrease of pH in antagonist-treated flowers was also observed (Fig. 5.11). In addition under low-phosphate and low-pH conditions, an antibacterial product of E325 with high specificity to *E. amylovora* was able to suppress the pathogen growth at low concentrations. The antagonistic activity was heat stable and it was not lost due to treatment with amino acids, iron or enzymes known to inactivate antibiotics effective against *P. agglomerans.* The results suggest that the strain E325 might suppress the development of the fire blight pathogen, not only by competing for nutrients on the stigma and producing antibiotics, but also by modifying flower stigma environment (Pusey et al. 2008). The mechanism of biocontrol activity of *Pantoea agglomerans* strain E325 against the fire blight

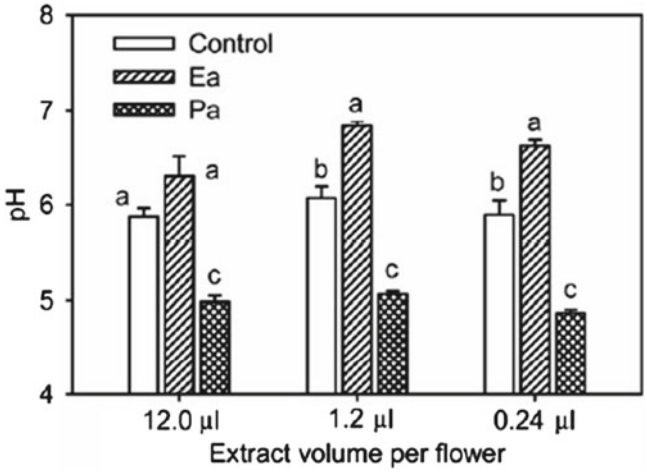

Fig. 5.11 Bacterial modification of pH of exudates extracted from 'Gala' apple stigmas treated with *Pantoea agglomerans* E25 (*Pa*) and inoculated with *Erwinia amylovora* (Ea). *Vertical lines* represent standard error within each bar group; means with same letter are not significantly different according to the least significant difference ($P \leq 0.05$) (Courtesy of Pusey et al. 2008 and with kind permission of The American Phytopathological Society, MN, USA)

pathogen *Erwinia amylovora* was studied. Antibiosis was evaluated as a mode of antagonism on flower stigmas using two antibiosis-deficient mutants. Mutants were tested against *E. amylovora* on stigmas of detached flowers of crab apple (*Malus mandshurica*) in growth chambers and apple (*Malus domestica*) in the orchard. Epiphytic fitness of the antibiosis-negative mutants was similar or greater than the wild-type strain as reflected by relative area under population curve (RAUPC). Both mutants were less effective in inhibiting pathogen development in in vitro assays and under orchard conditions. The mutants and the parent strain showed distinct differences in the colony morphology. However, E325 and the mutants induced similar level of decrease in pH in a broth medium, indicating acidification of the medium which was not related to antibiosis. The results provided evidence for the involvement of antibiosis as the mechanism of the biocontrol activity of *P. agglomerans* E325 against *E. amylovora* (Pusey et al. 2011).

The mechanism of biocontrol activity of *Pantoea agglomerans* strain CPA-2 effective against the postharvest pathogens *Penicillium digitatum* and *P. italicum* respectively causing green mold and blue mold diseases of citrus, was studied. The strain CPA-2 did not produce antibiotics or chitinolytic enzymes that could account for the suppression of the mold diseases. The antagonist did not induce the activation of defense-related enzymes PAL and PO in the orange peel at different periods after inoculation with the antagonist and/or the pathogen. *P. agglomerans* could effectively suppress the pathogens, only when it was in close contact with the pathogens. Competition for nutrients was assessed, using tissue culture plates with cylinder inserts which allowed competition for nutrients to be studied without competition for space. The physical contact between pathogen and the bacterial strain was

avoided by this setup. The germination of conidia of *Penicillium* spp. was substantially decreased by the antagonist, when the diluted orange peel extract or diluted potato extract or diluted potato-dextrose was the nutrient source. Direct contact between the pathogen and bacteria resulted in complete inhibition of conidial germination. The results indicated that although the competition for nutrients might be one mechanism of biocontrol activity of CPA-2, physical contact between the pathogen and the BCA was important for greater effectiveness of the biocontrol activity against the mold pathogens infecting citrus fruits (Poppe et al. 2003). The ability of *Pantoea agglomerans* strain CPA-2 to elicit resistance in oranges against *Penicillium digitatum* was investigated, by determining the generation of hydrogen peroxide (H_2O_2) and changes in the activities of superoxide dismutase (SOD) and catalase (CAT) activities at different periods after inoculation of oranges with the pathogen. At 3 days after inoculation, CPA-2 treated fruit showed an accumulation of H_2O_2. The activities of SOD and CAT also registered increases in oranges treated with CPA-2 strain, while infected fruit showed sharp decreases in the levels of all three enzymes. The results suggested that as a response to pathogen infection, H_2O_2 production, as well as activities of SOD and CAT, were suppressed, whereas the BCA strain accelerated the H_2O_2 generation and enzyme activities involved in the activation of disease resistant systems. The CPA-2 strain appeared to have multiple mechanisms of biocontrol activity against green mold pathogen *P. digitatum* infecting stored orange fruits (Torres et al. 2011).

The biocontrol potential of another strain 59–4 of *Pantoea agglomerans* in suppressing the development of green and blue mold diseases in mandarin oranges was assessed. The strain Pa 59–4 was isolated from soil samples from different locations in Korea. The concentration of cells of Pa 59–4 required for the inhibition of *Penicillium digitatum* (causing green mold) was less (10^5–10^6 cells/ml) than the concentration (10^8–10^9 cells/ml) required for inhibition of *P. italicum* (causing blue mold). Pa 59–4 suppressed infection by green and blue mold pathogens on wounded mandarins to the extent of 85–90 % and 75–80 % respectively. Higher concentrations of Pa 5904 provided better protection, indicating the dosage effect of the BCA. The ability to effectively colonize wounds on the peels of citrus fruit is important for the survival of Pa 59–4, because its antagonistic action was primarily based on physical contact with the pathogen in infection site and also on competition for nutrients. Pa 59–4 in wounded mandarin fruits increased more than ten times during 24 h of incubation at 20 °C. The results indicated that competition for nutrient might play a role in the inhibition of conidial germination, as the strain Pa 59–4 could not inhibit the mycelial growth of *Penicillium* spp. in plate confrontation assays (Yu et al. 2010).

The inconsistency of the performance of biocontrol agents to provide effective fire blight disease suppression may be due to instability of antagonist populations, environmental conditions or host physiological changes associated with flower development and senescence. Knowledge on these factors will be useful for improving the efficacy and consistency of performance of the bacterial biocontrol agents like *Pantoea agglomerans* strain E325 and *Pseudomonas fluorescens* A506 that have been reported to be effective against *Erwinia amylovora*. The flower stigma is of considerable

importance as the site of pathogen establishment and the site where antagonist adaptation is considered to be critical for effective biocontrol. Growth of *E. amylovora* on flower stigma is dependent on temperature as revealed by forecasting systems (Billing 2000). The temperature affects the duration of stigma receptivity to pollen and the stigma senescence is accelerated after pollination. Generally the pathogen reached higher population levels than the antagonists at temperatures from 20 to 32 °C. The populations of *P. agglomerans* strains C9-1 and E325 attained relatively high levels at these temperatures (Pusey and Curry 2004). The difference in temperature optima of strains A506 and C9-1 was considered to be a mode by which these two antagonists could complement each other when applied as a mixture. The dominant strain tended to be A506, during relatively low temperature years and C9-1 in high temperature years (Johnson and Stockwell 2000). Detached Manchurian crab apple flowers were inoculated with *E. amylovora* and subjected to a range of constant temperatures or various fluctuating temperature regimes. Maximum stigma age supporting bacterial multiplication decreased, as temperature increased and was reduced by pollination. Stigmas were receptive to bacteria at ages older than the age reported earlier, possibly because of less interference from indigenous organisms. The results indicated that antagonists could not grow on relatively old stigmas conducive to the pathogen. It may be possible to select antagonists that can develop on old stigmas rapidly to exclude the pathogen from such ecological niche (Pusey and Curry 2004).

5.1.8 Agrobacterium *spp.*

The mechanism of suppression of crown gall disease caused by *Agrobacterium tumefaciens* by the non-pathogenic *Agrobacterium rhizogenes* (*A. radiobacter*) strain K84 is unique. Differentiation of the pathogen and the biocontrol agent is based only on the presence of the plasmid with T-DNA responsible for infection in the pathogen and its absence in the BCA. On the other hand, the BCA possesses another plasmid responsible for the production of a bacteriocin termed agrocin 84 which suppresses the development of *A. tumefaciens* and gall formation in susceptible host plants (Kerr 1980). The strain K84 was not found to be effective against all strains of *A. tumefaciens*. *A. vitis* causing crown gall disease of grapevine was not sensitive to agrocin 84. Agrocin 84 targeted a tRNA synthetase in tumorigenic *Agrobacterium* strains (Reader et al. 2005). *Agrobacterium tumefaciens* causes crown gall disease in large number of host plants including crop plants. *Agrobacterium* strains D286, HLB2 and K1026 produced agrocins effective against nopaline strains of *A. tumefaciens*. Strain K84 was effective only against nopaline strains of *A. tumefaciens,* but not against octopine strains causing crown galls on grapevine. A nonpathogenic strain of *A.vitis* E26 was found to be effective against *A. vitis* causing crown galls on grapevine and also against *A. tumefaciens* and *A. radiobacter* infecting peach and cherry respectively. An antibacterial compound Ar26 with MW 76-kDa was isolated from the culture supernatant of the strain E26. The compound Ar26 strongly inhibited the growth of *A. vitis* MI3-2 and *A. tumefaciens* CY4 on culture plates (Wang et al. 2003).

Another strain of *Agrobacterium vitis* F2/5 inhibited the growth of most tumorigenic strains of *A. vitis* in vitro and inhibited crown gall development on grapevine in stem-wounding experiments in the greenhouse. The strain F2/5 also produced a bacteriocin and effectively inhibited tumor formation at wound sites on grapevine stems inoculated with a tumorigenic strain of *A. vitis* (Burr and Reid 1993). Suppression of tumor development in grapevine by the strain F2/5 was not dependent on the production of agrocin, since the agrocin-minus mutant of the BCA was able to reduce gall production as effectively as the wild-type strain (Burr et al. 1997). A new strain K1026 was constructed by using recombinant DNA techniques. The new strain was identical to K84 apart from 5.9-kb deletion overlapping the Tra region of pAgK84. The strain K1026 was unable to transfer its mutant agrocin 84 plasmid, designated pAgK1026 to other agrobacteria, but it remained antagonistic to strains sensitive to agrocin (Jones and Kerr 1989). Agrocin mutants of nontumorigenic *A. vitis* strain F2/5 suppressed grape crown gall development as the wild-type strain, indicating agrocin was not the major factor in the mechanism of biological disease suppression. Relative levels of attachment to grape cells by tumorigenic and biocontrol strains were measured. Attachment of tumorigenic strains (CG49 and K306) and biocontrol strains (F2/5 and agrocin mutant 1077) was frequently reduced, when mixtures of the strains were applied. However, high populations (10^3–10^5 CFU/ml) of all strains attached following mixed inoculation, suggesting that competition for attachment sites was not a factor in the mechanism of biocontrol activity of the BCA strains tested. Transfer of T-DNA to grape by CG49 was prevented or greatly inhibited in the presence of F2/5 or 1077 as measured by expression of the GUS reporter gene. Sonicated and autoclaved preparation of F2/5 and 1077 did not suppress crown gall development in grapevine or inhibit the T-DNA transfer to grapevine cells. Suppression of crown gall disease by the strain F2/5 was specific to grapevine, since it was ineffective against gall formations on tomato and sunflower (Burr et al. 1997).

A nonpathogenic *Agrobacterium vitis* strain VAR 03–1 capable of producing a bacteriocin, greatly inhibited tumor formation on stems of tomato and grapevine seedlings caused by several tumorigenic *A. vitis* strains. When roots of grapevine, rose and tomato were soaked in a cell suspension of VAR 03–1, before planting in soil infested with tumorigenic *A. vitis, A. rhizogenes* and *A. tumefaciens* respectively, the antagonist treatment significantly reduced the number of plants with tumors and disease severity in the plant species tested. The inhibitory effects of treatment with VAR 03–1 on grapevine was more effective than that of treatment with K84, whereas the effects of treatment with both BCA strains on rose and tomato were almost similar. The strain VAR 03–1 was bacteriocinogenic, producing a halo of inhibition against all the three species of *Agrobacterium*. Under field conditions, the strain VAR 03–1 was highly effective in reducing the incidence of the crown gall disease in grapevine. This strain could establish populations averaging 10^6 CFU/g of roots in the rhizosphere of grapevine and persisted on roots for 2 years. The results indicated that the strain VAR 03–1 could be applied for effective control of crown gall diseases caused by *Agrobacterium* spp. (Kawaguchi et al. 2008).

5.1.9 Myxobacteria

Myxobacteria are Gram-negative, unicellular bacteria with rod-shaped vegetative cells. They are soil dwelling, gliding bacteria that form fruiting bodies containing myxospores. Thick-walled myxospores are resistant to desiccation, high temperature and UV-radiation and hence, they are responsible for the survival of myxobacteria. These bacteria specialize in biodegradation of biomacromolecules and are considered as micropredators, because the antibiotics and/or enzymes produced by them can kill microorganisms and lyse cells from which bio-macromolecules are then scavenged (Dawid 2000). Myxobacteria produce many different classes of antibiotics, cell wall-degrading enzymes, lipases, nucleases, polysaccharidases and proteases. The antibiotic pyrrolnitrin produced by myxobacterial species is of agricultural importance (Rosenberg and Varon 1984).

Myxococcus spp. were isolated from soils in organic and conventionally managed strawberry production and transplant fields that were not treated with methyl bromide or chloropicrin which virtually eliminated these bacteria. Six *Myxococcus* spp. were evaluated for the antagonistic activity against fungal pathogens and two well known fungal biocontrol agents *Gliocladium virens* and *Trichoderma viride*. The pathogen *Phytophthora capsici*, *Pythium ultimum, Rhizoctonia* spp., *Sclerotinia minor* and the BCA *T. viride* were entirely inhibited by all of the *Myxococcus* spp. tested. *M. coralloides* inhibited the growth of *Cylindrocarpon* spp. *Fusarium oxysporum* f.sp. *apii, Verticillium albo-atrum* and *V. dahliae,* in addition to the fungal pathogens that were inhibited by other *Myxococcus* spp. The BCA *Pseudomonas fluorescens* CHA0 was protected by secondary metabolite production regulated by *gacS* from lysis by myxobacteria. Phenazine antibiotics were responsible for the protection of *P. aureofaciens* strain 30–84 from lysis by the myxobacteria (Bull et al. 2002). *Myxococcus* spp. strain KYC 1126 inhibited completely the spore germination of *Botrytis cinerea*, *Colletotrichum acutatum* and *Magnaporthe grisea*. The activity of the bioactive compounds present in the culture supernatant was found to be fungicidal, but the liquid culture filtrate of KYC 1126 did not affect protoplast reversion in *C. acutatum.* Application of culture filtrate on hot pepper plants reduced the incidence of anthracnose disease significantly under both greenhouse and field conditions (Kim and Yun 2011).

5.1.10 Achromobacter

Achromobacter spp. is a Gram-negative bacteria and catalase-positive. It can be obtained frequently from the rhizosphere. *A. xylosoxydans* isolate MM1 was isolated from a *Fusarium*-suppressive soil in Italy. The biocontrol potential of the isolate MM1 was assessed against Fusarium wilt disease of tomato caused by *F. oxysporum* f.sp. *lycopersici.* The bacterial strain was applied by dipping plant roots in bacterial cell suspension (1×10^8 CFU/ml). The disease incidence was

reduced by about 50 % under severe disease pressure conditions, while up to 81.3 % infection was recorded in the control treatment. In vitro assays, no chitinolytic activity or antibiotic production by the isolate MM1 could be detected and no inhibition of growth of the pathogen was observed in plate confrontation tests. The isolate produced siderophore in malt extract agar (MEA) medium supplemented with $FeCl_3$. The disease suppressive effect of isolate MM1 in vivo might be related to its ability to produce siderophores that limit the bioavailability of iron to the pathogen. In addition, the possibility of *A. xylosoxydans* eliciting induced systemic resistance (ISR) in tomato against the wilt pathogen was also suggested by Moretti et al. (2008).

5.1.11 Actinomycetes

Actinomycetes are generally saprophytic, soil dwelling bacteria, spending majority of their life cycle as spores. They represent a high proportion of the soil microbial biomass and have the potential to produce a wide range of antibiotics and extracellular enzymes. Several strains of actinomycetes are able to protect crop plants against diseases caused by microbial pathogens and some of them promote growth of plants too. The antibiotic geldanamycin produced by *Streptomyces hygroscopicus* var. *geldanus* suppressed the development of *Rhizoctonia solani* causative agent of Rhizoctonia root rot disease of pea. The methanol extracts of soils in which the antagonist was incubated for two or more days, inhibited the growth of *R. solani*. The concentration of geldanamycin was 88 µg/g of soil at 7 days after incubation. The period of incubation necessary for antibiotic production and disease suppressed matched with each other (Rothrock and Gottlieb 1984). In a later study, *S. hygroscopicus* var. *geldanus* strain EF-76 was shown to protect potato against common scab disease, indicating the vital role of geldanamycin production by this BCA strain in its ability to control the disease (Beauséjour et al. 2001).

Streptomyces diastatochromogenes strain PonSSII produced antibiotics inhibitory to pathogenic strains of *S. scabies* causing potato scab disease and reduced the number lesions formed on tubers significantly. The inhibitory effect was directly correlated to the more vigorous growth and larger inhibition zones of the strain PonSSII against pathogenic strains, compared with another BCA strain PonR in in vitro tests. Production of antibiotic by the strain PonSSII could be stimulated or repressed by the presence of other *Streptomyces* strains including pathogenic strains. It appeared that interspecies/strain communication could be of significance in pathogen suppression (Becker et al. 1997). *S. violaceusniger* strain YCED9, isolated from the rhizosphere, suppressed the development of damping-off disease of lettuce caused by *Pythium ultimum* (Crawford et al. 1993). The strain YCED9 produced nigericin, geldanamycin and a fungicidal complex of polyene-like compounds designated AFA (anti-*Fusarium* activity) that included guanidylfungin A. Production of these antimicrobial compounds was significantly influenced by the composition of media on which the strain YCED9 was grown. The actinobacteria strain provided effective

protection against the turfgrass pathogens *Colletotrichum graminicola, Sclerotinia homeocarpa, Gaeumannomyces graminis* and *Rhizoctonia solani* (Trejo-Estrada et al. 1998). *Streptomyces* spp. isolated from the soil was evaluated for its ability to suppress the growth of *Sclerotium rolfsii* and development of damping-off disease in sugar beet. Among the four isolates, J-2 was the most efficient in inhibiting the mycelial growth of *S. rolfsii*. Inhibition of sclerotial germination occurred in soils treated with biomass inoculum and culture filtrate of the isolate J-2, the percentage of inhibition being 93 and 88 % respectively. Seed treatment with the isolate J-2 resulted in significant reduction in disease severity in the seedlings. The results suggested that antibiosis might be the mechanism of biocontrol activity of *Streptomyces* spp. against *S. rolfsii* (Errakhi et al. 2007).

Streptomyces rimosus MY02, isolated from soil, produced antifungal metabolites in the culture medium. An antifungal metabolite SN06 was extracted from the culture of the strain MY02 using n-butanol and purified by silica gel column chromatography. The minimum concentration of SN06 inhibitory to *Fusarium oxysporum* f.sp. *cucumerinum* was 12.5 μg/ml. Using light microscope, the changes in morphology of *F. oxysporum* f.sp. *cucumerinum* treated with SN06 were determined. Some of the hyphal cells began to dilate and formed some strings of heads. The cytoplasm oozed out of the cells and most of hyphal cells were empty. The hyphae were cleaved into many segments and then collapsed after 48 h. Damage to mycelial cell membrane and leakage of nucleic acid from the damaged fungal cells were indicated by scanning, using ultraviolet spectrophotometer and absorption at 260 nm (Liu et al. 2009a, b). The *Streptomyces* strain (AS1) inhibited both germination and growth of *Aspergillus flavus* producing the mycotoxin aflatoxin B_1(AFB_1) both directly or via secondary metabolites. The core extracts of AS1 metabolites at 50 and 100 ppm entirely inhibited germination of conidia of *A. flavus* over 48 h. The metabolites extractable with ethylacetate were the most effective. Concentrations of 50, 200 and 500 ppm of AS1 metabolites significantly inhibited the populations of *A. flavus* on stored peanuts at two water stress levels (0.90, 0.93 a_w) at 25 °C over 14-day storage periods. The amounts of AFB_1 produced by *A. flavus* on peanuts were also reduced by application of AS1 metabolites, indicating the inhibitory effects of metabolites on the population of *A. flavus* and the amount of mycotoxin produced (Sultan and Magan 2011).

Streptomyces hygroscopicus strain SRA14 was assessed for its potential to produce extracellular hydrolytic enzymes that may be involved in its biocontrol activity against *Colletotrichum gloeosporioides* and *Sclerotium rolfsii.* Production of extracellular chitinolytic and β-1,3-glucanolytic enzymes by the strain SRA14 was determined at different growth phases. The level of chitinase was sharply increased during the exponential phase and dramatically declined, when the cells entered the stationary phase. The highest level of β-1,3-glucanase was produced at 2 days after incubation period and decreased slightly during the stationary phase. Inhibition of mycelial growth of the fungal pathogens *C. gleosporioides* and *S. rolfsii* was due to the activities of chitinase and β-1,3-glucanase secreted by the strain SRA 14 (Fig. 5.12). Observations under light microscope to determine the changes in the fungal morphology, following exposure to cell-free culture

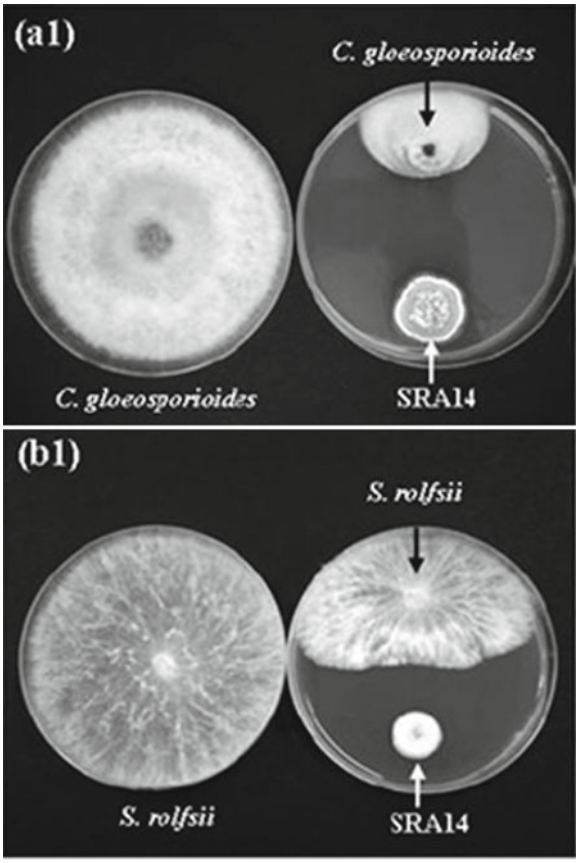

Fig. 5.12 Inhibition of mycelial growth of *Colletotrichum gloeosporioides* and *Sclerotium rolfsii* by *Streptomyces hygroscopicus* SRA14 in dual culture plate assay (Courtesy of Prapagdee et al. 2008 and with kind permission of Ivyspring International Publisher)

filtrate of SRA14 revealed hyphal swelling, distortion and cytoplasm aggregation, indicating adverse effects of extracellular metabolites on the pathogens growth (Prapagdee et al. 2008).

The biocontrol agents and the phytopathogens have to compete with each other for nutrients and space available in the habitat. Some actinomycetes are able colonize inside the roots and other plant tissues and they are known as endophytic actinomycetes. *Streptomyces griseorubiginosus* strain S96 colonized healthy roots of banana and its biocontrol potential against *Fusarium oxysporum* f.sp. *cubense* (*Foc*) race 4, the causal agent of Panama (Fusarium) wilt disease was assessed. The antagonism of strain S96 against *Foc* was conditioned by the bioavailability of iron. There was no antagonism, when the pathogen and BCA were inoculated onto banana tissue extract (BTE) medium containing more than 0.05 % $FeC1_3$. But the BCA was antagonistic in the same medium containing less than 0.05 % $FeC1_3$, indicating that iron-limiting condition favored the antagonistic activity of the strain S96. In addition, the antagonism of strain 96 was lost, when FeCl3 was introduced into the inhibition zone. In vivo biocontrol assays demonstrated that the disease severity

index (DSI) was significantly reduced and mean fresh weight increased in plantlets treated with strain S96, compared to untreated control plantlets (Cao et al. 2005).

Rhizosphere competence of the biocontrol agents is one of the critical requisites for effective suppression of soilborne pathogens. *Streptomyces griseoviridis* has been reported to be a good rhizosphere colonizer. *S. griseoviridis* was found to be an efficient antagonist of fungal pathogens, causing diseases such as Fusarium wilt disease of carnation, damping-off disease of Brassica and root rot disease of cucumber (Tahvonen and Lahdenpera 1988). The mechanism of root colonization by *Streptomyces* spp. may be affected by different factors. Various plant species produce different types and concentration of compounds in their root exudates which may significantly influence root colonization by various kinds of microorganisms including actinomycetes. The potential of *S. griseoviridis* applied as seed treatment for barley and spring wheat was assessed against foot rot disease. Yields of wheat were increased to a greater extent by seed treatment with *S. griseoviridis* than that of barley, probably because the BCA colonized wheat roots more effectively (Weller 1988). *Streptomyces globisporus* JK-1 was evaluated for its antifungal activity against the postharvest pathogen *Penicillium italicum* causing blue mold disease of citrus fruits. The strain JK-1 inhibited the spore germination and mycelial growth of *P. italicum*. Sporulation and disease incidence on pathogen-inoculated fruit were suppressed in the presence of volatiles produced by the strain JK-1. Treatment of naturally infected citrus fruit with the BCA reduced the disease incidence from 25 to 7.5 %. Scanning electron microscopic observations revealed inhibition of spore germination on the Shatang Mandarin orange and abnormal morphology of conidiophores and hyphae exposed to the volatiles of JK-1. Gas chromatography/mass spectrophotometric analyses indicated the presence of 41 organic compounds in the volatiles of JK-1. These volatiles showed inhibitory effect to varying degrees. Dimethyl disulfide or dimethyl trisulfide showed antifungal activity in in vitro assays and also effectively reduced the disease incidence on Shatang Mandarin, when applied at a concentration of 100 µl/l of air space in treatment containers. Acetophenone was effective in suppressing disease development only at higher concentration (1,000 µl/l). The results indicated the potential of the volatiles produced by *S. globisporus* JK-1 for effective suppression of development of blue mold disease of citrus caused by *P. digitatum* (Li et al. 2010).

The effect of volatiles produced by *Streptomyces globisporus* JK-1 on the development of *Botrytis cinerea,* causing gray mold of tomato fruit was investigated. *S. globisporus* was grown on autoclaved wheat seed. The mycelial growth, conidial germination and sporulation of *B. cinerea* and *Sclerotinia sclerotiorum* were suppressed by the volatile compounds produced by the strain JK-1. Incidence and severity of gray mold disease on wound-inoculated tomato fruit were inhibited, when fumigated with 120 g wheat seed culture of JK-1 per liter of air space in treatment containers. Scanning microscopy was used to follow the infection process of *B. cinerea* on tomato fruit. Inhibition of conidial germination and appressorial formation on tomato fruit were observed as well as abnormal morphology of appressoria and conidia. Using the vital stain fluorescein diacetate (FDA) and propidium iodide (PI),

the viability of conidia, after exposure of the disease lesions to the volatiles, was assessed. Reduction in conidial viability varied from 46 to 48 % depending on the concentration of the BCA culture. Transmission electron microscopy (TEM) of fumigated and untreated *B. cinerea* showed excessive vesiculation or thickened cell walls in exposed conidia and increased vesiculation or strong retraction of plasma membrane in exposed hyphae. The results indicated that the mode of action of *S. globisporus* JK-1 could be antagonism, due to the fumigant action of the volatiles produced by the BCA which has the potential for the control of postharvest gray mold disease of tomato fruits (Li et al. 2012).

The efficacy of native *Streptomyces* isolates C and S2 was evaluated for the control of fungal pathogens *Rhizoctonia solani* AG-2, *Fusarium solani* and *Phytophthora drechsleri* associated with sugar beet root rot disease. The isolate C inhibited the mycelial growth of *R. solani*, *F. solani* and *P. drechsleri* by 45, 53 % and 26 % respectively. Incorporation of NaCl into the medium enhanced the biocontrol activity of soluble and volatile compounds of the isolate C and S2. The inhibition percentages of all the pathogens were increased by NaCl addition to the medium. The mechanism of antagonism of *Streptomyces* isolates C and S2 was investigated by analyzing the activities of protease, chitinase, β-glucanase, cellulase, lipase and amylase. Biosynthesis of siderophore by both isolates was also detected. Production of siderophore and activity of protease and amylase by both isolates were increased after adding salt. In contrast, chitinase activity decreased significantly. Production of SA, β-1,3-glucanase and lipase by isolate S2 and biosynthesis of cellulase by isolate C were observed in the presence and absence of NaCl. Soil treatment with *Streptomyces* isolate C reduced the incidence of root rot disease of sugar beet caused by the fungal pathogens. The results indicated that these two isolates of *Streptomyces* could effectively suppress the development of sugar beet root rot disease especially in saline soils (Karim et al. 2012).

The mechanism of biocontrol activity of *Bacillus subtilis* CPA-8 with ability to suppress the development of *Monilinia laxa* and *M. fructicola* infecting peach fruit was investigated. The cell-free supernatants and butanolic extracts of the cultures of the strain CPA-8 showed strong antifungal activities against both fungal pathogens. The presence of fengycin, iturin and surfactin lipopeptides in the butanolic extracts from cell-free supernatants were identified by employing thin layer chromatography (TLC), indicating that antibiosis could be a major factor involved in the biocontrol activity of CPA-8. Strong antifungal activity could be linked to fengycin lipopeptides, as indicated by the results of TLC-bioautography analysis. The transformants from CPA-eight lacking fengycin had reduced or suppressed antifungal activity. Pathogenicity experiments confirmed that fengycin-defective mutants and their cell-free supernatants lost their ability to suppress the development of peach brown rot disease, compared with wild-type CPA-8 strain or Serenade Max®, a commercial product based on *B. subtilis*. However, the fengycin-deficient mutant survived in wounds in peach fruit equally well as the wild-type strain. The results showed that the biocontrol activity of *B. subtilis* CPA-8 depended primarily on its ability to produce fengycin-like lipopeptides for the control of brown rot disease of peach fruits (Yánez-Mendizábal et al. 2012).

Appendix 5.1: Visualization of Effects of the Metabolite 2,4-diacetylphloroglucinol (2,4-DAPG) of *Pseudomonas* spp. on Fungal Pathogen Using Confocal Laser Scanning Microscope (CLSM) (Islam and Fukushi 2010)

A. Preparation of fungal hyphal cells for visualization

i. Grow the fungal pathogen (*Aphanomyces cochlioides*) on potato dextrose agar (PDA) at room temperature (25 °C); cut out agar disks (6 mm diameter) from the growing edges; place the disk individually 30 cm apart from the colony of bacterial biocontrol agent (*Pseudomonas fluorescens* ECO-001) in four replicates: prepare control plates without the BCA and incubate at 25 °C for 5 days in the dark

ii. Harvest the fungal hyphae, using a sterile corkborer (6 mm diameter) from the colony edges growing toward the BCA colonies for observations under confocal laser scanning microscope (CLSM)

iii. Prepare different concentrations of DAPG and latrunculin B (0.1, 0.5, 1.0 and 5.0 μg) in acetone; place the solutions on individual sterile paper disks (8 mm diameter × 1.5 mm thickness) (Advantec Toyo, Japan) and dry the disks by evaporating acetone under vacuum

iv. Place the disks 2 cm apart in petriplates containing PDA inoculated with mycelial plugs (6 mm diameter); cut from the edge actively growing fungal pathogen colony and incubate at 25 °C

B. Preparation of specimen and observation under CLSM

i. Remove mycelial plugs using a sterile cork borer (6 mm diameter) from the edge of the actively growing hyphae of the pathogen paired with bacterial colony or DAPG or latrunculin B-treated paper disks and use plugs removed from untreated plates as control.

ii. Fix the mycelial plugs with 6 % paraformaldehyde in 60 mM 1,4-piperazine- diethanesulfonic acid buffer (Sigma) pH 7.0 with 100 μM MBS (*m*-maleimidobenzoyl *N*-hydroxyl succinimide ester, Pierce) for 30 main at room temperature; rinse three times in a buffer solution and transfer to glass slides for sectioning

iii. Section the upper portion of the agar plug uniformly (0.25 mm thickness) with a sterilized stainless blade

iv. Stain the sections for 30 min in 0.66 μM RP in 60 mM Pipes buffer (pH 7.0); rinse in buffer and mount in 50 % glycerol with 0.1 % p-phenylene-diamine and observe under CLSM

v. Use scan time perframe 1.08 s; to obtain a DIC image and perform averaging at four scans per frame

vi. Repeat the experiment three times with three replicates for each experiment

Appendix 5.2: Assessment of Effect of Phenazines on Microsclerotial Germination of *Verticillium* spp. by Microplate Assay (Debode et al. 2007)

i. Place nylon mesh filters (pore size 41 µm, diameter 25 mm, Millipore, USA) in the wells of 96-well microplate; embed the microsclerotial preparation (20 µl) in the well (approx. 50 microsclerotia/well); add aliquots of 180 µl bacterial suspension (2×10^7 and 2×10^9 CFU/ml); use sterile physiological solution (180 µl) for controls and incubate for 2 days at 24 °C
ii. Retrieve the filters from the wells separately; place them on sterile filter paper under sterile conditions and dry the filters
iii. Place the filters on soil-pectate-tergitol agar (SPTA) plates containing each 50 mg/l of chloramphenicol, tetracycline and streptomycin sulfate and incubate for 10 days
iv. Examine under the dissecting microscope and record the effect on percentage of microsclerotial germination and formation of secondary microsclerotia in different treatments
v. Maintain five replications and repeat the experiment once

Appendix 5.3: Assessment Antagonistic Activity of Bacterial Antagonists Against *Agrobacterium* spp. (Dandurishvili et al. 2010)

A. Dual-culture 'sandwich' assay

i. Fill a petridish with suitable medium seeded with the test antagonist strain and fill another petridish similarly with medium inoculated with the target pathogen strain (overnight culture) at appropriate dilution (10^5 cells/ml)
ii. Join the open plates together and tightly seal with parafilm maintain suitable control (without the antagonistic strain)
iii. Incubate the plates at 28 °C and examine the samples taken at 24-h interval

B. Bioassays for tumorigenicity in the greenhouse

i. Raise the tomato seedling nursery in plastic seed trays for 25–30 days at 25 °C in a growth chamber; transplant the seedlings at 2–3 leaf stage into bigger containers and placed in the greenhouse
ii. Grow the bacterial pathogen and the BCA strains separately in suitable medium for 48 h at 28 °C in a shaker; centrifuge the suspension at 8,000 rpm for 15 min; resuspend the bacterial cells in tap water and adjust the concentration to 10^8 cells/ml
iii. Soak the roots of tomato seedlings in glass vessels containing the BCA cell suspension at 10^7 cells/ml and transplant the seedlings into containers placed in the greenhouse

iv. Make wounds by scratching on the stem surface of seedlings with a needle at two sites (second and third internodes); inject into the wound sites with pathogen suspension (10 µl, 2–5 x 10^8 cells/ml) and treat the control plants similarly only with the pathogen cell suspension
v. Inoculate tomato seedling in the wound sites first with the antagonist cell suspension; after 7 days inoculate the pathogen in the same wounds by injecting the pathogen suspension (10 µl/wound site)
vi. Record the tumor formation in tomato plants inoculated by two different methods and express the disease incidence in terms of tumor fresh weight in different treatments

Appendix 5.4: Assessment of Activities of Enzymes in Papaya Fruits Treated with *Pseudomonas putida* MGY2 (Shi et al. 2011)

A. Phenylalanine-ammonia lyase (PAL) (EC4.3.1.5) activity

i. Perform all steps at 4 °C; homogenize pericarp tissue samples (1 g) in 2 ml of extraction buffer (50 mM Tris–HCl buffer, pH 8.8 containing 15 mM B-mercaptoethanol, 5 mM EDTA, 5 mM ascorbic acid, 1 mM PMSF and 0.15 % PVP (w/v)); filter the homogenate through cheesecloth (four layers); centrifuge at 12,000 g for 20 min at 4 °C and use the supernatant as crude enzyme
ii. Prepare the reaction mixture (3 ml) containing 16 mM L-phenylalanine, 50 mM Tris–HCl buffer (pH 8.8), 3.6 mM NaCl and 0.1 ml of enzyme solution and incubate at 37 °C for 40 min
iii. Stop the reaction by adding 500 µl of 6 mM HCl; centrifuge at 12,000 g for 10 min to precipitate the denatured protein
iv. Record the absorbance at 290 nm using a spectrophotometer before [step (ii)] and after incubation
v. Use cinnamic acid (analytical grade) as standard; one unit of PAL activity is equal to the amount enzyme that produced 1 µmol of cinnamic acid within 1 h
vi. Express the results as µmol of cinnamic acid/mg protein/h

B. Catalase (CAT) (EC1.11.1.6) activity

i. Grind pericarp tissues (5 g) from five papaya fruits in 20 ml of 0.05 M sodium borate buffer (pH 7.0, containing 5 mM mercaptoethanol) with 0.5 g polyvinyl polypyrrolidone (PVPP).
ii. Add 0.2 ml of enzyme preparation to 2.8 ml of 40 mM H_2O_2 (dissolved with 50 mM sodium phosphate buffer, pH 7.0) as a substrate.
iii. Measure the decomposition of H_2O_2 by the decline in absorbance at 240 nm
iv. One unit of CAT is equal to the amount of enzyme per microgram protein that decomposed 1 mol of H_2O_2/min at 30 °C

C. Peroxidase (POD) (EC 1.11.1.7) activity

i. Homogenize the pericarp tissues (1 g) in 4 ml of extraction buffer (0.2 M sodium phosphate buffer, pH 6.4 containing 0.2 % PVP (w/v)) at 4 °C; centrifuge at 12,000 g for 20 min and use the supernatant as crude enzyme
ii. Incubate the mixture containing 0.5 ml enzyme extract and 2 ml of guaiacol substrate (100 mM sodium phosphate, pH 6.4, containing 8 mM guaiacol) for 5 min at 30 °C
iii. Determine the increase in absorbance at 460 nm after adding 1 ml H_2O_2 (24 mM)
iv. One unit of POD activity is equal to the amount of enzyme per microgram protein that increased 0.01 in absorbance at 460 nm in 1 min

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Chapter 6
Detection and Identification of Viral Biological Control Agents

Viruses can infect all organisms from human beings to prokaryotic bacteria. The viruses may induce specific symptoms of infection or some of the hosts may remain asymptomatic even when infected by viruses. Fungal and bacterial pathogens that cause serious economically important diseases of crops may be infected by viruses. The possibility of employing these viruses as biocontrol agents of crop pathogens has attracted the attention of researchers in different countries. The presence of virus-like particles or viruses in fungi and bacteria has been detected and they have been characterized for assessing their biocontrol potential against microbial plant pathogens.

6.1 Viruses Pathogenic to Fungal Plant Pathogens

Reduction in pathogenic potential of fungal pathogens due to infection by viruses is designated hypovirulence. Hypovirulence as a natural phenomenon that limited the chestnut blight epidemics in Italy was first discovered by Biraghi (1953). Hypovirulence was shown to be not associated with resistance of chestnut tree, but to hypovirulence of the fungal pathogen *Cryphonectria parasitica* (= *Endothia parasitica*) which causes the destructive blight disease of chestnuts (*Castanea* spp. and *Quercus* spp.). Hypovirulence is considered to play a role in counterbalancing plant disease in nature. Mycoviruses are known to infect all major taxa belonging to the kingdom Fungi. The mycoviruses may have double-stranded (ds)-RNA or single-stranded (ss)-RNA as their genomes. The mycoviruses containing ds-RNAs are classified into three families based on the number of genome segments, capsid structure and nucleotide sequences with some ds-RNA viruses remaining unclassified (Ghabrial 1998). The isometric ds-RNA mycoviruses are classified into two families *Totiviridae* and *Partitiviridae* which include viruses with nonenveloped isometric particles of 25–50 nm diameter and typically induce latent infection in their host fungi. *Totiviruses* have non-segmented genomes, while *Partitiviruses* have segmented genomes

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(Ghabrial 1998). Hypoviruses included in the family *Hypoviridae* lack conventional virions and their ds-RNA are enclosed in the host-encoded vesicles (Dawe and Nuss 2001). A geminivirus-related DNA mycovirus conferring hypovirulence to a plant pathogenic fungus *Sclerotinia sclerotiorum* was first isolated and characterized by Yu et al. (2010).

6.1.1 Biological Properties of Mycoviruses

The phenomenon of hypovirulence is expressed in some fungal plant pathogens which become less virulent due to a ds-RNA-mediated morphological, physiological and biochemical modifications. The potential of hypovirulence induced by ds-RNA viruses was demonstrated as a biological disease management approach for chestnut blight disease. The determinant for hypovirulence of *Cryphonectria hypovirus*1 was shown to be inherited cytoplasmically (Grente and Surete 1969). Infection of *Cryphonectria parasitica*, causative agent of chestnut blight disease by *Cryphonectria hypovirus* (CHV) induces reduction in fungal virulence, ranging from avirulence to almost normal virulence (Rigling et al. 1989). Fungal isolates containing the European-type hypovirus (CHV1) exhibited reduced sporulation and pigmentation, resulting in a typical white cultural appearance. *Diaporthe RNA virus* (DaRV) induced hypovirulence in *Diaporthe perjuncta,* infecting grapevines in South Africa. Apple stem pieces inoculated with virus-free *D. perjuncta* produced perithecia containing ascospores. But DaRV-transfected *D. perjuncta* isolate and the naturally infected isolate produced neither ascospores nor perithecia (Moleleki et al. 2003). Different subtypes of CHV1 were found to be distributed in different European countries (Allemann et al. 1999). The hypovirus CHV2 was isolated in North America and later some variants of *Cryphonectria hypovirus* 3 and 4 (CHV3 and CHV4) were distinguished in the isolates occurring in the eastern North America (Linder-Basso et al. 2005).

Cryphonectria hypovirus-CHV1 has been the only example available for utilization of mycoviruses as biocontrol agents, under field conditions, against the fungal pathogens in which they are able to induce hypovirulence. The success of biological control with hypoviruses depends on their ability to reduce the virulence of target fungal pathogen. Hypoviruses are transmitted from a hypovirulent strain by hyphal fusion (anastomosis), when these two strains are compatible vegetatively. Hypoviruses, however, cannot be transmitted by any other mode, as they cannot remain infectious in an extracellular state. It is well known that closely related fungal strains are vegetatively compatible. Hence, vegetative incompatibility among many fungal species in agricultural ecosystems is recognized as a major barrier to the use of hypoviruses as biological control agents (Leslie 1993). *Cryphonectria hypovirus-1* (CHV1) was successfully used as a biocontrol agent of *C. parasitica* in Europe. CHV1-infected strains of *C. parasitica* exhibited reduced virulence, reduced asexual and sexual sporulation and reduced pigmentation. But CHV1 was not effective as a

biocontrol agent in North America, because the host fungus in North America had multiple vegetative compatibility groups (VCGs) that did not allow hyphal anastomosis and consequently the spread of the virus was seriously hampered (Nuss 2005). The failure of mycovirus transmission due to vegetative incompatibility could be overcome by employing protoplast fusion technology in vitro (Lee et al. 2011). Among *Fusarium* species, ds-RNA mycoviruses have been detected in *Fusarium graminearum, F. poae* and *F. solani* f.sp. *robiniae*. The ds-RNA elements of the same electrophoretic mobility isolated from vegetatively compatible strains of *F. poae* were similar or identical in many cases. In the case of *F. graminearum*, occasionally colonies with unusual morphology, slow growth, amoeboid mycelia and increased pigmentation were observed. It was not possible to reliably attribute phenotypic characteristics to the presence of the mycovirus (Xu et al. 1992; Chu et al. 2002). Significant differences in mycelial growth rates of *Fusarium graminearum* isolates containing the ds-RNA were recognized. Derivatives of strain DK21, free of the ds-RNA, required only 3 days to cover the plates as against 9 days needed for virus-infected isolates. Conidial production by the hypovirulent isolates was reduced substantially. Loss of ds-RNA through conidial passage led to faster mycelial growth and less pigmentation. The ds-RNA of *F. graminearum* isolates that had the ds-RNA, was transferred to only about 50 % of the conidia. Isolates carrying ds-RNA also produced much less mycotoxins (DON), compared to the ds-RNA-free isolates. In addition, ds-RNA-containing *F. graminearum* isolates caused much slow disease development. There are several vegetative compatibility groups (VCGs) for *F. graminearum* and this condition might serve as a major constraint for transmission of ds-RNA to virus-free isolates (Chu et al. 2002). The fungi carrying ds-RNA mycoviruses may be cured by exposing the hyphae to cycloheximide which inhibits RNA synthesis. *Fusarium graminearum* hyphae containing ds-RNA were transferred to PDA medium containing cycloheximide (5–70 ppm). The ds-RNA infected mycelial plugs of six isolates were inoculated on PDA containing 60 ppm of cycloheximide and incubated at 25 °C for 10 days. The hyphal tips (12) of each isolate were transferred to fresh PDA and incubated for 10 days at 25 °C. The nucleic acids extracted from the mycelial plugs from colony margins were purified and separated by electrophoresis. The hyphal tip cultures were confirmed to be free of ds-RNA after exposure to cycloheximide. *F. graminearum* isolates carrying ds-RNA produced much less mycotoxins (DON) than the ds-RNA- free isolates, possibly resulting in the reduction in disease severity compared to the ds-RNA-free isolates (Aminian et al. 2011).

Many fungal species have been shown to intraspecifically transmit the hypovirulence-associated ds-RNA by anastomosis following transfection. The ascospores progeny derived from the debilitated strain Ep1PN of *Sclerotinia sclerotiorum* exhibited normal growth rate and typical colony morphology, indicating the failure of *Sclerotinia sclerotiorum* debilitation-associated RNA virus (SsDRV) passing through sexual reproductive cycle. The homothallic nature of sexual reproduction of *S. sclerotiorum* would also impede the transmission SsDRV through hyphal anastomosis. The ascospore progeny was similar in virulence to the wild-type strain Sunf-M, when allowed to colonize detached leaves of oilseed rape. The debilitated strain Ep-1PN could survive on leaves of oilseed rape for more than

1 week and it could protect leaves from attack by Ep-1PNA1, a virulent ascospore progeny of Ep-1PN with normal colony morphology. The debilitation phenotype of Ep-1PN also could be transmitted to Ep-1PNA1 in the soil and subsequently protected seedlings against invasion by normal virulent strains (Xie et al. 2006). Transmissible hypovirulence associated with ds-RNA has been reported in *Sclerotinia sclerotiorum* which has high levels of mycelial incompatibility. On the other hand, *S. minor* has fewer mycelial compatibility groups (MCGs). Interspecific transmission of a hypovirulence-associated ds-RNA and hypovirulent phenotype was attempted from hypovirulent isolates Ss275 of *S. sclerotiorum* to five virulent isolates of *S. minor* ds-RNA. The hypovirulent phenotype was successfully transmitted to one of the five isolates, Sm10. Three putative converted isolates of Sm10 had all cultural characteristics of the hypovirulent phenotypes and pathogenicity as well. In the northern hybridizations, ds-RNA isolated from one of the converted isolates, Sm10T hybridized with a digoxigenin-labeled cDNA probe prepared from ds-RNA isolated from Ss275. Random amplified polymorphic DNA (RAPD) analysis confirmed that the isolate Sm10T was derived from Sm10 and not from Ss275 or a hybrid of the two species. The results indicated clearly that intraspecific transmission of ds-RNA was possible (Melzer et al. 2002). In a later study, it was demonstrated that *S. sclerotiorum* hypovirulence-associated DNA virus1 (SsHADV-1) could be transmitted from strain DT-8 to vegetatively incompatible *S. sclerotiorum* strain with relatively high frequency (Yu et al. 2010).

Sclerotinia homeocarpa isolates (116) collected from various locations in Ontario, Canada, were examined for the presence of a hypovirulence-associated mitovirus, *Ophiostoma mitovirus 3a* (OMV3a). Nucleic acid extraction with gel electrophoresis (NAE-GE) and reverse transcription-polymerase reaction (RT-PCR) techniques were applied for the detection of OMV3a. Phenotype, growth rate and virulence of isolates were also determined. Four isolates detected by NAE-GE method, displayed reduced colony growth and virulence and they were considered to be symptomatic, hypovirulent isolates infected by OMV3a. RT-PCR assay detected OMV3a in 53 of remaining 112 isolates. These isolates had typical colony growth and virulence and hence, they were considered as asymptomatic infections by the virus. OMV3a was detected in the majority of locations (8 of 10) and the virus present in 17–100 % of *S. homeocarpa* isolates. Vegetative compatibility groups (VCGs) have been differentiated among isolates of *S. homeocaropa*. OMV3a was detected in 66, 54 and 19 % of the isolates belonging to VCG B, A and G respectively. OMV3a was present in isolates of *S. homeocarpa* with and without characteristic symptoms of hypovirulence and the virus was widely distributed, especially as symptomatic infections, in fungal plant pathogens (Melzer et al. 2005).

Phytophthora nicotianae and *P. palmivora* are the causal agents of citrus Phytophthora root rot disease in Florida. *P. nicotianae* isolate Pn117 was characterized as hypovirulent on citrus roots. The efficacy of this hypovirulent isolate for biocontrol was indicated by the nonrequirement for additional applications to sustain rhizosphere activity for 7 months after citrus trees were planted. The isolate Pn117 induced less severe disease symptoms, compared with virulent isolate of *P. nicotianae*, Pn198 and *P. palmivora* Pp99. When all rootstocks were inoculated

with the hypovirulent Pn117 for 3 days prior to inoculation with virulent isolates, preinoculated seedlings developed significantly less disease and produced greater amounts of roots, compared with seedlings inoculated with virulent isolates alone. Space and resource competition between the hypovirulent and virulent isolates may play a role in the biological control interactions. The virulent strain Pp99 recovery from coinoculated citrus was reduced, if Pn11 was preinoculated, compared with the single inoculation with *P. palmivora* alone. Pre-establishment of the hypovirulent isolate in root cortex might consume nonstructural carbohydrates, reducing subsequent colonization by *P. palmivora*. The isolate *P. nicotianae* Pn117 could be a promising biological control organism in citrus, because the hypovirulent isolate effectively colonized the host roots and persisted with soil amendments or repeated application. On the other hand, the antagonistic biocontrol agents *Pseudomonas putida* and *Trichoderma viride* required weekly augmentations or addition of organic amendments (Colburn and Graham 2007).

The physiological modifications in the hypovirulent strain of *Cryphonectria parasitica* have been studied. Investigations to determine the influence of virus infection on fungal vesicular secretory pathways showed that virus-infected hypovirulent strains contained electron-dense materials (possibly ds-RNA) in the vesicles. In addition, accumulation of a number of proteins secreted through vesicles was down-regulated in virus-infected fungal strains (Newhouse et al. 1990; McCabe and Van Alfen 1999). The ds-RNA virus actively interfered with vesicular secretion of cargo proteins through as yet unknown mechanisms. The CHV-1 could be involved in interference with secretory pathways. Among the proteins initially identified by different experiments were a laccase, a pheromone-inducing gene and cryparin, a cell wall hydrophobin. These compounds were found to be down-regulated in the presence of CHV-1. A working model was constructed to explain the replication of the virus. The virus hijacks a subset of the secretory vesicles for its own replication and protection, resulting in vesicle build-up (Kazmierczak et al. 1996; Zhang et al. 1998). Cryparin was present in high concentrations and its function in the fungal infection cycle was determined. Absence of cryparin prevented the eruption of stromal pustules through the bark of chestnut plant. A pulse-chase experiment revealed that cryparin was secreted more slowly by virus-infected strains than the wild-type strains, suggesting possible functional link between the presence of virus and perturbation of secretion (Turina and Rostagno 2007).

6.1.2 Molecular Characteristics of Mycoviruses

The presence of several viruses in *Cryphonectria parasitica*, the causative agent of chestnut blight disease has been detected. *Cryphonectria hypovirus1*, family Hypoviridae is the most virulent one rendering *C. parasitica* incapable of causing the severe blight disease in European and American chestnut trees (*Castanea sativa* and *C. dentata*). Grente (1965) isolated atypical strains of *C. parasitica* from healing cankers of chestnuts in France. These strains appeared almost white on potato

dextrose medium (PDA) with less pigmentation and sporulation and reduced virulence. When these strains were coinoculated with normal strains of *C. parasitica*, most of the cankers were healed and the inoculated trees survived. A high molecular weight ds-RNA was detected in the hypovirulent strains (Day et al. 1977). It was observed that this ds-RNA was transferred from the hypovirulent strain to the virulent strain of *C. parasitica* via hyphal anastomosis (Anagnostakis and Day 1979; Anagnostakis 1981). The patterns of distribution of mycoviruses in the pathogenic fungus *Rosellinia necatrix* were determined by employing the colony-print immunoassay (CPIA), using an anti-ds-RNA antibody. Four unrelated mycoviruses with ds-RNA genomes, a partitivirus RnPV1, mycoreovirus RnMyRV3, megabirnavirus RnMBV1 and an unidentified virus (RnQV1) were detected in the mycelia of the white root rot pathogen *R. necatrix*. CPIA technique revealed the different distribution patterns within single colonies for each virus. Both RnPV1 and RnMBV1 were distributed throughout single colonies, while RnMyRV3 was not present in some colony sectors and RnQV1 exhibited varied accumulation levels between sectors. RnMyRV3 and RnQV1 were transmitted to the recipient virus-free colonies of virus-infected and virus-free colony pairs more slowly than the other two viruses. The presence of RnMyRV3 in recipient colonies restricted horizontal transmission of RnPV1 and RnMBV1. The results indicated that different mechanisms may operate in virus-infected fungus host, permitting different patterns of virus distribution and that virus-virus interactions may positively or negatively restrict the spread of mycoviruses within and between colonies of fungal pathogens (Yaegashi et al. 2011).

The *Cryphonectria hypovirs* has been well characterized. The entire genome of this virus is encoded on a large ds-RNA (L-dsRNA with 12,712-bp) which has two open reading frame (ORF A and ORF B). The ORF A encodes for a proteinase, whereas ORF B contains a RNA polymerase and a RNA helicase motif. Transformation tests indicated that ORF A caused reduction of pigmentation and sporulation in the host fungus, but not hypovirulence (Choi and Nuss 1992). In addition to L-dsRNA, smaller ds-RNA parts (M- and S-dsRNA) which are internally deleted forms of the L-dsRNA of the L-dsRNA, may also be present. These multi-segmented ds-RNA consists of a larger 12–13 kb segment. The smaller segments are derived by the internal deletions from the largest segment so that ds-RNA segments within isolates examined have conserved regions and share terminal sequences (Shapira et al. 1991). The largest segment contains all genetic information required for virus replication and maintenance (Choi and Nuss 1992). Many ds-RNA isolated from *Cryphonectria parasitica* have been recognized on the genomes of different members of the family *Hypoviridae* (Hillman et al. 1995). These viruses from the hypovirulent strains of *C. parasitica* are phylogenetically more closely related to potyviruses infecting several crops than to other ds-RNA viruses infecting fungi (Koomin et al. 1991). The *Cryphonectria hypovirus1*-EP713 originally isolated from France has been characterized (Shapira et al. 1991; Choi and Nuss 1992). Hypoviruses HV2-NB58 from New Jersey (Hillman et al. 1994) and CHV3-GH2 from Michigan (Smart and Fulbright 1951) have also been identified. European isolates belong to a single species of ds-RNA. CHV1 and the ds-RNA of this type CHV1 did not hybridize to North American ds-RNAs (Hillman et al. 1992; Enebak et al. 1994).

The ds-RNA CHV2-NB58 cross-hybridized weakly with North American ds-RNAs (Hillman et al. 1992). Among the 166 isolates of ds-RNAs from *C. parasitica* prevalent in the United States, three hybridization groups were recognized. None of the ds-RNAs of these three groups hybridized to CHV1 from Europe, although CHV1 had been released in many locations in the Eastern North America for the control of chestnut blight disease (Peever et al. 1997).

Four distinct virus species in the family *Hypoviridae* have been well characterized. They are taxonomically related, but have different genome organization. The most commonly occurring viruses in Europe are isolates of *Cryphonectria hypovirus 1* (CHV-1). A similar virus *Cryphonectria hypovirus 2* (CHV-2) was observed in New Jersey. *Cryphonectria hypovirus 3* (CHV-3) was the one more frequently recorded in North America. Both have a monocistron genome. CHV-4 is similar, but its presence in *C. parasitica* does not induce hypovirulence. Two viruses included in the genus *Mycoreovirus* have 11 segments of ds-RNA in the fungal cytoplasm. These viruses resemble the viruses in the family *Reoviridae. Cryphonectria mycoreovirus 1* strain 9B21 induced hypovirulence without interfering with other developmental processes such as pigmentation and sporulation. The strain 9B21 could establish infection, following the introduction of purified virus particles into fungal protoplasts (Hillman et al. 2004; Turina and Rostagno 2007). A modified procedure was developed for rapid and efficient isolation of ds-RNA from *C. parasitica* and this method required only small quantities of fungal mycelium (Lázló et al. 2010).

The presence of ds-RNA mycoviruses has been detected in *Fusarium* species such as *F. solani* and *F. poae.* The ds-RNA elements of the same electrophoretic mobility isolated from vegetatively compatible strains of *F. poae* were similar or identical in many isolates. Morphological alterations or signs of degeneration of *F. poae* were not seen in any of the ds-RNA-containing isolates (Compel et al. 1999). Field strains (286) of *F. graminearum*, causative agent of Fusarium head blight disease isolated from maize were examined for the presence of ds-RNAs. Double-stranded RNAs (7.5 kb) were detected in 13 strains of *F. graminearum* associated with altered fungal morphology. Loss of ds-RNA of one strain DK21 through conidial passage resulted in faster mycelial growth and less pigmentation, whereas its acquisition through hyphal anastomosis led to the abnormal ds-RNA-containing DK21 phenotypes. In addition to the 7.5 kb ds-RNA, smaller ds-RNAs of 5.5–6.0 kb were also detected in some isolates. The presence of the smaller RNAs did not have any influence on the *F. graminearum* phenotypes. The ds-RNA could be transferred to virus-free strains by hyphal fusion and the recipient strain acquired the virus-associated phenotype of the donor strain. The ds-RNA was transmitted to approximately 50 % of conidia and only colonies resulting from conidia carrying the mycovirus had the virus-associated phenotype. Partial nucleotide sequences of the purified ds-RNA were used to identify an RNA-dependent-polymerase sequence and an ATP-dependent helicase that are closely related to those of *Cryphonectria hypovirus* and *Barley yellow mosaic virus.* The results suggested that the ds-RNA isolated from *F. graminearum* might encode traits for hypovirulence (Chu et al. 2002). Twelve isolates of *F. graminearum* obtained from wheat had more than three

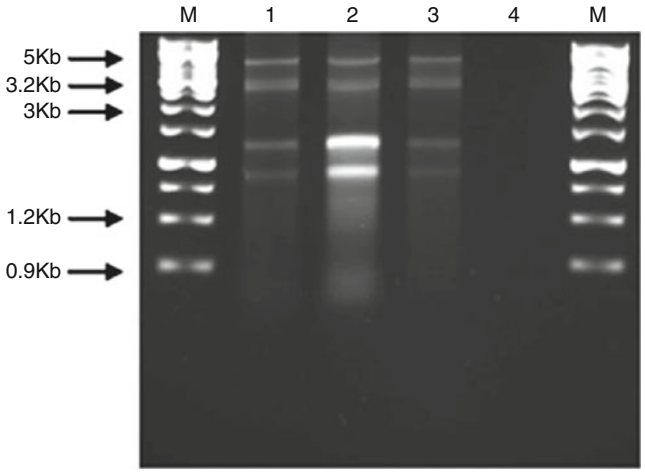

Fig. 6.1 Gel electrophoresis analysis of ds-RNA in isolates of *Fusarium graminearum. Lane M*: 1-kb DNA ladder; *Lane 1*: isolate F-42; *Lane 2*: isolate F-118; *Lane 3*: isolate F38; *Lane 4*: ds-RNA-free F-38 isolate (Courtesy of Aminian et al. 2011 and with kind permission of Journal of Plant Protection Research)

fragments measuring 0.9, 1.2, 3.2 and 5.0 kb (Fig. 6.1). The nature of ds-RNA was determined by treating with DNase I and RNase A. The bands of fragments were sensitive only to RNase A and resistant to DNase I (Aminian et al. 2011).

Fusarium boothi (=*F. graminearum* strain DK21) was found to be affected by FgV1-DK21 virus and it reduced mycelial growth, increased its pigmentation and reduced its virulence on wheat (Chu et al. 2002). The nucleotide-coding strand (6,621-bp) is polyadenylated and contains four ORFs (1–4). Pairwise sequence comparisons of the nucleotide and deduced amino acid sequences of ORF2 through four revealed no close relationships to other protein sequences available in GenBank. On the other hand, phylogenetic analysis of the deduced amino acid sequences of ORF1 which encodes a putative RNA-dependent-RNA polymerase (RdRp) and those other mycoviruses indicated that this virus forms a distinct virus clade with some hypoviruses and it is more distantly related to other mycoviruses. The FgV1-DK21 does not encode a coat protein, but the genome organization and accumulation of at least two subgenomic RNAs (sgRNAs) indicated that FgV1-DK21 belonged to a new, as yet unassigned genus of mycoviruses (Kwon et al. 2007). The presence of the ds-RNA mycovirus FgV-ch9 in *Fusarium graminearum* (Fg) isolate China-9 infecting wheat and maize was detected and the hypovirulence-related characteristics were studied. Single conidia-originating cultures of Fg China-9 contained high, medium or low amounts of viral ds-RNAs. At high and medium ds-RNA levels, the Fg China-9 isolates exhibited reduced mycelial growth rate and conidiation capacity, abnormal colony morphology and disorganized cytoplasm. Virulence of the infected isolates showed significant reduction, when inoculated onto wheat and maize plants. No obvious symptoms could be observed, when the

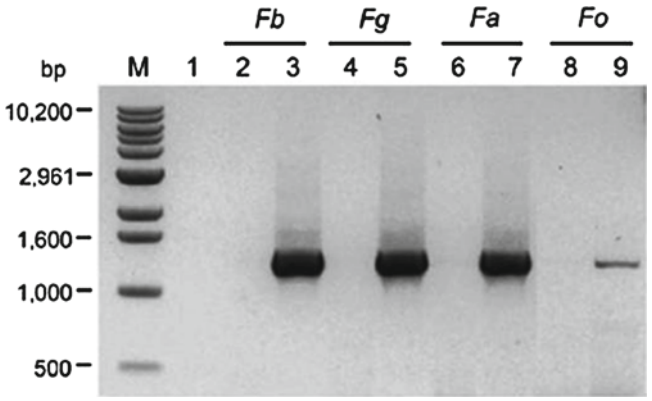

Fig. 6.2 RT-PCR analysis of ds-RNA in strains of *Fusarium boothi* (Fb), *F. graminearum* (Fg), *F. asiaticum* (Fa) and *F. oxysporum* f.sp. *lycopersici* (Fo). *Lane M*: 1-kb DNA size marker; *Lane 1*: negative control (no DNA template); *Lanes 2, 4, 6* and *8*: virus-free strains. Note the presence of viral ds-RNA in *lanes 3, 5, 7* and *9* (Courtesy of Lee et al. 2011 and with kind permission of Plos ONE, www.plosone.org)

pathogen isolates contained low amounts of viral ds-RNAs. However, the presence of the RNA segments of FgV-ch9 could be detected by RT-PCR technique. Transfection of the virulent *F. graminearum* PH-1 isolate with purified virus-like particles (VLPs) of FgV-ch9 resulted in reduced sporulation, perithecial production and pathogenicity to wheat and maize by several folds. The results showed that FgV-ch9 was the cause of hypovirulence of *F. graminearum* (Darissa et al. 2012).

Hypoviruses can be transmitted from a hypovirulent strain to a virulent strain of the fungal pathogen by hyphal fusion (anastomosis), when two vegetatively compatible strains come together. But hypoviruses cannot be transmitted when applied by extracellular routes (Chen et al. 1996). Vegetative incompatibility among many fungal species of agricultural importance, is a major barrier to the use of hypoviruses as biological control agents (Leslie 1993). The possibility of employing protoplast fusion technique was assessed to transmit FgV1-DK21 virus which is associated with hypovirulence of *F. boothi* to *F. graminearum, F. asiaticum, F. oxysporum* f.sp. *lycopersici* and *Cryphonectria parasitica.* The experiments demonstrated that FgV1-DK21 functioned as a hypovirulence factor, thereby resulting in morphological changes and hypovirulence in recipient strain. Protoplast fusion system can be used as a means of studying hypovirus-mediated alterations and strain development potential. Relative to virus-free strains, the FgV1-DK21 recipient strains had reduced growth rates, altered pigmentation and reduced virulence. The transmitted FgV1-DK21 virus replicated in the new hosts, though protoplast regeneration capacity was different between strains tested (Fig. 6.2). Hypovirulence induced in *C. parasitica* and measured in apple inoculation was greater with FgV1-DK21 ds-RNA than with CHV1. The results indicated that protoplast fusion could be employed to introduce FgV1-DK21 ds-RNA into other *Fusarium* spp. and

into *C. parasitica* and FgV1-DK21 could be used as a hypovirulence factor and hence, as a biocontrol agent (Lee et al. 2011).

Sclerotinia sclerotiorum has wide host range of over 450 plant species. Hypovirulence of *S. sclerotiorum* due to the presence ds-RNA was first reported by Boland (1992) and Boland and Hall (1994). The presence of ds-RNA in *S. homeocarpa*, causative agent of dollar spot disease of turf grass was detected and it was associated with reduction or absence of disease symptoms, when assessed 4 weeks after inoculation (Boland and Smith 1992). *S. minor* was also found to carry ds-RNA capable of inducing hypovirulence (Melzer and Boland 1996). Three ds-RNA segments designated L, M and S ds-RNA with estimated sizes of 7.4, 6.4 and 1.0 kbp respectively were shown to be associated with the debilitated strain. Of these three ds-RNA segments, only the M segment of ds-RNA was consistently detected in association with the debilitation phenotype (Li et al. 1999a, b). Three ds-RNA segments L, M and S were isolated from *S. sclerotiorum* strain Ep-1PN and the M ds-RNA was coincident with hypovirulence and debilitation of the fungal host. The complete nucleotide sequence of the M ds-RNA of 5,419 nt, excluding the poly(A) tail, was determined. Sequence analysis revealed the occurrence of a single ORF (nt 93–5,195) encoding a protein with significant similarity to the replicase of the 'alphavirus-like' supergroup of positive-strand RNA viruses. The M ds-RNA encoded putative replicase protein contained the conserved methyl transferase, helicase and RNA-dependent RNA polymerase (RdRp) domains characteristic of the replicases of potex-like plant viruses (flexiviruses) and *Botrytis virus F* (BVF), a flexuous rod shaped mycovirus infecting *Botrytis cinerea*. The ascospore descendents derived from the debilitated strain EP-1PN were devoid of ds-RNA and exhibited normal colony morphology. The debilitation phenotype was transmitted from the parental debilitated strains to its normal ascospore progeny via hyphal anastomosis. The M ds-RNA from strain Ep-1PN might be derived from the genomic RNA of a positive-strand RNA virus. This virus was designated *Sclerotinia sclerotiorum debilitation-associated RNA virus* (SsDRV) (Xie et al. 2006).

A unique geminivirus-related ss-DNA mycovirus that conferred hypovirulence to *Sclerotinia sclerotiorum* with potential for use as a biocontrol agent was isolated. The genome of this ss-DNA virus designated *Sclerotinia sclerotiorum* hypovirulence-associated DNA virus1 (SsHADV-1) has 2,166 nt, coding for a replication initiation protein (Rep) and a coat protein (CP). Although phylogenetic analysis of Rep showed that SsHADV-1 was related to geminiviruses, it is notably distinct from geminiviruses both in genome organization and particle morphology. Polyethylene glycol-mediated transfection of fungal protoplast was successful with either purified SsHADV-1 particles or viral DNA isolated directly from infected mycelium. The experiments showed that SsHADV-1 could be transmitted from strain DT-8 to vegetatively incompatible *S. sclerotiorum* strains with relatively high frequency. The SsHADV-1 isolates were detected in other *S. sclerotiorum* hypovirulent strain which were isolated from the same rapeseed field, indicating the natural spread of SsHADV-1 under field conditions. The results indicated that SsHADV-1 could be an effective biocontrol agent against fungal pathogen *S. sclerotiorum* (Yu et al. 2010).

Diaporthe ambigua causing cankers on apple, pear and plum trees and their rootstocks is responsible for the slow death of mature trees and rapid death of nursery stocks. Differences in the morphology and virulences of isolates of *D. ambigua* were observed. The hypovirulences of isolates was coincidental with the presence of a single species of ds-RNA (about 4 kb) in the fungal mycelia (Smit et al. 1996). Single-stranded ds-RNA elements of about 4 kb were isolated from the hypovirulent *D. ambigua* isolate CMW3407. This linear ds-RNA genetic element most probably represented a replicative form of a positive strand RNA virus which was designated *Diaporthe ambigua RNA virus* (DaRV). The putative proprotein p125 was possibly translated from the DaRV genome. The nucleotide sequence of the genome was 4,113-bp long and had a GC content of 53 %. Two large ORFs were present in the same reading frame. A significant homology could be observed to the nonstructural proteins of carmoviruses of the positive strand virus family *Tombusviridae*. These proteins included the conserved RNA-dependent RNA polymerase (RdRp) domain. The most significant difference in the genome organization of DaRV and the carmoviruses was that the DaRV encoded neither a coat protein (p38), nor small movement proteins (p7, p8 or p9) that are present in the carmoviruses. The results indicated that DaRV with its unique genome organization showed distant relationship to the plant virus family *Tombusviridae* (Presig et al. 2000).

Diaporthe perjuncta is an important fungal pathogen of grapevines. A double-stranded (ds) RNA virus was recovered from the slow-growing, non-sporulating and hypovirulent stains of *D. perjuncta*. These strains exhibited hypovirulence-associated traits such as reduced laccase activity. The ds-RNA was sequenced, characterized and named as *Diaporthe RNA virus* (DaRV). The effects of DaRV on a host fungus following transfection of a virus-free isolate were studied. A virus-free isolate of *D. perjuncta* from a South African grapevine was transfected with in vitro-transcribed positive strands of DaRV. Based on RT-PCR and partial sequence analysis the transfected virus was identified as DaRV. The in vitro-transcribed RNA transcripts used to transfect fungal spheroplasts contained parts of the vector at their distal ends. These vector sequences were separated from DaRv genome during replication in the new host. The transfected isolate had morphological features that differed from those of the isogenic virus-free strain, including production of a yellow pigment, a decreased growth rate and lack of sporulation. The pathogenicity tests on apple did not reveal any difference in virulence between virus-free and DaRV-infected isolates (Moleleki et al. 2003). *Monilinia fructicola* infects stone fruits in the orchards and also during storage. The presence of dsRNAs was detected in 36 of 49 isolates of *M. fructicola*. The ds-RNA profiles were highly variable even between isolates from a single tree. Partially purified extracts from isolates were examined under transmission electron microscope (TEM). One isolate showed the presence of isometric particles (45 nm), showing similarity in appearance to totiviruses and partitiviruses. Another isolate contained rigid rod-shaped particles (250 × 25 nm), similar to those of plant pathogenic tobraviruses and furoviruses (Tsai et al. 2004). In the case of *Alternaria alternata* infecting many crops, ds-RNA1, ds-RNA2, ds-RNA3 and ds-RNA4 were detected in the fungal strain EG35-193 which

exhibited an impaired growth phenotype. Exposure to cycloheximide during hyphal tip isolation resulted in curing of the virus-infected isolate. The ds-RNA mycovirus might be involved in modulating traits of *Alternaria alternata*. The ds-RNA encoded a single ORF (3,447 nt) containing the conserved motifs of viral RNA-dependent RNA polymerase (RdRp) which was related to the ORF encoded by ds-RNA1 of *Aspergillus* mycovirus 341. All of the encoding strands of the four ds-RNA genomes had 3′-poly(A) tails ranging from 33 to 50 nt in length. This virus was designated *Alternaria alternata virus-1* (AaV-1) (Aoki et al. 2009).

6.2 Viruses Pathogenic to Bacterial Plant Pathogens

Bacteriophages are viral pathogens capable of infecting prokaryotes. Bacteriophages or simply phages were discovered by Twort (1915) and d'Herelle (1917). They independently reported about their ability of lysing of bacterial cells. d'Herelle's bacterial virus theory received experimental evidence from the investigations for the control of anthrax and staphylococcus infection (Brunoghe and Maisin 1921). Later on, bacteriophages were evaluated for their effectiveness as biocontrol agents against bacterial plant pathogens. An extract from decomposed cabbage was shown to have a principle (filterable virus) capable of inhibiting the growth of *Xanthomonas campestris* pv. *campestris* infecting cabbage (Mallmann and Hemstreet 1924). Many investigations have indicated that bacteriophages have great potential for use as biocontrol agents and to replace the chemicals to some extent. Further, they can be used effectively as a component of integrated disease management (IDM) systems. The relative ease of preparing phages and low cost of production of the phages offer advantages over other alternative methods of disease management aimed to limit the use of chemicals.

6.2.1 Biological Properties of Bacteriophages

The purified phage virus particles, like other viruses, are metabolically inert. Infection of susceptible bacterial cells by phage begins with adsorption of phage particles to the bacterial cell wall, followed by capsid (protein coat of the phage) binding to the cell surface and subsequent genome uptake into the cell cytoplasm. Following infection, the phages exhibit two distinct life cycles. Active or lytic infection involves production and assembling of viral components (nucleic acid and capsid protein), within the cytoplasm of infected bacterium. When the phage particles mature, the bacterial cell wall is ruptured releasing the progeny particles which can cause infection in fresh bacterial cells. Lysis kills the infected bacterium and terminates the phage infection cycle. In the second type of life cycle known as lysogenic infection, the phage genome integrates (as a prophage) into the bacterial

Table 6.1 Biocontrol of bacterial plant pathogens using bacteriophages

Crop	Bacterial pathogen	References
Apple	*Erwinia amylovora*	Schbabel et al. (1999) and Gill et al. (2003)
Apple and Pear	*E. amylovora*	Boulé et al. (2011)
Begonia	*Xanthomonas campestris* pv. *begonia*	Kaesberg (2009)
Cabbage	*Xanthomonas campestris* pv. *campestris*	Mallmann and Hemstreet (1924)
Carrot	*Erwinia carotovora* subsp. *carotovora*	Coons and Kotila (1925)
Corn (maize)	*Pantoea stewartii*	Thomas (1935)
Cotton	*X. axonopodis* pv. *malvacearum*	Massey (1934)
Peach	*X. campestris* pv. *pruni*	Civerolo and Keil (1969)
Potato	*E. carotovora* subsp. *atroseptica*	Kotila and Coons (1925)
Tobacco	*Ralstonia solanacearum*	Tanaka et al. (1990)
Tomato	*X. campestris* pv. *vesicatoria*	Momol et al. (2002) and Balogh et al. (2003)
	Ralstonia solanacearum	Fujiwara et al. (2011)
Tomato and potato	*Ralstonia solanacearum*	Murugaiyan et al. (2010)
Walnut	*X. campestris* pv. *juglandis*	McNeil et al. (2001)

chromosome as a giant gene complex. The lysogenic bacterium carrying the phage genome has to be induced to produce virion progeny prophage. Induction may occur as a stochastic process or may be forced by applying DNA damaging agents. Many phages designated temperate may exhibit either active infection or reduction to lysogeny upon bacterial infection. The process of release of phage progeny called extrusion, allows phage progeny release to be leaked into the extracellular environment. The filamentous phage particles are moved out of the bacterial cell through extrusion. Only lytic phages are selected for assessing their biocontrol potential against bacterial plant pathogens by observing the formation of plaques representing areas on the bacterial lawn, free of bacterial cells. Obligately lytic phages are the phages of choice for the use as biocontrol agents against bacterial plant pathogens. Bacteriophages may be isolated from plant parts, soil and water (Abedon et al. 2001). The effectiveness of bacteriophages for controlling plant disease has been assessed for some plant pathosystems (Table 6.1).

Phages are abundant in most natural ecosystems and their usefulness for controlling bacterial populations has been recognized. As the total number of phages outnumbers the total cell count of prokaryotes in nature, a huge diversity of phages can be expected (Rowher 2003). Phages are likely to be most prevalent in environments where there is a high density of metabolically active bacteria. The plant rhizosphere supports a broad diversity of bacterial species including plant pathogens and antagonists. The viral diversity has been analyzed by metagenomic approach in soils and plant rhizosphere (Fierer et al. 2007; Kim et al. 2008). By assessing the extent of

phage diversity, it would be possible to select appropriate and effective phage for the control of target bacterial plant pathogen(s). This approach has been applied effectively for the control of plant bacterial diseases such as peach bacterial spot, apple fire blight and tomato bacterial spot diseases (Jones et al. 2007). The effectiveness of the phage therapy for the suppression of grapes Pierce's disease caused by *Xylella fastidiosa* was assessed. A functional *Xylella* phage named as *Xfas53* was isolated, propagated and characterized. A plate overlay method was developed for isolation and propagation of the phage specific *to X. fastidiosa* isolates. This method was employed to screen a 30×30 matrix, using each isolate as an indicator to test supernatant of isolates grown on PW-M broth. Plaque production due to dissolution of *X. fastidiosa* cells could be observed on plates seeded with host bacterial cells. Individual plaques were excised from the overplay, suspended in phage buffer and titered. High titer lysates (10^{10} PFU/ml) were obtained by harvesting overlays of plates exhibiting confluent lysis. Observations under transmission electron microscope revealed that phage *Xfas53* belonged to the family *Podoviridae* with a head diameter of 55 nm and a short noncontractile tail with a diameter of 12 nm. The isolates of *X. fastiosa* from different locations were sensitive to the phage *Xfas53*. The results indicated the potential of the phage *Xfas53* as a biocontrol agent capable of suppressing the development of the Pierce's disease of grapes and its incitant (Gonzalez et al. 2008).

Soil samples for phage isolation were collected from fields of tomato, hot pepper and tobacco crops. Soil samples after passing through sieve (1 mm) were placed in flasks and the moisture level was adjusted to 40 % of water holding capacity with distilled water. Each flask was seeded with overnight cultures of host bacterial strains (*Ralstonia solanacearum*) and incubated for 48 h. The soil samples were suspended in phosphate-buffered saline, centrifuged and the supernatant containing the phage was passed through a membrane filter (0.2 μm pore size). The presence of phages was assayed by soft agar overlay method. Bacterial lawns were prepared by seeding CPG soft agar (3 ml) with bacteria (10^8 CFU) mixed with the phage solution of 10^5/ml PFU in SM buffer containing Tris–HCl (50 mMol/l, pH 7.5), NaCl (100 mMol/l), $MgSO_4$ (10 mMol/l) and 0.01 gelatin. The plates were incubated at 30 °C for 24 h to assess plaque formation (Murugaiyan et al. 2010). Bacteriophages infecting *Ralstonia solanacearum* were isolated from pepper, tomato and tobacco plant rhizospheres infected with *R. solanacearum*. A host specificity analysis of the isolated phages using nine strains of *R. solanacearum* including race1 (biovars 3 and 4) and race 3 was performed. A filamentous phage PE226 had a relatively wide range of host bacterial infection. PE226 and TM227 generated clear plaques on all nine of the *R. solanacearum* strains tested on a bacterial lawn in soft agar. The morphological properties using transmission electron microscope (TEM) and the genomic characteristics of the two phages showed that they are probably closely related, although they were isolated from pepper and tomato respectively. Both PE226 and TM227 had long, flexible, filamentous shape which was different from other phages infecting *Ralstonia* species. A filamentous phage ØRSS1 infecting *R. solanacearum* strains was reported earlier by Kawasaki et al. (2007). But the genome organization of PE226 was different from that of the

phage ØRSS1. As the phage PE226 consistently formed clear plaques on the nine strains of *R. solanacearum* tested, it might be a typical temperate phage carrying properties of both lysis and lysogeny types of life cycles (Murugaiyan et al. 2010).

Phages are natural components of the biosphere and hence, their use against bacterial plant pathogens will be ecologically acceptable. Phages can be targeted against bacterial receptors that are essential for pathogenesis, resulting in attenuation of virulence (Kutter 1997). Phages are non-toxic to plants and animals. They may be desirable for use in situations where chemical control is not permissible, owing to legal regulations as in the case of peach fruits that cannot be treated with chemicals before harvest (Zaccardelli et al. 1992). Phages are specific to host bacterial species/strains, destroying only target bacteria without affecting other organisms that are beneficial to plant growth. This specificity of phage activity allows their use in combination with antagonistic bacteria that are being employed as biocontrol agents. The combined application may increase the potential of the antagonistic activity against bacterial pathogens. It is fairly easy to prepare the phages for large scale application at low cost. They can be stored at 40 °C for 7 months without loss of phage activity. Field application can be performed using regular farm equipment without any need for special attachment or modification. As the phages are not affected by most agrochemicals, the phage preparation can be tank-mixed with other plant protection chemicals with significant reduction in phage concentration (Zaccardelli et al. 1992; Balogh et al. 2005). Bacteriophages are rapidly degraded under field conditions and practically disappear from the tomato canopy at 2 days after application for the control of bacterial spot caused by *Xanthomonas campestris* pv. *vesicatoria*. In order to improve the efficacy of the phage, three formulations that significantly increase phage longevity on the plant surface were developed. The formulations (i) 0.5 % pregelatinized corn flour (PCF), (ii) 0.5 % casecrete NH-400 + 0.5 % sucrose + 25 % PCF and (iii) 0.75 % powdered skim milk + 0.5 % sucrose were tested under greenhouse conditions. The nonformulated PCF-, casecrete- and skim milk-formulated phage mixtures reduced disease severity on plants, compared with the controls by 1, 30, 51 and 62 % respectively. These three formulations increased the concentrations of the phage populations respectively by 4,700-, 38,500- and 100,000-fold at 2 days after application. Use of protective formulations significantly increased the efficiency of phage treatments for suppressing the development of bacterial spot disease of tomato (Balogh et al. 2003). *Pantoea agglomerans* effective against *Erwinia amylovora*, causative agent of apple fire blight disease, was sensitive to phages. This BCA was employed to deliver and propagate the phages on leaf surfaces. The effectiveness of disease suppression was comparable to the level obtained by applying streptomycin (Svircev et al. 2006). The efficacy of *Pantoea agglomerans* Eh21-5 (another biocontrol strain) as a carrier of the phages ØEa1337-26 and ØEa2345-6 was assessed. The infection by *Erwinia amylovora*, causing fire blight disease was reduced by 84 and 96 % respectively, when tested on detached pear blossoms (Boulé et al. 2011).

Use of phages for control of crop diseases seems to be fast- expanding approach with great potential to replace chemical measures. The efficacy of three lytic phages ØRSA1, ØRSB1 and ØRSL1 in inhibiting tomato bacterial wilt disease was

assessed. Infection with ØRSA1 and ØRSB1 either alone or in combination with other phages resulted in a rapid increase in the host cell density. Cells that were resistant to infection by these phages could be recognized at 30 h after addition of phage to the bacterial culture. In contrast, infection by ØRSL1, resulting in lysed the bacterial cells and lower host cell density (1/3 of control) were maintained over a long period, indicating the susceptibility of *Ralstonia solanacearum* to ØRSL1 phage. Pretreatment of tomato seedlings with ØRSL1 drastically limited penetration, growth and movement of root-inoculated bacterial cells. Tomato plants treated with ØRSL1 phage did not exhibit symptoms of wilting during the experiment duration of 18 days, while all tomato plants without phage treatment wilted. Phage ORSL1 was relatively stable in soil, especially at higher temperatures (37–50 °C). Persistence of ØRSL1 was indicated by the recovery of the phage from roots of treated tomato plants and soil at 4 months postinfection. As all host bacterial cells were not killed by ØRSL1, the coexistence of bacterial cells and phage may be expected to provide effective prevention of wilting (Fujiwara et al. 2011). The filamentous phage ØRSM infected *Ralstonia solanacerum*, causal agent of tomato bacterial wilt disease and inactivated its virulence. Inoculation of oRSM3-infected cells into tomato plants did not cause bacterial wilt. But ØRSM3-infected cells enhanced expression of pathogenesis-related (PR) genes, including PR-1, PR-2b and PR-7, in tomato plants. Further, pretreatment with oRSM3-infected cells protected tomato plants against infection by virulent *R. solanacearum* strains. The effective dose of ØRSM3-infected cells for disease prevention was approximately 10^5 CFU/ml. As the bacterial cells infected by ØRSM3 could grow and produce infectious phage particles continuously under appropriate conditions, it would be possible to develop the approach of using phages as a management strategy for tomato bacterial wilt disease (Addy et al. 2012).

Development of bacterial strains resistant to phages may become a major limiting factor for successful disease management by using bacteriophages. A process was patented to prevent occurrence of phage-resistant mutants by employing a mixture of host range mutant phages (h-mutants) for disease control. The h-mutants could lyse all bacterial strains that were resistant to the wild-type strain, while still being capable of lysing the wild-type bacterial pathogen strain. The phage mutants had an extended host range, compared to the parent phage alone. This approach was applied for the control of tomato bacterial spot disease caused by *Xanthomonas campestris* pv. *vesicatoria*. A mixture of four phages including wild-type and h-mutant phages, were applied twice weekly in the early morning prior to sun rise. The phages were provided more effective control compared to copper-mancozeb treatment (Jackson 1989). Likewise, a mixture of h-mutants and parent phages was applied for protecting geranium plants against *Xanthomonas campestris* pv. *pelargonii (Xcp).* Foliar application of the phage mixture daily, significantly reduced the spread of the disease and the disease incidence was significantly less than in plots treated with recommended chemical (Flaherty et al. 2001). Bacteriophages infecting *Streptomyces scabiei* isolated from soil samples were investigated by analyzing their genomes. The DNA fragment profiles obtained from restriction enzyme digestions were compared. Two genetically different phages infecting *S. scabiei* were

differentiated and designated Stsc1 and Stsc3. Phage morphology was compared using electron microscopy. The phages Stsc1 and Stsc3 had a binal symmetry with an icosahedral head and a long, striated tail. The phages Stsc1 and Stsc3 infected respectively 88 and 75 % of the pathogenic *S. scabiei* strains tested. In the in vitro tests, the phages prevented development of symptoms due to infection by *S. scabiei* in radish seedlings. The results suggested the possible use of bacteriophages for suppressing bacterial diseases of plants (Goyer 2005).

Burkholderia glumae and *B. plantarii* cause seedling rot and seedling blight respectively in rice. The possibility of employing bacteriophages specific to these bacterial pathogens was explored. Two phages could lyse both bacterial pathogens and suppress disease development. The phage BGPP-Ar suppressed the disease development more effectively than available bactericides. The ratio of seedlings exhibiting infection to the total number of seedlings examined after treatment with BGPP-Ar [1×10^8 plaque-forming units (PFU).ml] was a 0.0 for seedling rot and 2.0 for seedling blight. For treatment with ipconazole/copper (II) hydroxide, the ratios were 14.3 and 15.0 respectively for seedling rot and seedling blight diseases. BGPP-Ar phage treatment effect for sterilizing seed was achieved indoors to avoid phage inactivation by UV-irradiation. Seed treatment with phage was effective in reducing seedborne infection by *B. glumae* and *B. plantarii* (Adachi et al. 2012).

6.2.2 Molecular Characteristics of Bacteriophages

Transmission electron microscope (TEM) has been used to study the phage morphology, while biochemical and nucleic acid-based techniques have been employed to study the molecular characteristics of the bacteriophages. The phages are composed of nucleic acid and protein coat to protect the nucleic acid from inactivation. Phages have double-stranded (ds)-DNA, single-stranded (ss)-RNA as their genome. The protein coat may have different shapes ranging from small hexagonal structures consisting of a head and tail. Six phages isolated from blighted nuts, leaves, buds and petioles of walnut trees were effective against *Xanthomonas campestris* pv. *juglandis* were examined under the electron microscope. Three different types of particles viz., hexagonal with and without a short tail were recognized. The phages in walnut canopy appeared to vary extremely in form and infectivity to the bacterial pathogen (McNeil et al. 2001). A functional *Xylella* phage *Xfas53* effective against *Xylella fastidiosa (Xf),* causing Pierce's disease of grapevine was isolated, propagated and characterized. TEM observations with purified *Xfas53* phage revealed the morphology of the phage. The *Xfas53* belonged to the family *Podoviridae* with a head diameter of 55 nm and a short non-contractile tail having a diameter of 12 nm. The genome size of the *Xfas53* phage was estimated by pulsed-field gel electrophoresis to be 36,673-bp with a GC content of 57 %. Bioinformatic analysis of the *Xfas53* genome predicted a total of 46 protein coding genes (Gonzalez et al. 2008). The bacteriophages obtained from apple and pear orchards in British Columbia, Canada, effective

against *Erwinia amylovora*, belonged to the order *Caudovirales*, the tailed phages and included members of the families *Myoviridae* and *Podoviridae.* The phages were characterized by digestion of the phage DNA with four restriction endonucleases and two sets of PCR primers. Two novel groups, RFLP groups 7 and 8 were identified based on differences in restriction fragment patterns (Boulé et al. 2011).

Two phages PE226 and TM227, isolated from pepper and tomato fields infested with *Ralstonia solanacearum* were observed under transmission electron microscope (TEM). Both phages were virtually identical in morphology and genomes. The phage PE226, an Inovirus had a long flexible filamentous particles carrying a circular (+) sense single-stranded (ss) DNA genome of 5,475 nucleotides. The DNA sequences of PE226 had nine open-reading frames (ORFs) that were not very similar to those of other phages infecting *R. solanacearum.* The phage most similar to PE226 was p125 isolated from *R. pickettii*. The filamentous phage ØRSS1 infecting *R. solanacearum* strains was among the phage types similar to PE226, but its genome organization was different. The result suggested that PE 226 genome was similar to a mosaic structure of genomes of two phages of *R. solanacearum* and *R. picketti* respectively. The *zot* gene which was highly homologous among PE226, p12J and ØRSS1 was involved in phage assembly and exited from the bacterial cell as a NTPase homologue. The *zot* gene was detected in *R. solanacearum* SL341. However, PE226 was still highly virulent to SL341. The presence of *zot* in SL341 might be a consequence of phage genome integration to form a PE226 prophage which might have left some genes behind, when it split from the host bacterial genome (Murugaiyan et al. 2010).

6.3 Mild Strains of Plant Viruses as Biocontrol Agents

Nonpathogenic isolates or strains of fungal and bacterial plants have been demonstrated to protect plants against virulent strains of the pathogens. Likewise, the mild strain-inoculated plants do not develop symptoms of infection by severe strains of the same virus. This phenomenon is known as cross-protection. The extent of protection provided depends on the relatedness of the mild strain with the severe strain. Cross-protection as a disease management strategy has been effectively applied in the case of some viral diseases affecting perennial crops like citrus and annuals like tomato. The mild strains may be selected from the naturally-occurring strains or they may be artificially produced by exposing the viruses or virus-infected plants to chemicals or unusual temperature (high or cold) regimes. Systemic infection of a plant with one strain of a virus, generally a mild or attenuated strain, inhibits or restricts secondary infection by a related or more virulent viral strain. Cross-protection tests were earlier performed to establish relationship between virus strains or isolates. Absence of additional symptom due to the challenging virus was regarded as the protecting and challenging virus isolates to be related, while production of distinct symptoms of the challenging virus isolate was considered to indicate that the interfering virus strains or viruses to be unrelated (Narayanasamy 2002).

6.3.1 Use of Naturally Occurring Mild Strains

Several plant viruses like *Citrus tristeza virus* (CTV), *Potato virus Y* (PVY) and *Tobacco mosaic virus* (TMV) are known to exist in the form of many strains that vary in their virulence. The strain that induces mild or no visible symptom on its natural host is identified and its ability to protect the susceptible plant against the severe strain of the same virus. The phenomenon of cross-protection was first demonstrated by McKinney (1929) who observed that tobacco plants infected systemically by a mild strain of *Tobacco mosaic virus* (TMV) were not affected by subsequent infection by a severe strain of TMV which induced yellow mosaic symptoms in healthy tobacco plants. Tobacco inoculated with a mild strain of *Potato virus X* (PVX) became immune to severe strains of the same virus, but not to unrelated viruses like TMV or PVY (Salman 1933). With related strains, reciprocal cross-protection of varying degrees of completeness or unilateral cross-protection or no cross-protection may be observed as in *Tobacco streak virus* (TSV) in tobacco (Fulton 1978). Later, cross-protection of several crop plants by mild strains has been reported. Further, cross-protection as a criterion for studying the relationship between viruses and their strains or closely related viruses was suggested. However, strains of some viruses like *Beet curly top virus* (BCTV) (Bennett 1963) and *Maize dwarf mosaic virus* (Paulsen and Sill 1970) did not protect the plants against severe strains of the same virus.

Occurrence and degree of cross-protection produced by virus isolates representing five strains or serotypes of *Barley yellow dwarf virus* (BYDV) namely PAV, MAV, SGV, RPV and RMV were investigated by employing enzyme-linked immunosorbent assay (ELISA), cDNA dot blot hybridization and transmission by aphids involved in their natural transmission. Generally, the degree of cross-protection was positively correlated with the serological relatedness between the isolates. A high degree of cross-protection occurred between NY-MAV and MAV-PS1, two isolates of MAV serotype. The use of serological and nucleic acid probes provided unequivocal quantitative evidence that cross-protection occurred between isolates of BYDV within group1, but not between isolates of group 2 or between isolates of group1 and group 2. The persistence of cross-protection depended on the interval between inoculations with protecting and challenging viruses/strains; longer inoculation intervals enhanced the persistence of cross-protection. The results suggested that cross-protection might require continuous interference between protecting and challenging viruses. The results of ELISA and dot blot hybridization tests indicated that cross-protection affected both viral capsid and RNA synthesis (Wen et al. 1991).

One of the most successful commercial exploitations of cross-protection for viral disease management is that of employing a mild strain of *Citrus tristeza virus* (CTV). In Brazil, among the 45 mild strains tested, three strains were found to provide satisfactory level of protection against severe strains of CTV in Pera sweet orange, two strains in Galigo lime and one strain in Ruby grapefruit cultivars. There was no evidence of breakdown of cross-protection provided to about 5,000,000 pre-immunized citrus planted during a period of 10 years (Costa and Muller 1980).

Cross-protection employing mild strains of CTV was adopted on a commercial basis in several countries such as Australia, Japan and South Africa. Ieki et al. (1997) reported that Navel orange plants protected with mild strains were more vigorous and produced higher yields, compared with unprotected control trees. Rocha-Peña et al. (1995) suggested that cross-protection could be considered as a component of the integrated disease management (IDM) approach to extend economic productivity, but it may not be an effective method of offering long-term protection against severe strains of CTV. Such a view appears to be valid, because mild strains lost the ability to protect Navel orange against severe strains of CTV after a period of 7–9 years of immunization with a mild strain of CTV (Ieki et al. 1997). Cross-protection against severe strain achieved through the use of mild strains of *Citrus tristeza virus* (CTV) has to be applied as a disease management strategy with much caution. Constant monitoring of the strain composition in a geographical location has to be carried out. Efficient and precise procedures have to be applied to identify the presence of CTV isolates including the potentially dangerous one. Identification of the CTV isolates is carried out by biological indexing on selected indicator plants (Garnsey et al. 2005). Serological and molecular markers have been employed to characterize different CTV isolates. Some markers were useful to separate mild and severe isolates. But their limited sensitivity/specificity of the detection procedures have hampered their practical use to determine their pathogenic potential of unknown isolates (Sieburth et al. 2005).

Cross-protection provided by mild strain of *Citrus tristeza virus* (CTV) has been used extensively to reduce the losses due to stem-pitting isolates of CTV in Australia (Braodbent et al. 1995). Some mild isolates of CTV from apparently healthy Marsh and Thompson grapefruit trees in orchards declining with CTV stem-pitting, when grafted into virus-free Marsh grapefruit, protected them against stem-pitting isolates transmitted by the aphid vector *Toxoptera citricida*. The degree of protection varied with the CTV isolate and environmental conditions. Pre-immunization with mild CTV isolate (PB61) could protect Marsh grapefruit trees against stem-pitting isolate for 35 years (Braodbent et al. 1995). The efficacy of the mild strain PB61 to protect seedlings against super-infection with a severe grapefruit stem-pitting isolate PB219 or two orange stem-pitting (OSP) isolates PB155 or PB235 was assessed in the glasshouse. Symptom development was monitored and the presence of each isolate was followed using isolate-specific restriction fragment length polymorphism (RFLP) analysis of amplicons generated by RT-PCR and multiplex RT-PCR assays. Pre-immunization with PB61 provided partial protection against super-infection using aphid-inoculation and delayed super-infection, when challenge was by graft inoculation. Pre-immunization with PB61 did not ameliorate the expression of OSP symptoms, once super-infection with OSP-inducing isolates was initiated. The results suggested that mild strain cross-protection (MSCP) did not prevent movement of the challenge virus, since super-infection has occurred once, the challenge virus was detected both in the shoots and feeder roots, indicating systemic movement of the challenge virus (Zhou et al. 2002).

The mild strain of *Citrus tristeza virus* (CTV) PB61 protected pre-immunized seedlings more effectively against OSP isolate PB235 than PB155. The presence of

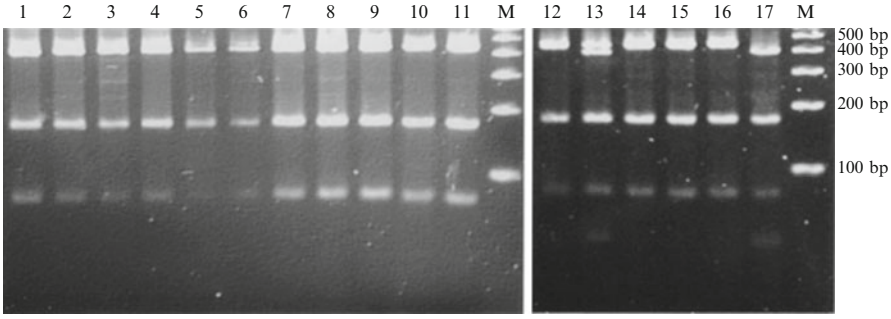

Fig. 6.3 Detection of the preimmunizing isolate PB61 and challenge strain of severe OSP subisolate PB235 of *Citrus tristeza virus* (CTV) at 90 days after post-challenge inoculation in Symons sweet orange (SSWO) by CP/*Hinf*I RFLP profiles. *Lane M*: 100-bp ladder DNA marker; *Lanes 1–15*: SSWO seedlings preimmunized with PB61 and challenged with severe OSP isolate PB235; *Lane 16*: a preimmunized SSWO seedling subsequently "mock inoculated" with 50 virus-free aphids; *Lane 17*: a SSWO seedling inoculated with PB235. Note all preimmunized plants except No. 13 remained free from superinfection by severe strain PB235 by 90 days post-inoculation (dpi) (Courtesy of Zhou et al. 2002 and with kind permission of International Organization of Citrus Virologists, Florida, USA)

the severe isolate PB235 and the protecting isolate PB61 was detected by the CP/*Hinf*I RFLP profiles (Fig. 6.3). The levels of nucleotide sequence identity between PB61 and PB235 (97.6 %) were much greater than between PB61 and PB155 (91.2 %). The variation in sequences of coat protein genes coding for coat proteins p18 and p23 appeared to have a bearing on the efficiency of protection offered against severe isolates. Cross-protection appeared to be more effective, if the nucleotide sequence identity is greater between the interacting CTV isolates. A model of RNA-mediated defence was proposed to explain the cross-protection between CTV isolates. Infection with pre-immunizing isolate may trigger the host to produce a ds-RNA-specific nuclease which targets the viral RNA for degradation to low levels. This might result in the appearance of small nucleotide fragments such as those observed during post-transcriptional gene silencing (PTGS). Once PTGS is established, other transcripts homologous to the silenced gene are also subsequently degraded, if they infect the plant (Zhou et al. 2002).

Citrus tristeza virus (CTV) isolates induce severe symptoms of seedling yellows (SY) and/or stem pitting (SP) in grapefruit or sweet orange. These isolates producing severe symptoms have to be differentiated from mild isolates that can be employed to protect the citrus seedlings by pre-immunization. Quantitative real-time RT-PCR assay was employed to identify 56 biologically characterized CTV isolates from 20 countries. A general primer set and three TaqMan locked nucleic acid (LNA) probes were used to target sequences characteristic of severe, mild (non-SY, non-SP) and T36 (occurring in Florida)-like isolates. Successful amplification was achieved from fresh or silica-desiccated CTV-infected samples and all isolates except one reacted with one or more probes. Standard curves using RNA transcripts homologous to the three probes allowed a reproducible quantitative assay with a

wide dynamic range of detection starting with 10^2 copies. RT-PCR assays with homologous and heterologous transcript RNA mixes showed that each probe reacted only with its cognate sequence. Analysis of the diverse isolates revealed that mild isolates reacted only with the mild probe, whereas SP and SY isolates reacted with severe-SP or the T36-like probes respectively and often with a second probe. This procedure enabled estimating the ratio of different genotypes of CTV present in the viral population (Ruiz-Ruiz et al. 2009).

Grapevine fan leaf virus (GFLV) causes the fan leaf degeneration responsible for serious losses in grapevine cultivation. It is transmitted by the ectoparasitic nematode *Xiphinema index* from vine to vine. Mild isolates of GFLV and *Arabis mosaic virus* (ArMV), a closely related virus were identified by comparative performance analysis of grapevines infected with different strains and comparative severity of symptoms on *Chenopodium quinoa*, a systemic herbaceous host of GFLV (Huss et al. 1989; Legin et al. 1993). Protection with mild GFLV strains against GFLV infection on *Gomphrena globosa* was also demonstrated (Bianco et al. 1988). In a later study, healthy scions were grafted onto rootstocks that were healthy or infected with mild protective strains of GFLV-GHu or ArMV-Ta. Challenge GFLV infection via *X. index* was monitored over nine consecutive years in control and ArMV-Ta cross-protected vines by DAS-ELISA, using GFLV-specific antibodies. In the case of GFLV-GHu cross-protected vines, GFLV infection was monitored by characterizing coat protein gene of superinfecting isolates by immunocapture (IC)-RT-PCR-RFLP analysis. Cross-protected vines had significantly reduced challenge infection rate consistently. However, both mild strains reduced fruit yield by 9 % (ArMV-Ta) and 17 % (GFLV-GHu) over 8 years and had a limited effect on fruit quality. Hence, utilization of the mild strains to protect vines against GFLV does not appear to be a feasible alternative for the management of grapevine fan leaf virus disease (Komar et al. 2008).

A mild isolate of *Sweet potato feathery mottle virus* (SPMV) 10–0 that could protect sweet potato (*Ipomoea batatas*) from the severe strain SPFMV-S was identified. The isolate 10–0 rarely caused skin discoloration and did not cause russet crack disease on storage roots that are typical of infection by the severe strain of SPFMV-S. RT-PCR-RFLP analysis showed that SPFMV-S did not cause any latent infection of sweet potato plants protected by the mild strain 10–0. Further, the yield of the 10-0-infected sweet potato ranged from 92 to 105 % of yield of healthy plants. Because of the narrow host range, the strain 10–0 might be spread by the aphid vectors under natural conditions. Based on the nucleotide sequence of its 3′ noncoding region and CP gene, the strain10-0 was genetically more similar to the strain SPFMV-O than to SPFMV-S. The CP gene of 10–0 encoded 315 amino acids and had sequence identities of 91.1 % at the nucleotide level and 95.6 % at the amino acid level with corresponding region of SPFMV-S. The results indicated that the mild strain 10–0 had the potential for use against the severe strains of SPFMV infection of sweet potato (Yamasaki et al. 2009).

Pepino mosaic virus (PepMV) originally isolated from *Solanum muricatum* infects tomato seriously in glasshouses. Four genotypes of PepMV namely LP, CH2, EU and US1 have been distinguished based on their nucleotide homology. An RT-PCR-RFLP-based method was developed to differentiate the genotypes of

PepMV (Hanssen et al. 2008). Isolates belonging to the LP genotype have been shown to cause only mild symptoms in tomato indicating the possibility of using them to protect tomatoes against severe strains. A mild isolate of LP genotype was identified and used for cross-protection of tomato in the Netherlands (Brakeboer 2007). In a later investigation, the cross-protection potential of a mild LP, EU, and CH2 isolates was assessed against infection by the aggressive CH2 isolate. After challenge inoculation, enhanced symptom severity was observed in tomato plants that were pre-inoculated with a protector isolate belonging to a different EU or LP genotype isolate from challenge isolate. A quantitative genotype-specific TaqMan assay showed that in these plants, accumulation of the challenge isolate slowed down only temporarily. In contrast, efficient cross-protection was achieved by using the mild isolate of CH2 genotype and the challenge isolate was barely detectable in the pre-inoculated plants. The incidence of PepMV causing typical of fruit symptoms was remarkably reduced. Furthermore, the titer of the CH2-mild isolate was significantly higher than that of the CH2-aggressive isolate which was not detectable by cloning and by specific conventional RT-PCR procedure. The results suggested that the interaction between PepMV isolates largely depended on RNA sequence homology and the post-transcriptional gene silencing (PTGS) might play an important role in cross-protection (Hansssen et al. 2010). In another investigation, greenhouse experiments were conducted under conditions similar to commercial tomato production. Two mild strains causing mild leaf symptoms were selected from field isolates, in addition to two aggressive strains of *Pepino mosaic virus* (PepMV). The severe isolates reduced the tomato yields by 8–24 % in single infections. On the other hand, yield losses were reduced to 0–3 % in tomato plants protected by the mild strains and challenged with severe strain. The quality of the fruits was not affected in mild-strain-protected plants. After challenge inoculation, levels of virus accumulation and symptom severity were significantly reduced in cross-protected tomato plants (Schenk et al. 2010).

6.3.2 Use of Virus Strains with Attenuated Virulence

Virulence levels may be altered by exposing the infected plants to high or low temperatures for predetermined periods. Physical agents like UV rays or chemicals like nitrous acid have also been used to generate mutants with altered virulence potential. The variants with reduced virulence are evaluated for their efficacy in protecting the plants against infection by severe strains.

6.3.2.1 Generation of Virus Mutants by Chemical Treatment

A symptomless *Tomato mosaic virus* (ToMV) mutant designated MII-16 was artificially produced by treating the purified virus preparation with nitrous acid. The mutant MII-16 was used to protect tomato seedlings. The tomato seedlings were

mechanically inoculated with MII-16 using a spray gun with adequate pressure. Failure of protection in some cases was attributed to ineffective inoculation of seedlings artificially (Fletcher and Rowe 1975). The mild strain has been very widely used to protect tomatoes grown in glasshouses in England and Netherlands. Yield increases up to 10 % were realized by using mutant strain (Channon et al. 1978). In Japan, an attenuated strain of TMV LIIA was found to be effective in protecting tomatoes against severe strains (Motoyoshi and Nishiguchi 1988). *Papaya ringspot virus* type W (PRSV-W), [formerly known as *Watermelon mosaic virus-1* (WMV-1)] affected cucurbit production seriously in Taiwan. The mild strain PRSV HA5-1 was derived from a severe Hawaii PRSV type strain (PRSV-HA) by nitrous acid treatment (Yeh and Gonsalves 1984). The mutant PRSV HA5-1 provided high levels of cross-protection against the severe strains under greenhouse and field conditions (Yeh and Gonsalves 1984; Yeh et al. 1988).

6.3.2.2 Generation of Virus Mutants by Temperature Treatment

Soybean mosaic virus (SbMV) causes chronic damages to black soybean (*Glycine max* cv. Shin Tambaguro) as the virus is seedborne. The soybean seedlings inoculated with SbMV were maintained at 15 °C for 14 days or alternatively for 30 days. Two attenuated isolates designated Aa15-M1 and Aa15-M2 had serological and biochemical properties similar to that of the parent wild type isolate Aa of SbMV. But these two mutants differ in the type of symptoms produced, aphid and seed transmissibility and accumulation in soybean plants. The attenuated isolates protected soybean plants from virulent strains of SbMV, when the pre-inoculated plants were challenged after 6–8 days. The pre-inoculated plants produced mild mottle symptoms on seeds with a normal-colored seed coat (Kosaka and Fukunishi 1993). A promising attenuated isolate M11 of *Bean yellow mosaic virus* (BYMV) was obtained after the low-temperature treatment of a severe isolate originally obtained from gladiolus. An efficient method for inoculating mericlone gladiolus seedlings with M11 isolate was developed to prevent severe symptoms on gladiolus (Nakazono-Nagaoka et al. 2004). The efficacy of the isolate M11 in cross-protecting against infection by gladiolus, broadbean (*Vicia faba*) and white clover (*Trifolium repens*) was assessed by applying western blotting and RT-PCR assays. The level of cross-protection varied depending on the challenge isolates. Cross-protection was complete against BYMV isolates from gladiolus, but incomplete against BYMV isolates from other hosts. A comparison of the nucleotide sequence of M11 and other BYMV isolates showed higher homology among gladiolus isolates than the homology between gladiolus isolates and non-gladiolus isolates. A comparison of the amino acid sequences between M11 and its parental isolate IbG and analysis of recombinant infectious clones between M11 and IbG revealed that an amino acid at position 314 was involved in the attenuation of BYMV. The results indicated that the isolate M11 was a promising candidate to be considered for the protection of gladiolus against BYMV (Nakazono-Nagaoka et al. 2009).

The effectiveness of cross-protection provided by the attenuated strains obtained from severe parental strains of *Cucumber mosaic virus* (CMV), *Zucchini yellow mosaic virus* (ZYMV) and *Watermelon mosaic virus-2* (WMV-2) against respective virus infection was assessed. No significant differences could be seen between single and multiple inoculations in the cross-protection by the attenuated virus isolates against their respective virulent strains, in spite of virus accumulation. This indicated that the cross-protection effect of an attenuated virus isolate was probably not affected by the presence of unrelated attenuated virus isolates in the same plant. In addition, the protecting strain exhibited its efficacy, when it had reached a minimum concentration or before it reached its highest concentration. The degree of cross-protection was correlated with the concentration of the protecting strain in the plants. The cross-protection effectiveness of CMV-mild and ZYMV-mild was not as effective as that of WMV-mild which was complete under greenhouse conditions. Multiple inoculation of cucumber with mild isolates could be desirable for the season, when economic losses due to concurrent incidence of CMV, WMV-2 and ZYMV might be greater than the yield loss due to multiple inoculations of mild isolates (Kosaka and Fukunishi 1997).

In another investigation, a new attenuated strain designated ZYMV-2002 was developed from a virulent isolate of *Zucchini yellow mosaic virus* (ZYMV), after repeated low temperature treatments at 12.5–15 °C for about 2 months, followed by five cycles of single-plant transfer. The isolate ZYMV-2002 did not induce any local lesions on *Chenopodium quinoa* and it produced very mild or no symptoms on cucurbit plants. The cucumber plants inoculated with ZYMV-2002 apparently delayed and suppressed the expression of mosaic symptoms and/or deformed leaves characteristic of challenge viruses. Plants inoculated with ZYMV-2002 had very similar fruit productivity to healthy control plants under field conditions. The isolate ZYMV-2002 could be differentiated from other ZYMV isolates by RT-PCR analysis. By using the primers AZ199-F and IZR, amplification of the DNA fragment of the expected size (199-bp) occurred only from the cucumber leaves infected only with ZYMV-2002. In contrast, amplification with primers SZ199 and IZR yielded DNA fragments in Z5-1 and KAMO-infected plants, but no detectable products from ZYMV-2002-infected plants. Poor aphid-transmissibility of the isolate ZYMV-2002 was considered as a desirable trait of attenuated viruses to minimize the risk of economic loss due to natural spread of the mild isolate to other crops in which it might become virulent (Kosaka et al. 2006). The efficacy of an attenuated strain of Japanese yam *mosaic virus* (JYMV) was assessed to protect the Chinese yam (*Dioscorea opposita*) against infection by severe strains of JYMV. The Chinese yam plants grown from virus-free tubers were checked for freedom from JYMV by employing the PCR-based assay. The tubers were pre-inoculated with T-3 strain before planting in the field. JYMV was detected in only one of the 50 plants pre-inoculated with the attenuated strain during the experiments for 6 years, indicating that T-3 strain was able to protect the Chinese yam plants effectively over a long period under field conditions. In addition to the protection against virus infection, pre-inoculation with T-3 strain significantly increased the yield of tubers per plant compared with unprotected plants (Kajihara et al. 2008).

6.3.2.3 Generation of Virus Mutants by Genetic Modification

Cross-protection provided by the mild strain against severe strains appears to be highly strain- or isolate-specific and the specificity may be determined by the genetic relatedness of the challenge viruses. Generation of an attenuated strain from a locally prevalent severe strain may result in higher level of cross-protection to pre-inoculated plants. Three amino acid changes $Arg_{180} \rightarrow Ile_{180}$ (GA mutation), $Phe_{205} \rightarrow Leu_{205}$ (GB mutation) and $Glu_{396} \rightarrow Asn_{396}$ (GC mutation) of the conserved motifs of the helper component, protease (HC-Pro) of a severe strain TW-TN3 of *Zucchini yellow mosaic virus* (ZYMV) were generated from an infectious cDNA clone that carried a green fluorescent protein (GFP) reporter. These three mutants caused milder mosaic symptoms on squash plants. Single substitution in these three amino acids apparently affected the virulence of the mutants and also significantly reduced accumulation of GFP. The GAC mutant conferred complete cross-protection against the parental virus carrying a mite allergen as a reporter. The cross-protection reaction was completely induced at 5 days after the protective inoculation. The absorbance values of ELISA tests on the challenge virus were inversely related with the increase in the length of protection. The GAC mutant was stable, as reflected by the observation that no revertants with severe strain sequences were found after nine passages through squash plants after a period of 9 months. The results showed that the mutant GAC had the potential for providing cross-protection against ZYMV infection in squash plants (Lin et al. 2007).

In a later study, the attenuated mutant without the GFP reporter *Zucchini yellow mosaic virus* (ZYMV)AC (ZAC) derived from GAC mutant was evaluated for its aphid-transmissibility and cross-protection effectiveness in cucurbits. Apterous aphids (*Myzus persicae*) were allowed a 10-min acquisition access on plants inoculated with the severe TN3 or attenuated ZAC strain. The results indicated that the mutant virus ZAC lost its aphid transmissibility even though the level of virus accumulation was as high as that of the severe strain. Squash plants protected with ZAC strain remained symptomless and grew normally. Complete protection provided by ZAC, as indicated by ELISA-negative results, was also observed, when challenge inoculation was performed at 5 and 10 days after protective inoculation. The ELISA absorption values also demonstrated that replication of the challenge TN3 virus was entirely inhibited in protected plants. The ZAC mutant was able to effectively protect the major crops watermelon, oriental melon and cucumber widely grown in Taiwan. The results revealed that the mutant ZAC could provide effective cross-protection to important cucurbits against ZYMV and the lack of aphid transmissibility of the mutant added additional basis for its suitability for use against the economically important virus disease (Chao et al. 2010).

6.3.3 Mechanisms of Cross-Protection Induced by Viruses

The phenomenon of cross-protection operating in plants inoculated with a mild strain of a virus, leading to reduction in the adverse effects of the severe strain of the same virus on plant growth and yield has been demonstrated in many pathosystems.

Different mechanisms of cross-protective activity of mild strains have been proposed to explain the beneficial interaction between the mild and severe strains of plant virus diseases that are not amenable for control by other methods.

6.3.3.1 Exhaustion of Precursors of Virus Replication

When the protecting strain is allowed sufficient time for reaching high concentrations, before the challenge virus is inoculated, all available precursors required for the challenging strain become limited, resulting in the failure of severe strain development to attain the concentration to express its presence by the producing characteristic symptoms. Evidence to support this suggestion was presented by Hageman (1964). A significant correlation was observed between the decrease in the amount of microsomal RNA and the degree of cross-protection between two strains of *Tobacco mosaic virus* (TMV). However, other reports indicated that the availability of precursors might not be a limiting factor for the development of severe strains. The mild strains generally reach only low concentration, but they are able to effectively protect plants against severe strains, as in the case of *Tobacco streak virus* (TSV) (Fulton 1978). All viruses and their strains are capable of replication in susceptible plants utilizing the same amino acids and nucleotides available in the plant species. The possibility of unavailability of these basic materials for virus replication being a limiting factor appears to be remote.

6.3.3.2 Competition for Specific Sites Between Virus Strains

The strain inoculated first may gain access to the specific site(s) required for its replication and thereby it may exclude the challenge strain gaining access to the same site(s), resulting in failure of infection by the challenge strain. On the other hand, unrelated viruses requiring different sites for multiplication, may be able to infect the same susceptible cell (Bawden 1950). The virus-specific RNA polymerase may be a site for which there may be competition between protecting strain and challenge strain and the latter may be at a competitive disadvantage with the established strain (Barker and Harrison 1978). The specificity of ribosome-binding may be altered by the virus-induced polypeptide in favor of the protecting viral RNA. The mRNA of the protecting strain may occupy most of the available ribosomes, creating an unfavorable situation for the development of challenging strain (Ziemiecki and Wood 1976).

The ability of *Papaya ringspot virus* (PRSV)-W-C severe strain to compete for replication sites in plants previously infected with the mild strain PRSV-W-1 was evaluated as a potential mechanism for cross-protection against the severe strains of the virus. It is necessary to study the systemic movement of PRSV-W starting from different inoculation points to identify possible virus-free sites. The severe strain PRSV-W-C was detected in the cotyledonary leaves by plate-trapped antibodies (PTA)-ELISA format at 8 days after inoculation into the first true, partially expanded leaf of the plants. The fact that the virus was not detected in the cotyledonary leaves

of test plants could be, due to the absence of the virus, as a result of competition for replication sites. The failure to detect the PRSV-W-C may also be due to the very low virus concentration that was not detectable by PTA-ELISA test. The results indicated that competition for replication sites at the infection point was involved in the protection between PRSV-W strains, especially in the first days after protective inoculation with the mild strain. After some time, there seemed to exist some degradation mechanism of the severe strain which could be of the gene silencing type that is considered to operate in plant-virus interactions (Frietas and Rezende 2008). The fact that cross-protection occurs generally between closely related virus or strains, implies that virus nucleotide sequence similarity is required for competition. The results of the investigation on cross-protection between strains of *Grapevine fan leaf virus* (GFLV) support the hypothesis of competition between virus strains as a mechanism of cross-protection. The rate of GFLV infection was markedly lower in virus initially infected with GFLV-GHu (19 %) than in those initially infected with ArMV-Ta strain (40–55 %) (Komar et al. 2008).

Among the three mutants of *Zucchini yellow mosaic virus* (ZYMV) involving amino acid changes, the GAC mutant conferred complete cross-protection against the parental strain of ZYMV. The squash plants became resistant to ZYMV at 5 days after the protective inoculation with GAC mutant. Cross-protection induced by the mild strain could be differentiated into three stages: initiation, resistance and maintenance. The defensive reaction is triggered, as the mild strain initiates the infection process (within 5 days after protective inoculation). After the host plant's defense systems are stimulated, the defective (mutated) HC-Pro proteins of the GAC strain is unable to arrest the plant's defense responses, leading to sharp reduction in accumulation of mild strain. However, the mild strain perhaps retains some ability to help itself to survive, as low levels of mild strain can be detected at this stage. The low titer of mild strain continues to stimulate and maintain the plant defense responses, resulting in high level of protection against the severe strain of ZYMV. In the initiation stage, the protection may result from competition for replication factors that are already used by the protective strain. At the maintenance stage, cross-protection is virus-dependent and a low titer of the protective virus is needed for stimulating the plants to continuously synthesize host factors for maintaining the protection mechanism at the required level (Lin et al. 2007; Chao et al. 2010).

6.3.3.3 Post-transcriptional Gene Silencing (PTGS)

Post-transcriptional gene silencing (PTGS) appears to be the hypothesis currently used to explain the phenomenon of cross-protection. The protective strain induces RNA silencing against its own RNA and homologous sequences such as those occurring in closely related strains of the same virus. Thus, the protective strain may act as an elicitor of a natural antiviral response, RNA silencing which underlies other natural resistance phenomena, such as recovery and green island formation (Goldbach et al. 2003). Post-transcriptional gene silencing (PTGS) as a functional

mechanism for cross-protection of mild strains against severe strains of the same virus was proposed by Ratcliff et al. (1999). In the case of *Citrus tristeza virus* (CTV), pre-immunization with the strain PB61 protected the sweet orange seedlings more effectively against orange stem pitting (OSP) isolate PB235 than OSP isolate PB155, possibly because PB235 had closer nucleotide sequence homology to PB61 than to PB155. The hypothesis based on RNA-mediated defense was proposed to account for the cross-protection provided by the mild isolates of CTV. Infection with the protective strain triggered the host to produce a ds-RNA-specific nuclease which targets the viral RNA for degradation to low concentrations. This may lead to the formation of small nucleotide fragments such as those detected during PTGS. Other transcripts homologous or ones similar to the silenced gene are also degraded, if they infect the plant, after the establishment of PTGS initiated by the mild strain of CTV (Zhou et al. 2002). *Tobacco rattle virus* (TRV) and *Potato virus X* (PVX) constructs share a common sequence and one viral construct could suppress the other through RNA-mediated cross-protection in co-infected plants. Based on the results, it was suggested that cross-protection was mediated by pre-activation of the RNA-induced silencing complex (RISC) with small interfering RNA (siRNA) derived from the protector virus RNA, thus inhibiting replication of the challenge isolate. In contrast, co-infection of two viruses with limited sequence similarity could lead to synergism, mediated by inhibition of the PTGS defense mechanism by viral silencing suppressors (Ratcliff et al. 1999; Gal-On and Shiboleth 2006).

Many plant viruses have adapted to resistance of host plants, mediated by RNA silencing by acquiring silencing suppressor proteins that enable them to evade or blunt the effect of this defense mechanism. Viral suppressor proteins target different points of the machinery regulating induction, amplification and maintenance of RNA silencing (Palukaitis and Mac Farlane 2005). *Cucumber mosaic virus* (CMV) encodes a suppressor of RNA silencing, the 2b protein which can also act as a symptom determinant. It induces disease symptoms by interfering with micro-RNA mediated gene regulation (Lewsey et al. 2007). A CMV mutant of subgroup IA strain Fny (Fny-CMVΔ2b) which could not express the 2b silencing suppressor protein, cross-protected tobacco and *Nicotiana benthmiana* plants against the wild-type strain Fny-CMV. In addition, FnyCMVΔ2b also protected plants against infection by TC-CMV, a subgroup II strain which was not closely related to Fny-CMV. In situ hybridization analysis revealed that Fny-CMVΔ2b and Fny-CMV were able to co-exist in the same tissues, but these tissues contained zones of Fny-CMVΔ2b-infected host cells from which the parental strain appeared to be excluded. The results indicated that infection with the mutant strain resulted in the generation of a strong systemic silencing signal directed against CMV-specific RNA sequences. It is more likely that protection occurred through either induction of very highly localized RNA silencing or by competition between strains for host cells or resources (Ziebell et al. 2007).

The attenuated isolate M11 of *Bean yellow mosaic virus* (BYMV) exhibited different levels of cross-protection in gladiolus, depending on the challenge virus isolates. Cross-protection was complete against isolates from gladiolus, but incomplete

against BYMV isolates from other hosts. Post-transcriptional gene silencing (PTGS) was considered to be functionally involved in the cross-protection. A computational estimation system to predict potential targets for PTGS was developed (Xu et al. 2006). With estimation system for potential RNA segments were recognized in the M11 genome (+ sense). Of the 876 RNA segments, 866 were found in the genome of the parental isolate IbG. When the M11 and the gladiolus isolates Gla and Gl that M11 completely cross-protected were compared, 447 and 411 common interference RNA segments respectively were found, even though G1 was molecular biologically the most distantly related to M11 among the three gladiolus isolates. In contrast, less than 100 common interference RNA segments were found between M11 and nongladiolus isolates, against which M11 partially cross-protected. The CS isolate which is the most distantly related to M11, shared only ten RNA segments. *Clover yellow vein virus* (ClYVV)-No.30, a virus distinct from BYMV, but was partially protected by M11, shared two RNA segments with M11. These results suggest that PTGS is likely to be involved in the cross-protection by mild (attenuated) strain of BYMV against severe strains infecting gladiolus (Nakazono-Nagaoka et al. 2009). A mild isolate of the CH2 genotype of *Pepino mosaic virus* (PepMV) efficiently cross-protected tomato plants against infection by aggressive isolate. The challenge isolate was barely detectable in the pre-inoculated tomato plants. The nucleotide sequence homology between the mild and aggressive CH2 isolates of PepMV was 99.4 %. The results suggested that RNA sequence homology could be a determining factor in PepMV cross-protection efficiency in tomato and PTGS might play an important role in cross-protection of tomato plants by mild strain of PepMV (Hansssen et al. 2010).

6.3.4 Cross-Protection by Engineered Mild Strains of Viruses

The possibility of enhancing the effectiveness of cross-protection by mild strains against severe strains by modifying viral genome was explored. Chimeric mild strains of *Papaya ringspot virus-W* infecting Cucurbitaceae, were constructed from HA5-1 to carry a heterologous coat protein (CP)-coding region and or a M′ UTR (untranslatable region HA5-1Wep3u provided much higher level of cross-protection against the type P virus P-HA in cucurbits. The results indicated that broad-spectrum protection against two strains (differentiated only by their ability to infect (PRSV-R) or not (PRSV-W)), can be achieved by using a chimeric mild potyvirus, carrying a heterologous 3′ genomic region, since the 3′ UTR of the potyvirus is involved in the replication for positive-strand viral RNA. Sequence of homology dependent PTGS is considered an antiviral defense mechanism in virus-infected plants, playing an important role in cross-protection against the same virus or related strains (Voinnet 2001). The chimeric mild virus HA5-1Wep3u provided more effective protection against W-CI in horn melon and squash plants than HA5-1 could provide. This may be due to a consequence of the effect of the identical sequences of the CP region and 3′ UTR between HA5-1Wep3u and W-CI through a PTGS mechanism. The high

degrees of protection provided by all chimeric mild strains against P-HA in horn melon and squash plants could be triggered by PTGS induced by long homologous RNA segments shared by individual chimeric mild strains and the P-HA strain. The results demonstrated that the chimeric attenuated virus strains have great potential for providing high degree against cross-protection of different strains of the same virus varying in their host range (You et al. 2005).

6.4 Subviral Agents for Biological Disease Management

Subviral entities like the satellite RNAs and viroids have only nucleic acids and lack the protein component present in the viruses. The satellite RNAs are unable to replicate independently in plant cells in the absence of a specific helper virus. In contrast, viroids can multiply independently in susceptible plants and induce specific disease symptoms.

6.4.1 Satellite RNAs as Biocontrol Agents

Cucumber mosaic virus (CMV)-associated satellite RNA is the best documented among the satellite RNAs. CMV-associated RNA5 (CARNA5) was first described by Kaper and Waterworth (1977). The basic genome of CMV consists of three separately encapsidated ss-RNA molecules (RNA1, 2 and 3) and all these three RNAs are required for virus replication. RNA4 is encapsidated with the three major RNA molecules, but it is not required for virus replication. CMV-associated RNA5 (CARNA5) is a satellite depending on the CMV genome for its replication and encapsidated along with other genomic RNAs. It is produced in different quantities, depending on the host plant species. CARNA5 induces different biological effects, depending on the host plant species. In tobacco, the titer of CMV was greatly reduced and the symptoms of infection became milder, as large amounts of satellite RNA accumulated (Kaper and Tousignant 1977). In contrast, increased disease intensity resulting in lethal necrosis was observed in tomato, when CARNA5 was added to the CMV inoculum (Waterworth et al. 1978). The effectiveness of using CARNA5 as a biocontrol agent against CMV was assessed. CARNA5 was employed to protect tomato plants against infection by two severe strains of CMV (D and 16). The satellite RNA-mediated protection was more effective and could be established earlier, compared to conventional cross-protection provided by mild strains. In the field trials conducted in locations of high CMV incidence in Italy, inoculation of tomato plants with CMV-S containing CARNA5 effectively protected more than 95 % of the plants. Fruit yields registered a 100 % increase over unprotected control plots (Gallitelli et al. 1991; Montasser et al. 1991). A combination of a benign satellite RNA and a mild strain of CMV, when used for pre-inoculation, offered effective

protection to pepper and melons against two severe strains of CMV both in the glasshouse and field experiments (Montasser et al. 1998).

Satellite RNAs associated with plant viruses can affect the replication, pathogenesis and symptom expression in plants infected by respective viruses. A DNA construct was introduced into 'UC82' tomato plants whose transcript contained a single full-length copy of *Cucumber mosaic virus* (CMV)-Tfn-sat-RNA, benign variant of CMV satellite RNA. Tomato plants expressing the sat-RNA did not exhibit any symptoms of CMV, when challenged with sat-RNA-free strain of CMV (CMV-FL). In transgenic plants inoculated with CMV-FL, the symptomless phenotype was correlated to the down-regulation of CMV by Tfn-satRNA, amplified from the transgene transcripts. The delayed resistance to CMV-77 (strain supporting a necrogenic variant of sat-RNA) in transgenic lines was mediated by a degradation process that targets sat-RNAs in a sequence-specific manner. It appeared that there could be correlation between a reduced accumulation level of transgenic messenger Tfn-sat RNA, the accumulation of small RNAs (approximately 23 nucleotides) with sequence homology to sat-RNAs, the progressively reduced accumulation of 77 sat-RNAs in infected tissues and the transition in infected plants from diseased to healthy. The events resulting in the degradation of sat-RNA sequences indicated a role of RNA silencing as the second mechanism determining resistance of transgenic tomato lines (Cillo et al. 2004).

6.4.2 Viroids as Biological Control Agents

Viroids are subviral pathogens consisting entirely of a short strand of RNA with a molecular weight ranging from 10,000 to 140,000. They can replicate independently, reaching high concentrations, when inoculated into a susceptible plant species and cause characteristic symptom. Viroids have small covalently closed circular RNA molecules with a highly base-paired, rather stiff rod-like native conformation (Diener 1979; Henco et al. 1979). Mixtures of viroids from citrus were evaluated for their ability to protect citrus plants against the root rot disease caused by *Phytophthora nicotianae* and *P. citrophthora*. The viroid mixtures consisting of *Citrus exocortis viroid* (CEVd), *Hop stunt viroid* (HSVd), *Citrus viroid III* and *Citrus viroid IV* reduced the number of sporangia of *Phytophthora* produced on the bark, leaves and roots used as baits to varying degrees. Infection by *Phytophthora* was also suppressed significantly by the citrus viroids. The source plant 13E containing CEVd, HSVd and CVd-III suppressed the sporangial production to the maximum extent. The suppressive effect could not be related to any of the viroid specifically. The results suggested that there could be a possible relationship between the concentrations of the phenolic acids in viroid-infected plants and suppression of sporangial production. Similarly changes in the contents of salicylic acid, flavones and quercetin dihydrate might have some role in viroid-induced resistance to *Phytophthora* infection of citrus (Thomas et al. 2010). The effects of viroid infection on the growth and yield of citrus plant do not seem to have been assessed in this investigation.

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Chapter 7
Genetic Engineering for Improving the Performance of Biotic Biological Control Agents

Various species and strains of organisms have been assessed for their efficacy in suppressing the development of the phytopathogens and the diseases caused by them. The principal obstacle facing widespread use of the biocontrol agents (BCAs) is that the level of control provided under commercial conditions frequently falls short of chemical control. A superior antagonist is yet to be identified through conventional screening of the naturally occurring microflora. Enhancing the biocontrol potential of microbes could be the most important factor in their acceptance for management of diseases affecting various crops at pre- and postharvest stages on commercial scale. The genetic potential of a BCA can be expected to play the crucial role in its antagonistic activity against microbial plant pathogens. The genetic diversity of a biocontrol agent has to be studied by collecting all available strains and isolates and subjecting them to analysis by different molecular techniques such as random amplification polymorphic DNA (RAPD), DNA fingerprinting and polymerase chain reaction (PCR)-based assays. If genes controlling the biosynthesis of antimicrobial compounds or enzymes can be identified, such information can facilitate the identification of methods to enhance the biocontrol potential of the microorganism(s) selected for genetic manipulation. When the efficacy of the naturally occurring species/strains is not up to the required level, genetic modification through biotechnological methods has been attempted to improve the performance of the biocontrol agents. Identifying the most efficient strain(s) among the naturally existing strains of the biocontrol species, genome analysis, identification of genes controlling the production of metabolites with antifungal and antibacterial activity, characterization of gene products, induction of mutation, deletion or transfer of gene(s), increasing the copy number of basic gene(s), and protoplast fusion are some of the aspects studied for the enhancement of the efficacy of the biocontrol agents.

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7.1 Fungal Biological Control Agents

7.1.1 Selection of Efficient Fungal Species/Strains

Selection of strains or isolates with specific characteristics to suit a certain environment is the first approach made to increase the effectiveness of the biocontrol agents. Random fragment length polymorphism (RFLP) technique has been employed to identify the specific isolate of the BCA with highest efficiency. A hybridizing band of 1.1-kb of diagnostic value was identified in *Trichoderma harzianum* by RFLP analysis. By using this hybridization probe, the isolate C65 of *T. harzianum* was differentiated from other closely related *Trichoderma* spp. and strains that were less effective, compared with the strain C65 (Bowen et al. 1996). In an extensive study based on RFLP analysis, the worldwide collections of *Ampelomyces quisqualis* effective against powdery mildew pathogens were classified into seven RFLP groups. Isolates belonging to one RFLP group were distributed in different continents and in one location genetically distinct isolates of the BCA could be detected. Genetic differences might influence the effectiveness of the isolates as biocontrol agents against powdery mildew diseases, indicating the need for selecting the most effective isolates or strains for genetic modification (Kiss 1997).

Selection of strains or isolates with greater tolerance or resistance to fungicides or bactericides is essential for control of diseases affecting fruit and vegetable crops which generally receive frequent applications of chemicals. The biocontrol agents (BCAs) compatible with fungicides are either applied as a mixture or alternated with fungicide sprays as in the case of *Trichoderma harzianum* (Elad et al. 1994; Shtienberg and Elad 1997). Likewise, by selecting cold-tolerant isolates of *Gaeumannomyces graminis* var. *tritici* and *Phialophora* sp. (lobed hyphopodia), the effectiveness of biocontrol of take-all disease of wheat could be significantly enhanced (Wong et al. 1996). A new strain of *Sporothrix flocculosa* tolerant to high doses of dodemorph acetate (300 µg/ml) was obtained by repeated exposure of the BCA to the fungicides and it retained this tolerance trait, after several subcultures in fungicide-free medium. This fungicide-tolerant strain was equally effective in suppressing rose powdery mildew pathogen *Sphaerotheca pannosa* var. *rosae* and its biocontrol activity was not affected, when applied as a mixture with dodemorph acetate (Benyagoub and Bélanger 1995). The genetic variability among eight isolates of *Trichoderma harzianum* was assessed by the random amplified polymorphic DNA (RAPD) technique. The ability of these isolates to antagonize the fungal pathogen *Sclerotium rolfsii* determined by employing dual culture assay was correlated with RAPD profiles. Eight oligodeoxynucleotide primers were selected for RAPD analysis and 86 bands were obtained for eight isolates of *T. harzianum*. The RAPD analysis showed that variability among isolates of *T. harzianum* was very high. Using the dual culture assay, *T. harzianum* isolates were classified into distinct classes of antagonism. *T. harzianum* isolates were screened for their ability to produce chitinase and ß-1,3-glucanase which have a role in the biocontrol activity against *S. rolfsii* (Sharma et al. 2009). Biocontrol potential of some of the fungal

biocontrol agents is dependent on the production of ß-1,3-glucanase which is an important cell wall degrading enzyme (CWDE) involved in mycoparasitism. Hence, precise assessment of ß-1,3-glucanase activity may be a reliable basis for assessing the efficacy of the biocontrol species/strain. A simple microplate-based method was developed to assay ß-1,3-glucanase activity of various isolates of *Trichoderma* spp. The sensitivity of the optimized micromethod was shown to be fourfold greater than the expensive tube assay method. Thus, by applying the sensitive method, it may be possible to select the most efficient strain(s) of the biocontrol agent (Ramada et al. 2010).

7.1.2 *Induction of Mutation in Specific Genes of Fungal Biological Control Agents*

Among the fungal biocontrol agents, strains of *Trichoderma* have been employed increasingly for the control of soilborne pathogens. The most frequently suggested mechanisms of biocontrol activity of *Trichoderma* include mycoparasitism, antibiosis, competition for nutrients and space, induction of systemic resistance against microbial pathogens or all of these mechanisms in different combinations, depending on the BCA-pathogen interactions. Other species of fungal BCAs also possess these different mechanisms of biocontrol activity against various plant pathogens. *Trichoderma harzianum* has a chitinolytic system consisting of five to seven distinct enzymes, depending on the strain of this BCA. The entire chitinolytic system may be essential for maximum efficiency as BCA. The ability of individual enzyme of the complex, the 42-kDa endochitinase (Ech42) has been investigated. Ech42 could hydrolyze *Botrytis cinerea* cell walls in vitro and inhibit spore germination and germ tube elongation of other fungi (De La Cruz et al. 1992). The gene *ech42* encoding this endochitinase was strongly induced during interaction with other fungi. Expression of *ech42* was repressed by glucose (Garcia et al. 1994). The role of Ech42 in the mycoparasitism of *T. harzianum* has been studied by manipulating the *ech42* gene. Several transgenic strains of *T. harzianum* carrying multiple copies of *ech42* and the corresponding gene disruptants were generated. Under inducing conditions, the level of extracellular endochitinase activity of multicopy strains dramatically increased up to 42 folds, compared to the parent strain. In contrast, the disruptant had lost the enzymatic activity entirely. However, no major differences in the efficacy of the transformants as BCA against *Rhizoctonia solani* and *Sclerotium rolfsii* could be observed in the greenhouse assays (Carsolio et al. 1999).

Mycoparasitism of *Trichoderma* has been shown to be due to the action of cell wall-degrading enzymes. Chitinolytic and glucanolytic enzyme systems involved in the mycoparasitism of *Trichoderma* have been studied. *Trichoderma* strains are known to produce extracellular proteases. The involvement of *T. harzianum* proteases in the biocontrol activity against *Botrytis cinerea* was suggested by Elad and Kapat (1999). Many acidic, neutral and basic extracellular proteases have been detected in the culture filtrates of *T. harzianum* (Delagada-Jarana et al. 2000).

The strain *T. harzianum* T334 had the ability to produce low levels of protease constitutively. To improve its antagonistic ability, UV-mutagenic experiments were performed. The mutant strains were generated by UV-irradiation and they were selected for *p*-fluorophenyl-alanine resistance or altered colony morphology. It was shown that by means of specific chromogenic substrates, it would be possible to elevate secretion of both trypsin-like and chymotrypsin-like proteases by mutant strains. The profiles of isoenzymes of mutants and parent strain were different, as indicated by gel filtration chromatography. Some of the mutants, col26, pfa37 and pfa5 were found to be more efficient in suppressing the mycelial growth of the soil-borne pathogen *Rhizoctonia solani*. The results indicated the possibility of generating mutants of BCA with improved constitutive extracellular protease secretion against fungal plant pathogens by UV mutagenesis (Szekeres et al. 2004).

Trichoderma harzianum 1295–22, exhibited strong rhizosphere competence and ability to control many fungal pathogens, including turf grass dollar spot disease caused by *Sclerotinia homoeocarpa*. Soil population of *T. harzianum* increased by 100 folds, following application of commercial granular or peat formulations and this resulted in significant reduction in the incidence of dollar spot disease of turf grass (Lo et al. 1996). However, root colonization did not appear to be essential for suppression of damping-off disease of cucumber caused by *Pythium ultimum*. The strain 17R1, a rifampicin-resistant mutant of *Escherichia coli* strain S17-1, protected the cucumber seedlings effectively against the pre- and post-emergence damping-off caused by *P. ultimum*, when applied as seed treatment. The buildup of the mutant population was very slow, reaching 65-fold increase in the soil, compared to the 24,000-fold increase in the population of *Enterobacter cloacae* strain 501R3, indicating that extensive root colonization of the host plant was not required for suppression of disease development, because of the brief period of susceptibility of cucumber to damping-off pathogen *P. ultimum* (Roberts et al. 1997). The fungal biocontrol agent *Talaromyces flavus* was shown to effectively suppress the development of the fungal pathogen such as *Verticillium dahliae* and parasitized *Sclerotinia sclerotiorum, Rhizoctonia solani* and *Sclerotium rolfsii*. The mutant of *T. flavus* BenRTF1, selected for benomyl resistance, over-produced extracellular enzymes, chitinase, ß-1,3-glucanase and cellulase. This mutant was more efficient in inhibiting *S. rolfsii* and *V. dahliae*. The antagonism of the mutant was attributed to the enhanced activity of chitinase (Madi et al. 1997). *Fusarium proliferatum* is a mycoparasite of grape downy mildew pathogen *Plasmopara viticola*. A cold-tolerant strain of *F. proliferatum* was isolated following UV mutagenesis of the G6 strain. The mutant strain 1505 exhibited radial growth two- to threefold that of the parent strain G6, when grown at 13 °C which was generally suboptimal for the growth of *Fusarium* spp., but desirable for its host *P. viticola*. The ability of the isolate 1505 to suppress the number of sporangia produced by *P. viticola* (by 85 %) at 25 °C was similar to that of the parent isolate G6, indicating the antagonistic activity of the isolate 1505 was not impaired by the mutagenesis procedure. In addition, the antagonistic activity of the isolate 1505 was significantly increased at low temperature (13 °C), compared with that of the parent strain G6. The mutant strain 1505 produced significantly higher levels of extracellular ß-glucosidase and endo-1,4-ß-glucanase in the culture filtrate relative

to that of parent isolate G6. The results indicated that the biocontrol potential of *F. proliferatum* could be improved by UV mutagenesis approach (Bakshi et al. 2001).

The possibility of inducing variants (mutants) lacking pathogenicity that may be employed as biocontrol agents against the same pathogen was explored. Mutants of *Colletotrichum gloeosporioides* isolate Cg-14, infecting avocado were generated by insertional mutagenesis by restriction enzyme-mediated integration (REMI) transformation. Among the 14 isolates showing reduced virulence, compared with the wild-type strain, the mutant Cg-M-142, when preinoculated, delayed symptom development by the wild-type strain. Furthermore, this mutant induced resistance to the wild-type strain and this phenomenon was accompanied by an increase in the levels of preformed antifungal compound, diene, from 760 to 1,200 μg/g fresh weight at 9 days after inoculation. Another mutant Cg-M-1150, failed to produce appressoria, but exhibited reduced macerating ability on mesocarp and no symptoms on the pericarp. This mutant neither altered the concentration of diene produced, nor delayed the appearance of decay symptoms on inoculated fruit. This study indicated that mutants with reduced pathogenicity may be used for biological control of anthracnose disease of avocado (Yakoby et al. 2001).

7.1.3 Transformation of Fungal Biocontrol Agents

Transformation of biocontrol agents by transferring specific genes has been attempted to improve the effectiveness of the biocontrol activity against target pathogens. A strain of *Trichoderma harzianum* was produced by transformation. The plasmid containing the *TUB2* gene from the yeast *Saccharomyces cerevisiae* conferring tolerance to carbendazim was transferred to *T. harzianum* which was able to tolerate high doses of carbendazim that was applied for disease control in the field (Yang and Zhao 1996). The effectiveness of biocontrol activity may be increased by enhancing the level of expression of desired genes. The simplest approach to improve the transcription level of the introduced gene is to increase its copy number. Biocontrol activity of *T. harzianum* was enhanced by increasing the copy number of the basic proteinase gene *prb1*. Transformants carried 2–10 copies of *prb1*, resulting in over-expression of the gene encoding proteinase. Production of proteinase was induced by the cell walls of *Rhizoctonia solani*, causing root rot diseases. The transformants significantly reduced the root rot disease in cotton, when incorporated into the pathogen-infested soil in the greenhouse (Flores et al. 1997)

Fungal biocontrol agents produce cell wall-degrading enzymes (CWDEs) and antibiotics which have important role in their biocontrol activities. The combination of hydrolytic enzymes and antibiotics of *Trichoderma* spp. has been shown to have an antifungal synergism. The gene encoding chitinase (ChiSH1) from *Kruthia zopfi,* a chitin digesting bacterium was introduced into cells of *Escherichia coli*. The transformed bacterium was applied on barley leaves inoculated with the powdery mildew pathogen, *Erysiphe graminis*. Growth of the pathogen was

effectively suppressed by the transformed BCA (Ikeda et al. 1996). Transformants of *Trichoderma longibrachiatum* with extra-copies of *egl1* gene encoding the ß-1,4-endoglucanase under the control of a constitutive promoter exhibited the highest enzymatic activity. The transformants, when applied to cucumber seeds sown in soil infested with *Pythium ultimum*, suppressed the damping-off disease more effectively than the wild-type strain (Migheli et al. 1998). Chitinase Chit33 from *Trichoderma harzianum* CECT2413 is encoded by *chit33* gene whose expression is repressed by glucose and derepressed by chitin, fungal cell walls or starvation conditions. Transformants of *T. harzianum* CECT2413 that overexpressed a 33-kDa chitinase Chit33 was cotransformed with the *amdS* gene and its own *chit33* gene under the control of the *pki* constitutive promoter from *T. reesei*. Southern blotting indicated that the *chit33* gene was integrated ectopically mostly in tandem. Some transformants showed the same restriction pattern, indicating preferable sites of integration. There was no correlation between the number of integrated copies and the level of expression of the *chit33* gene transformants. When grown in glucose, the extracellular chitinase activity of the transformants was up to 200-fold greater than that of the wild-type, whereas in the presence of chitin, the activity of both transformants were more effective in inhibiting the growth of *Rhizoctonia solani*, compared with the wild-type strain. The culture supernatants from the transformants and wild-type strain showed similar effects as the strains themselves (Limón et al. 1999).

Trichoderma harzianum, an efficient biocontrol agent applied for the control of several microbial plant pathogens, produces cell wall-degrading enzymes (CWDEs). The antagonism of *T. harzianum* correlates well with the production of antifungal enzymes such as chitinases. The chitinases Chit42 and Chit33 from *T. harzianum* CECT2413 do not have a chitin-binding domain. By incorporating a cellulose-binding domain (CBD) from cellobiohydrolase II of *T. reesei* to these enzymes, hybrid chitinases Chit33-CBD and Chit42-CBD with stronger chitin-binding capacity were engineered. The transformants of *T. harzianum* over-expressing the native chitinases exhibited higher levels of chitinase-specific activity, resulting in greater inhibition of growth of pathogens such as *Botrytis cinerea, Rhizoctonia solani* and *Phytophthora citrophthora*. The results reveal the importance of endochitinase in the antagonistic activity of *T. harzianum* strains and the effectiveness of incorporation of a CBD to enhance the biocontrol potential of the microorganisms against fungal pathogens (Limón et al. 2004). In another study, the vectors pBI121 and pCAMBIA1301 and cloning vector pUC18 were employed to successfully construct expression vector pCA-GChiV for filamentous fungi transformation mediated by *Agrobacterium tumefaciens* (At). The *ChiV* gene was transferred into *T. harzianum* with an efficiency of 90–110 transformants per 10^7 spores, using At-mediated transformation. Putative transformants were analyzed by Southern blot. The expression of *ChiV* was detected by RT-PCR format. The transformants were co-cultured to assay antifungal activities with *Rhizoctonia solani.* The inhibition rates of the transformants and wild-type strain were 98.56 and 84.42 % respectively on the 4th day. The results indicated the usefulness of the *ChiV* transformants that exhibited higher level of pathogen suppression (Yang et al. 2011).

The process of mycoparasitism is complex and lytic enzymes such as chitinases, ß-1,3-glucanases and ß-1,6-glucanases and proteases have been shown to have a role in this process. In vitro confrontation assays and gene expression studies have also implicated these enzymes in mycoparasitism (El-Katatny et al. 2001; Djonovic et al. 2006a, b). Constitutive over-expression of genes, encoding individual cell wall-degrading enzymes (CWDEs) has been demonstrated to improve the potential of biocontrol agents. Transformants of *Trichoderma virens* in which ß-1,3-glucanases and ß-1,6-glucanases genes *TvBgn2* and *TvBgn3* respectively were constitutively co-expressed in the same genetic *T. virens* GV29.8 wild-type background, were generated. The double over-expression transformants (doEs) grew and sporulated at a slower rate, compared with the wild-type (WT) strain. However, the reduction in growth did not appear to affect their mycoparasitic and biocontrol potential, since doEs displayed much higher levels of total ß-1,3- and ß-1,6- glucanase activity than the WT. The activity of doEs on growth of pathogens *Pythium ultimum* and *Rhizoctonia solani* was more effective than that of single over-expression strains (oEs). The proteins secreted by doEs significantly inhibited both pathogens. The inhibitory activity of the transformants was mainly due to higher ß-1,3- and ß-1,6-glucanase activity. The doEs greatly increased the level of protection against both pathogens (up to 312 % over that of WT), when disease pressure was higher. The higher enzymatic activity of doEs was positively correlated with enhanced bioprotection of cotton seedlings against *P. ultimum, R. solani* and *Rhizopus oryzae*, when applied as seed treatment. The results indicated that transformation of fungal BCAs with multiple lytic enzyme-encoding genes may result in providing more effective bioprotection (Djonovic et al. 2007a, b).

Trichoderma spp. are more resistant to the products of glucose oxidase activity than fungal pathogens, although the BCA does not possess a glucose oxidase orthlog (Kim et al. 1993; Mach et al. 1999). A transgenic strain SJ3-4 of *Trichoderma atroviride,* expressing the *Aspergillus niger* glucose oxidase-encoding gene *goxA*, under a homologous chitinase (*nag1*) promoter had increased capabilities as a fungal biocontrol agent. The transgenic strain differed only slightly from the wild-type in sporulation or the growth rate. The *gox4* expression occurred immediately after contact with the plant pathogen and the glucose oxidase was secreted. SJ3-4 had significantly less N-acetylglucosaminidase and chitinase activities than its nontransformed parent. Glucose oxidase-containing culture filtrates exhibited threefold greater inhibition of germination of spores of *Botrytis cinerea.* The transgenic strain overgrew more rapidly and lysed the plant pathogens *Rhizoctonia solani* and *Pythium ultimum*. In planta, SJ3-4 had no detectable improved effect against low inoculum levels of these pathogens. Beans planted in heavily infested soil and treated with conidia of transgenic *Trichoderma* strain germinated, but beans treated with wild-type spores did not germinate. SJ3-4 was more effective in inducing systemic resistance in plants. Further, beans with SJ3-4 root protection were highly resistant to leaf lesions caused by the foliar pathogen *B. cinerea*. The results showed that the transgenic use of biocontrol-related promoters associated with an appropriately selected heterologous gene is a powerful method to improve both biocontrol and the ability of soilborne fungal biocontrol agents to systemically induce resistance against foliar pathogens (Brunner et al. 2005).

Aspartic proteases constitute one of four main classes of proteinases, others being serine, cysteine and metalloproteinases, with a role in the biocontrol activity of diverse fungal biocontrol agents. Three novel proteases related to biocontrol response of *Trichoderma atroviride* growing on the cell walls of *Rhizoctonia solani* have been identified by a proteomic approach (Grinyer et al. 2005). Cloning, sequence analyses and heterologous expression of the aspartic protease gene of *Trichoderma harzianum* in a laboratory strain of *Saccharomyces cerevisiae* were performed. A gene *SA76* encoding an aspartic protease was cloned by 3′ rapid amplification of cDNA ends from *T. harzianum* T88. The coding region of the gene was 1,593-bp long, encoding a polypeptide of 530 amino acids with a predicted molecular mass 55-kDa and a pI of 4.5. The expression pattern of *SA76* was analyzed in the presence of cell walls of plant pathogenic fungi *Fusarium oxysporum, Rhizoctonia solani, Phytophthora sojae* and *Sclerotinia sclerotiorum*. Aspartic protease activity was induced in simulated parasitism by the presence of fungal cell walls. The increase in the activity was due to induction at the transcription level, because the transcripts accumulated abundantly shortly after induction (4 h). Assessment of the fungicidal activity of the culture supernatant of *S. cerevisiae* which expressed the aspartic protease showed that the culture supernatant inhibited the mycelial growth of *F. oxysporum* to a greater extent, while *S. sclerotiorum* was less sensitive. The inhibition of mycelial growth varied between 7 and 38 % among the fungal pathogens tested (Liu and Yang 2007).

Superoxide dismutase (SODs) are considered as the first and most important line of antioxidant enzyme defense systems by removing the superoxide anion, since the superoxide anion initiates a radical chain reaction that results in other reactive oxygen species (ROS) provoking damage to DNA, proteins and membrane lipids (Krutika and Subramanian 2007). The ability of the fungal biocontrol agents to develop in high temperature and salt environments for their biocontrol activity has to be enhanced to attain the required level for successful suppression of the target pathogens. Hence, *Trichoderma harzianum* was transformed with the SOD gene by applying *Agrobacterium tumefaciens* (At)-mediated transformation procedure with an efficiency of 60–110 transformants per 10^7 spores. The putative transformants were checked for the presence of SOD gene consisting of the 751-bp sequence of nucleotides by Southern blotting (Fig. 7.1). There was no obvious side-effect on the growth of transformants as well as the biocontrol activity against *Sclerotinia sclerotiorum*. Constructing genetically engineered microbial strain of *T. harzianum* by SOD gene recombinant technology increased the tolerance of the transformants to salt and heat which would be important for *T. harzianum* to survive in an adverse environment. Antifungal activities of the transformants were assessed under abiotic stresses. The transformants exposed to 40 °C for 3 days and 2 mol/l NaCl at 27 °C for 5–10 days were tested for their antifungal activity. The inhibition rates of transformants compared with wild-type strain were respectively 83.96 % after 40 °C and 60.13 % after 2 mol/l NaCl, indicating higher level of tolerance of the transformants to heat and salt stress (Yang et al. 2010).

Highly coordinated molecular dialogue occurs between plants and microbes during the early stages of their association. The signaling molecules play an essential role

Fig. 7.1 Detection of superoxide dismutase (SOD) in transformants of *Trichoderma harzianum* for hygromycin resistance gene by Southern blotting *W*: wild strain; 1: SOD transformant (Courtesy of Yang et al. 2010 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

in this association, determining the final outcome of the relationship which ranges from parasitism to mutualism. An elicitor produced by *Trichoderma virens* named Sm1 was identified and characterized, in order to have new insights into the events underlining the process of plant-*Trichoderma* interactions. Sm1 was found to be a novel proteinaceous non-enzymatic elicitor from this rhizosphere-competent biocontrol agent (Djonovic et al. 2006a). Sm1 was induced and secreted by the fungus at the early stages of plant-*Trichoderma* interactions, suggesting a signaling role for this protein. In fact, the purified Sm1 was shown to elicit the plant defense responses efficiently and systemic resistance against a foliar pathogen of cotton (*Gossypium hirsutum*) (Djonovic et al. 2006a, b). In a later study, maize seedlings were inoculated with *Trichoderma virens* Gv29-8 wild-type and transformants in which *SM1* was disrupted or constitutively over-expressed in a hydroponic system or in soil-grown maize seedlings with the pathogen *Colletotrichum graminicola*. As in the case of dicot plants, colonization of maize roots by *T. virens* induced systemic protection of the leaves inoculated with *C. graminicola*. This protection was associated with appreciable induction of jasmonic acid and green leaf volatile-biosynthetic genes. Neither deletion nor over-expression of *SM1* affected the normal growth or development of *T. virens*, conidial germination, production of gliotoxin, hyphal coiling, hydrophobicity or the ability to colonize maize roots. Maize plants grown with *SM1*-deletion strains exhibited the same levels of systemic protection as non-Trichoderma-treated plants, as indicated by plant bioassays. Furthermore, deletion and over-expression of *SM1* resulted in significantly reduced and enhanced levels of disease protection respectively, compared with the wild-type. The results showed that the activity of a functional Sm1 elicitor was required for *T. virens*-mediated induced systemic resistance (ISR) (Djonovic et al. 2007a, b).

A protoplast-based DNA-mediated transformation protocol for *Fusarium prolifieratum* effective against *Plasmopara viticola,* causing grapevine downy mildew disease, was adopted. Antibiotic resistance [bacterial hygromycin phosphotransferase gene (*hph*)] and ß-glucuronidase (GUS) reporter gene were introduced into the mutant 1505 of *F. proliferatum* obtained through UV-mutagenesis from the wild-type strain G6. Lesions were sprayed with microconidia of *F. proliferatum* G6 transformant (designated 27). The proliferation of the transformed strain was examined under the microscope after an interval of 7 days. Based on the expression of GUS in the hyphae of *F. proliferat*um, the characteristic coiling around of *P. viticola* sporangiophores by the BCA was recognized. Blue hyphae were observed within sporangiophores and even within the sporangia. By employing the GUS-engineered strains, the characteristic coiling and penetration of the host pathogen structures by the fungal BCA could be demonstrated (Bakshi et al. 2001).

Coniothyrium minitans was reported to be effective against *Sclerotinia sclerotiorum*. *C. minitans* was transformed to hygromycin resistance using protoplast produced by lysing enzymes from *Trichoderma* (Jones et al. 1999). Restriction enzyme-mediated integration (REMI) and *Agrobacterium*-mediated transformation (ATMT) producers were applied to transform the protoplasts or germinated conidia of the mycoparasite *C. minitans* to hygromycin resistance. The use of REMI with 5–20 U *Hind*III resulted in doubling of *C. minitans* transformation rates. *Agrobacterium*-mediated transformation of *C. minitans* germinated conidia was successfully applied. Up to 37.8 % hygromycin resistant transformants from 5×10^5 2-day old conidial germlings were obtained. Southern analysis of ATMT transformants indicated that 40 % of the transformants were the result of single-copy integrations, significantly higher than that obtained using REMI. Single-copy integrations facilitated recovery of specific insertional mutagenesis events. A novel microtitre plate-based test was used to expedite screening of 4,000 REMI and ATMT *C. minitans* transformants. Nine pathogenicity mutants that displayed reduced or no pathogenicity on sclerotia of *S. sclerotiorum* were identified. The frequency of pathogenicity mutants identified was approximately 0.23 % (Rogers et al. 2004). An *Agrobacterium tumefaciens* (AT)-mediated transformation system for *Coniothyrium minitans* strain ZS-1 was established to investigate the parasite interaction with the fungal plant pathogen, *Sclerotinia sclerotiorum.* T-DNA insertional transformation of strain ZS-1 mediated by *At* was obtained with optimization of spore maturity for transformation. The T-DNA insertional transformants of *C. mintans* strain ZS-1 was identified by employing PCR using pTFCM-F and pTFCM-R primers. DNA fragments with an expected size (1,165-bp) were amplified from all the eight candidates and binary vector pTFCM, but not from the parent strain ZS-1. Thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) – an essential technique required for amplifying the genomic DNA fragment flanking the site of DNA of the T-DNA insertion was employed to successfully isolate the flanking DNA of T-DNA insertional transformants of *C. minitans*. More than 3,000 transformants were obtained by the TAIlL-PCR procedure. The transformants were subjected to Southern blot analysis. More than 82.7 % of the transformants had single T-DNA insertions and 12.1 % of the

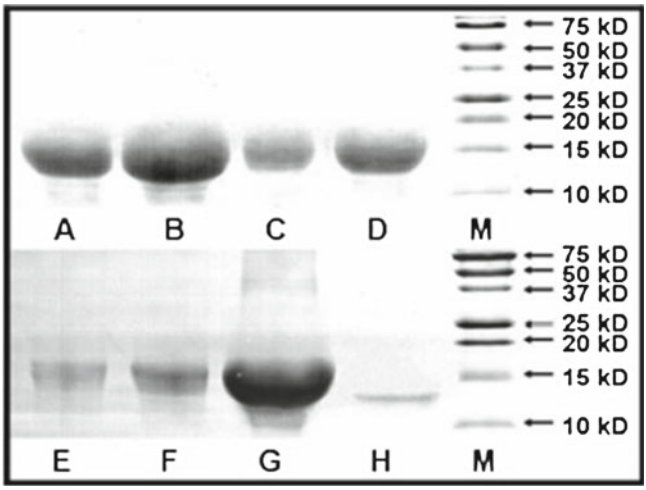

Fig. 7.2 SDS-PAGE analysis of purified fused wheat ns-LTPs expressed in yeast. *Lane A*: TaBs 108 F7; *Lane B*: TaLt19C10; *Lane C*: TaLt709LC; *Lane D*: BQ241565; *Lane E*: TaLt10H24; *Lane F*: TaBs116G9; *Lane G*: TaLt10F9; *Lane H*: TaLt10B6 and *Lane M*: protein standard marker (Courtesy of Sun et al. 2008 and with kind permission of The American Phytopathological Society, MN, USA)

transformants had two copies T-DNA insertions. Four types of mutants identified from the T-DNA library were compromised sporulation deficient mutants, pathogenicity mutants, pigment change mutants and antibiotic deficient mutants. The T-DNA insertional transformation library established in this investigation might be useful to unravel the interaction between the biocontrol agent and its host fungal pathogen (Li et al. 2005).

Plant nonspecific lipid transfer proteins (ns-LTPs) are abundant and ubiquitous, small, soluble and basic cystine-rich proteins. Several ns-LTPs have been implicated in plant defense responses to diseases. A barley type 1 ns-LTP, acting synergistically with thionin, inhibited the growth of bacterial pathogens in infected plants. Overexpression of a barley type 2 ns-LTP in transgenic tobacco and *Arabidopsis* was shown to enhance resistance to *Pseudomonas syringae* pv. *tabaci* and pv. *tomato* (Molina and García-Olmeda 1997). Eleven full-length wheat ns-LTP cDNA clones were isolated and their sequences at both nucleotide and amino acid levels were compared. Eight wheat ns-LTP genes from different clades were cloned into expression vector pPICZoc and transformed into *Pichia pastoris*. SDS-PAGE, western blotting and in vitro lipid binding activity assay confirmed that all the eight ns-LTPs were successfully expressed and capable of in vitro binding fatty acid molecules (Fig. 7.2). A comparative in vitro study on the toxicity of eight wheat ns-LTPs to mycelial growth or spore germination of wheat pathogens and non-wheat pathogens revealed differential toxicities among different ns-LTPs. Three ns-LTPs providing 50 % inhibition of mycelial growth or spore germination of six fungi were identified. Lipid binding activity and antifungal activity of these ns-LTPs were not correlated,

Table 7.1 LD_{50} values of selected wheat nonspecific lipid transfer proteins (ns-LTPs) for obtaining 50 % inhibition of mycelial growth and spore germination of wheat and nonwheat pathogens (Sun et al. 2008)[a]

	Lipid transfer protein (μM)		
Fungus species tested	TaBs108F7	TaLt19C10	TaBs116G9
Sclerotinia sclerotiorum	1.75	1.25	3.00
Low-temperature basidiomycete	–	–	1.00
Fusarium graminearum	1.50	5.00	4.00
Verticillium dahliae	2.75	5.00	7.00
Puccinia graminis	1.00	3.50	–
Tilletia tritici	–	3.00	–

[a]LD_{50} values were determined from dose–response curves representing percentage of growth inhibition versus protein concentration; – = no inhibition

suggesting that ns-LTP toxicity and lipid-binding activities might be independent of each other (Table 7.1). The results suggested that collectively the different inducible and constitutively expressed ns-LTPs could play a role in enhanced generalized nonspecific defense responses induced during abiotic and biotic stresses that provide transitional protection against a wider range of plant pathogens (Sun et al. 2008).

Histidine auxotrophs of *Candida oleophila* strain 1–182 were produced by using methane sulfonate. They were transformed with plasmids containing the *HIS3, HIS4* and *HIS5* genes of *Saccharomyces cerevisiae*. *HIS5* gene was transformed to complement histidine auxotrophy of *C. oleophila*. Integration of transgenes was verified by DNA-gel-blot analysis. No detectable physiological differences between the transformants and wild-type were observed. Further, the biocontrol potential of the transformants was not altered. A genetically marked transformant with a ß-galacturonidase gene could colonize wounds on oranges followed by increase in population under field conditions. Polymerase chain reaction (PCR) amplification of a portion of the ß-galacturonidase gene established the identity of this transformant with good survival under field conditions (Chand-Goyal et al. 1999). Over-expression of indigenous genes encoding lytic enzymes in the biocontrol agents may enhance the biocontrol potential of the transformed biocontrol agents. *CoEXG 1* gene encoding ß-1,3-glucanase (glucan 1,3-glucosidase) of *Candida oleophila* was cloned by colony hybridization of *Escherichia coli*, harboring partial genomic library of *C. oleophila*. A fragment of *CoEXG 1* obtained using degenerate oligomers constructed from *Saccharomyces cerevisiae* and *C. albicans* exoglucanase gene sequences, was used as a probe. *C. oleophila* secreted ß-glucanase continuously in vitro and the putative protein belonged to a family of highly conserved proteins in yeasts (Segal et al. 2001).

The possibility of transforming biocontrol agents, by incorporating genes that code for antimicrobial compounds from diverse sources such as insects, has been assessed. *Colletotrichum coccodes* causes fruit rot of tomato. Cecropin is an antimicrobial protein isolated from the giant silk moth *Hyalophora cecropia*. A cecropin A-based peptide inhibited the germination of conidia of *C. coccodes* at 50 μM. The DNA sequence encoding the peptide was cloned in the plasmid pRS413, using a *Saccharomyces cerevisiae* invertase (ß-fructo-furanosidase) leader sequence for secretion of the peptide and expression in yeast. The transformants inhibited the

growth of germinated conidia of *C. coccodes*. The decay development in tomatoes cv. Roma inoculated with *C. coccodes* was also inhibited. This investigation opens up a new approach for effective control of plant diseases by employing BCAs whose biocontrol potential can be enhanced by expression of antifungal peptides from unrelated organisms, even in animal kingdom (Jones and Prusky 2002). The antifungal activity of the recombinant rice chitinase was assessed for the control of gray mold rot of loquat caused by *Botrytis cinerea*. Rice class I chitinase was inserted into the vector pIC9 to construct pPIC9/Chi and then expressed in *Pichia pastoris* strain GS115. The expression level and antifungal activity of the recombinant chitinase against *B. cinere*a on loquat was analyzed. The chitinase was successfully expressed at a high level in the host yeasts and enzymatically active. In addition, the chitinase significantly arrested the spread of the gray mold on the rot region of loquat. This study revealed the effectiveness of the rice chitinase in suppressing the development of the loquat gray mold disease during storage (Yan et al. 2008).

The potential of fungal pathogens for the biocontrol of weeds has been demonstrated. A strain of *Colletotrichum gloeosporioides* marketed as COLLEGO (Encore Technologies, USA) was reported to be effective against the weed *Aeschynomene virginica,* which is difficult to control by chemicals in rice fields. The virulence of the mycoherbicide could be enhanced by transferring genes to the fungus, tipping the evolutionary balance. Two genes that sequentially convert tryptophan into the plant hormone indole acetic acid (IAA) were incorporated into *Fusarium* spp. that parasitize *Orobanche* spp. Over production of IAA by the transformant resulted in the enhancement of virulence of *Fusarium* spp. (Cohen et al. 2002). The *NEP1* from *Fusarium*, a gene that is responsible for the natural virulence of the species expressing it, was used to transform fungal pathogens. The protein product of Nep1is a potent phytotoxin, when injected into leaves or into the translocation stream of plants (Jennings et al. 2001). A strain of *Colletotrichum coccodes* was transformed with *NEP1* gene from *Fusarium* spp. and its virulence on the weed *Abutilon theophasti* in cotton fields was determined. Cotton and *Abutilon* were found to be resistant to the same selective herbicides, while *C. coccodes* could differentially infect *Abutilon*. The virulence of *C. coccodes* transformed with NEP1 gene was increased by ninefolds. Transgenic *NEP1 Colletotrichum* rapidly killed *Abutilon* through the stage of three true leaves at far lower doses than that was obtained with wild-type strain. Furthermore, the requirement for a long duration of humidity for the wild-type strain was reduced by introducing *NEP1* encoding a phytotoxic protein which was lethal to *Abutilon theophrasti*. Strategies of coupling virulence genes with fail-safe mechanisms to prevent spread and to mitigate transgene introgression into crop pathogens could be advantageous in the biocontrol of major weeds in row crops (Amsellem et al. 2002).

7.1.4 Protoplast Fusion Technique

Protoplast fusion technology has facilitated the manipulation of fungal biocontrol agents as enzyme producers and biocontrol agents. Protoplast fusion allows transfer of complex traits, without knowing much about the functions of the genes involved

and for genetic recombination between organisms that cannot undergo sexual recombination. Interspecific and even intergeneric crosses have been performed and protoplast fusants possessing more efficient biocontrol potential than the parent strains have been generated. Genetic modification of *Trichoderma* spp. strains was attempted by employing the protoplast fusion procedure for the biocontrol of *Botrytis cinerea, Sclerotinia sclerotiorum* and *Pythium ultimum.* However, the new strains produced by this technique did not show any significant increase in their antagonistic activity, compared with parent strains (Migheli et al. 1995). Isolation, fusion and regeneration of protoplasts from *Trichoderma harzianum* and *T. viride* were performed by an effective protocol, using Novozym 234 as lytic enzyme and potassium chloride as osmotic stabilizer. The interspecific and intraspecific fusion frequency was determined, using polyethylene glycol (PEG) as fusogen and the intrafusants were selected on the basis of mycelial growth, sporulation, pigmentation on chitin- and cellulose- amended media. On the other hand, the interfusants were selected on fungicide resistance, as a marker (Balasubramanian and Lalithakumari 2008).

Protoplast fusants between a strain of *Trichoderma virens* with remarkable biocontrol potential and a strain of *T. koningii* which had rapid germination after extended periods in storage were generated to combine the desirable characteristics, especially biocontrol activity and maintenance in storage from two species. Five of the fusants were morphologically similar to either of the parental species *T. koningii* or *T. virens*. Three fusants, TKG4, TKG12 and TKG18 morphologically similar to *T. koningii* retained the ability to grow on the selective medium containing fludioxonil + propioconazole on which the parental isolates were unable to grow. Two fusants, showing morphological similarity to *T. koningii*, exhibited better biocontrol activity than the parent against *Rhizoctonia solani* infecting cotton. On the other hand, one *T. virens*-like fusants gave significantly less control than the parent *T. virens*. Fusants differed from the morphologically similar parent in their ability to produce secondary metabolites. One of the fusants exhibited greater biocontrol potential during storage up to 1 year (Hanson and Howell 2002; Appendix 7.1). Self-fusion of protoplasts from *Trichoderma harzianum* was performed to enhance the level of production of chitinase and to determine the antagonistic potential of the fusants. Self-fusion of protoplasts was obtained by using 40 % polyethylene glycol (PEG) in STC buffer containing 0.6 M sorbitol, 10 mM Tris–HCl and 10 mM CaCl2, pH 6.5. The fused protoplasts were regenerated and 15 self-fusants were selected to determine the chitinase production and biocontrol activity. Enhanced chitinase production was assessed by quantitative assay. The chitinase activity was substantially increased in most of the self fusants (87 %), except two fusants, compared with the wild-type strain. Among the fusants, the strain SFTh 8 produced maximum chitinase with a twofold increase, as compared with the parent strain. The antagonistic activity of self-fusants against *Rhizoctonia solani* was also increased. Complete inhibition of mycelial growth of *R. solani* was effected by five fusants, while the parent strain inhibited the mycelial growth to an extent of only 67.6 %. The crude chitinase preparation of the self-fusant SFTh 8 caused lysis of pathogen mycelium at much higher level than that of the parent strain (Prabavathy et al. 2006).

Fungal biocontrol agents are applied for the management of crop diseases alone or in combination with fungicides or other chemicals. It is essential that the BCAs are tolerant to the fungicides to enable them to suppress the development of diseases to the desired level. The techniques UV mutagenesis and protoplast fusion were combined for the production of fungicide-resistant strains of the cold-tolerant strains of *T. harzianum* and *T. atroviride*. The parental strains were unable to grow in the presence of 20 µg/ml of benomyl and thiabendazole, whereas MBC-resistant mutants of both strains exhibited total cross- resistance to both fungicides, with a capacity to tolerate even the highest concentration of the fungicides tested. The possible mechanism of development of MBC resistance in UV-induced mutants could be a nucleotide substitution in the *tub2* ß-tubulin gene. Interspecific protoplast fusion was carried out between carbendazim- and tebuconazole-resistant mutants of both parental strains. Putative haploid recombinants with stable resistance to both pesticides were produced in the case of *T. atroviride*. The fungicide polyresistant fusants exhibited satisfactory level of antagonism against pathogenic fungi *Pythium debaryanum, Fusarium oxysporum, F. culmorum* and *Microdochium nivale*. The results indicated that the combination of UV-mutagenesis and protoplast fusion techniques might be a powerful tool for the improvement of multiple-fungicide resistance in *Trichoderma* strains with biocontrol potential against economically important plant pathogens (Hatvani et al. 2006).

In a later investigation, similar approach of combining UV-mutagenesis and interspecific protoplast fusion techniques was followed for improving the biocontrol potential of *Trichoderma* spp. against soilborne fungal pathogens. A combination of UV-irradiation and nitrate treatment was applied to generate three mutants of *Trichoderma koningii* and four mutants of *T. reesei*. Thirteen fusants were isolated from the interspecific protoplast fusion between *T. koningii* and *T. reesei* with a fusion frequency of 1.25 %. Most of the mutants and fusants were more effective in suppressing the mycelial growth of *Fusarium oxysporum*, *Pythium ultimum, Sclerotium rolfsii* and *Sclerotinia sclerotiorum*, causing root rot and wilt diseases of several crops, compared to parental strains. The results indicated the possibility of improving the biocontrol potential of fungal biocontrol agents through mutagenesis and protoplast fusion techniques (Mohamed and Haggag 2010).

7.1.5 Transformation of Host Plants with Genes from Fungal Biological Control Agents

Different species of *Trichoderma* constitute a potential source of antipathogenic genes. They are known to produce antibiotics and several enzymes such as chitinases, proteases, glucanases and mannanases that directly affect the growth of fungal pathogens. *Trichoderma* spp. secrete several lytic enzymes degrading chitin present on hyphal cell wall of several fungal plant pathogens. They include1,4-ß-acetylglucosaminidases, exochitinases and endochitinases. Transgenic tobacco and potato plants over-expressing an endochitinase (CHIT42) from *Trichoderma harzianum* were found to be highly

Table 7.2 Response of transformed and nontransformed Marshall McIntosh apple plants to apple scab pathogen *Venturia inaequalis* (Bolar et al. 2000)

Transgenic line	ThEn-42 activity[a]	Number of lesions[b]	% area of leaf infected[b]	Conidia recovered[b]
MM[c]	0.03	71.8a	57.5a	248,833a
T286 (vector)[d]	0.02	79.6a	58.4a	275,182a
T566	0.09	71.6a	51.8a	139,526a
T565	0.04	61.6ab	44.7ab	266,400a
T563	0.35	37.0bc	33.9bc	10,900ab
T562	0.57	32.8cd	25.7cd	21,440ab
T568	3.28	15.8c–e	12.5de	9,400bc
T561	112.50	17.3de	10.0de	ND
T564	2.08	4.3de	4.0de	1,500c
T560	78.60	0.0e	0.0e	4,750c
Liberty	0.01	0.0e	0.0e	0d

[a]ThEn-42 activity in nanometer MU per minute per microgram of protein

[b]Mean of four leaves/plant; values followed by the same letter in each column are not significantly different (P = 0.05)

[c]Nontransformed cv. Marshall McIntosh

[d]Transgenic cv. Marshall McIntosh line transformed with pBI121 that does not contain the endochitinase gene

tolerant to *Alternaria alternata, A. solani* and *Botrytis cinerea* (airborne pathogens) and *Rhizoctonia solani* (soilborne pathogen). In these transgenic plants, the *T. harzianum* signal peptide was correctly cleaved and extracellular accumulation of fungal enzyme was induced, suggesting signal sequences from fungi may be used to secrete fungal enzymes in plants which may be protected against fungal pathogens (Lorito et al. 1998). The chitinases of *Trichoderma harzianum* have been shown to suppress the development of *Venturia inaequalis*, causing apple scab disease in in vitro assays (Wong et al. 1999). With a view to improving resistance of apple to scab disease, the genes encoding chitinolytic enzymes from *T. harzianum* were transferred to apple cv. Marshal McIntosh. The endochitinase gene, as a cDNA and genomic clones was transferred by *Agrobacterium tumefaciens (At)*-mediated transformation. Apple lines (15) with varying levels of endochitinase expression were identified as transgenic by ELISA and PCR assays and confirmed by Southern analysis. The amount of nptII protein in the putative transgenic leaf tissue samples was determined by ELISA. Eight lines propagated as grafted and own-rooted plants were inoculated with *V. inaequalis*. Six of these transgenic lines expressing endochitinase were more resistant than the non-transformed Marshall McIntosh plants. The disease severity was reduced by 0–99.7 % (based on the percentage of leaf area infected) and 1–56 % (based on the conidia recovered) in the transgenic lines tested (Table 7.2). However, endochitinase had negative effects on the growth of both inoculated and uninoculated transgenic plants. A significant negative correlation was observed between the level of endochitinase production and both the amount of disease and plant growth (Bolar et al. 2000).

Various strains of *T. harzianum* produce endochitinases, CHIT31, CHIT33, CHIT36, CHIT37, CHIT42 and CHIT52 which vary in their molecular weight and structure and pI value (Markovich and Kononova 2003). The endochitinase gene, CHIT36, a 36-kDa protein was identified in *T. harzianum* TM. It showed 79 % nucleotide and 89 % amino acid homology to CHIT37 from *T. harzianum* CECT2413, but no significant similarity to other known *Trichoderma* endochitinases (Viterbo et al. 2001). Expression analysis revealed that *chit36* gene was induced by conditions of stress, colloidal chitin and N-acetyl glucosamine. Further, no direct contact of *T. harzianum* with the target pathogen was required for the activation of the *chit36* promoter (Viterbo et al. 2002). *T. harzianum* transformants overexpressing *chit36* gene regulated by constitutive pki1 promoter showed that the culture filtrate completely inhibited the germination of conidia of *Botrytis cinerea* and chitinase enzyme secreted significantly suppressed the mycelial growth of *Fusarium oxysporum* and *Sclerotium rolfsii* (Viterbo et al. 2001). In another investigation, the effect of combination of transgene-mediated defense mechanisms (*ech42* plus *nag70*) with conventionally based natural resistance and its ability to protect apple from strains of *Venturia inaequalis*, carrying different virulences were investigated. Transgenic lines of two genotypes of apple Galaxy (highly susceptible to apple scab resistance) were transferred with endochitinase and exochitinase genes derived from *Trichoderma atroviride.* The transgenic lines generated from two apple cultivars were analyzed for the expression of both genes and resistance to two races of *V. inaequalis*. The common race 1 and race 6 which could overcome resistance conferred by the *Vf* gene were used for challenge inoculation of transgenic lines. A negative correlation was noted between the growth of transgenic lines and endochitinase activity. Reduced growth appeared to be associated with high lignin content and high peroxidase as well as glucanase activity, suggesting that endochitinase activity might disturb the metabolism of the plant. Inoculation with races 1 and 6 of *V. inaequalis* performed in a growth chamber on 14 lines of normal vigor identified six lines with significantly enhanced resistance to scab pathogen. Ten lines with reduced vigor, when tested in vitro with a bioassay procedure on root shoots, exhibited a significant reduction in the intensity of scab symptoms. These transgenic lines expressed high endochitinase activity (Faize et al. 2003).

Enhancement of levels of resistance/tolerance against plant pathogens in plants transformed with chitinase genes was modest in most cases. Hence, the use of gene combinations was both considered to be a feasible approach to achieve substantial increase in resistance levels in transgenic plants. Transgenic tobacco plants overexpressing singly or in combination, two endochitinase from *Trichoderma harzianum* viz., CHIT33 and CHIT42 were generated. Five independent F3 homozygous pschit33 and pschit42 lines showed significantly enhanced resistance to both fungal pathogen *Rhizoctonia solani* and the bacterial pathogen *Pseudomonas syringae.* No synergistic effect in pschit33 × pschit42 lines could be recognized, but the level of resistance was intermediate to their parental lines. The results suggested that the increased resistance in tobacco lines over-expressing *pschit33* or *pschit42* may be due to the systemic induction of PR proteins and increase of cell wall-associated anionic peroxidase in the transgenic pschit lines (Dana et al. 2006). In a later

investigation, the effect of expression of CHIT36 in plant tissues was studied. Transgenic carrot (*Dacus carota*) plants constitutively expressing CHIT36 endochitinase were developed. A plasmid containing *chit36* gene under the control of CaMV 35S promoter and the *nptII* selection gene was introduced into polyethylene glycol (PEG)-treated carrot protoplasts. All transformed plants were checked for the presence of transgenes by polymerase chain reaction (PCR) assay with *nptII*- and c*hit36*- specific fragments respectively. The transgenic plants expressing CHIT36 were evaluated for their resistance to fungal pathogens *Alternaria dauci, A. radicina* and *Botrytis cinerea*. In vitro assays using detached leaves and petioles showed that the transgenic carrots had less severe disease symptoms. The resistance response depended on the transgenic clone, but all clones had significantly enhanced tolerance to *A. radicina* and *B. cinerea* on an average of 50 %. Slower disease progress due to infection by *A. dauci* could be seen only in two transgenic clones, while other clones showed higher level of susceptibility to this pathogen. The results revealed that CHIT36 endochitinase had the potential to enhance the level of resistance of transformed carrot lines to two fungal pathogens *A. radicina* and *B. cinerea* (Baranski et al. 2008).

Resistance to infection by fungal pathogen in plants mediated by a recombinant chitinase from an entomopathogenic and acaricide fungus *Metarhizium anisopliae* was reported for the first time. The *chit1* gene from *M. anisopliae* encoding the endochitinase CHIT42 was placed under the control of the CaMV 35S promoter and the resulting construct was transferred to tobacco. Seventeen kanamycin resistant transgenic lines were recovered and the presence of the transgene was confirmed by PCR assay and Southern blot hybridization. The number of *chit1* copies was determined to be varying from one to four. Copy number had no deleterious effects either on plant growth or development. Substantial heterogeneity concerning production of the recombinant chitinase and both general and specific chitinolytic activities were detected in leaf extracts from primary transformants. The highest chitinase activities were detected in plants with two copies of *chit1* inserts at different loci. Transgene segregation followed a Mendelian dihybrid ratio in the progeny of self-pollinated plants. Two selected plants expressing high levels of CHIT42 were consistently resistant to *Rhizoctonia solani*, suggesting a direct relationship between enzymatic activity and reduction in foliar area affected by fungal lesions (Kern et al. 2010).

7.1.6 Antibody-Mediated Protection to Plants

Antibodies, specific to the pathogenic *Fusarium* spp. and mycotoxins produced by them, have been produced. Antibody-mediated resistance in plants against Fusarium pathogens has been shown to be a promising strategy for the control of Fusarium head blight (FHB) disease of wheat and other cereals. Expression in plants of antibodies specific to mycotoxins-producing pathogens can restrict the spread of the pathogens under field conditions and eventually reduce mycotoxin-production load.

Monoclonal antibodies (MAbs) with high binding specificity to Fusarium mycotoxins may be isolated and the hybridoma producing the desired MAb may be cloned. In phage display technique, each phage displays a single antibody fragment, comprising the variable regions of the heavy and light chains that are specific for the Fv domains of antibodies. They are known as single-chain variable fragment (scFv). Phage display antibodies can be applied in the same range of applications as their hybridoma counterparts. Several single-chain antibodies specific to antigens are displayed on *Fusarium* cell surface. Western blot analyses and immunofluorescence (IF) labeling confirmed that these antibodies reacted strongly with cell wall-bound proteins and bind to the surface components of *F. asiaticum*, a predominant FHB species in China (Qu et al. 2008). One of the highly specific phage display scFv antibodies was demonstrated to protect plants against *Fusarium* pathogens (Li et al. 2008).

The *Fusarium*-specific single-chain antibody in planta was shown to confer resistance to *Fusarium* in transgenic *Arabidopsis* plants. Specific antibodies against *F. asiaticum*, infecting wheat in China were generated. *Fusarium* cell wall-bound proteins were selected as the target for protective antibodies. A chicken-derived phage display scFv antibody with a high affinity to cell wall-bound proteins from *Fusarium* pathogens was identified. This antibody conferred a significantly enhanced resistance in transgenic *A. thaliana* plants, upon infection with *F. asiaticum* at 14 days post-inoculation (dpi). When the coding sequence of this antibody was genetically fused to an antibody protein (AFP) like chitinase, the resulting AFP-scFv fusion protein displayed strong inhibitory effect on the growth of *Fusarium* spp. in in vitro assays (Peschen et al. 2004). Analyses of transgenic wheat plant showed that plants expressing an AFP-scFv fusion after single-floret injection and spray inoculation with *F. asiaticum* had significantly high resistance to FHB pathogen. Up to 86 and 79 % of reduction in spikelet infection were observed in T2 and T3 transgenic wheat plants respectively at 21 dpi with single-floret injection, compared with nontransgenic wheat plants, indicating resistance to spread of the pathogen as well as to the initiation of infection by *F. asiaticum*. In addition, the transgenic plants produced more grains compared to control plants. The results showed that *Fusarium*-specific antibodies fused to antifungal peptides could be ideal molecules for constitutive expression in cereal crops that grow under field conditions (Li et al. 2008).

7.2 Bacterial Biological Control Agents

7.2.1 Selection of Efficient Bacterial Species/Strains

Several fluorescent pseudomonads employ antibiosis as one of the mechanisms of biocontrol activity against microbial plant pathogens. Strains of *Pseudomonas* spp. exhibit wide variations in growth requirements and antibiotic-producing capacity. Strains of plant-associated *Pseudomonas* have to be selected on the basis of their ability to adapt

to the crop to be protected and soils to make their biocontrol activity more effective. Strains of *P. fluorescens*, producing one or more antibiotics, phenazines (Phz) and 2,4-diacetylphloroglucinol (Phl) or hydrogen cyanide (HCN) have been differentiated and selected for application (Duffy et al. 1996). The ability of producing *Phl* is an important factor determining the efficacy of *Pseudomonas fluorescens* strains in controlling soilborne fungal diseases. The DNA fragment with 6.5-kb in the strains Q2-87 contains the gene(s) controlling production of *Phl* and a red pigment distinct from *Phl*. Characterization of genomic locus required for the synthesis of *Phl* has been accomplished by Bangera and Thomashow (1996). Likewise, a cluster of four genes present in *P. fluorescens* strain BL-915 is required for producing pyrrolnitrin active against *Rhizoctonia solani*, causing root rot and sheath blight diseases in wheat and rice respectively. This cluster of four genes is present in the 32-kb genomic DNA fragment of the BCA. Deletion mutants, lacking even one of the four genes, did not have the ability to produce pyrrolnitrin, indicating that all four genes were essential for the biosynthesis of pyrrolnitrin. Transgenic *Escherichia coli* expressing the cluster of four genes acquired the capacity to produce pyrrolnitrin (Hammer et al. 1997).

Studies to elucidate the functions of genes controlling the production of compounds toxic to pathogens have been useful for understanding the mechanism of action of biocontrol agents. *Pseudomonas fluorescens* CHA0 strain produces antimicrobial metabolites, 2,4-diacetyl phloroglucinol (Phl), pyoluteorin (Plt) and hydrogen cyanide (HCN), the siderophores pyoverdin (Pvd), salicylic acid (SA) and pyochelin (Pch). A mutant derived from mutation in the global regulator gene *gacA* (GacA$^-$) could produce Phl, Plt and HCN, but over-produced Pch and Pvd. This mutant did not protect cress and cucumber against *Pythium ultimum,* causing damping-off diseases. However, this mutant protected wheat and maize against the take-all disease caused by *Gaeumannomyces graminis* var. *tritici*. The results suggested that a functional *gacA* gene might not be involved in the protection of dicotyledonous plants such as cucumber against root disease, but it might be required for the protection of monocotyledonous plants such as wheat and maize (Schmidli-Sacherer et al. 1997). Genes (*out*) are involved in the secretion of pectolytic enzymes of *Erwinia carotovora* subsp. *betavasculorum* Ecb168. Both Ecb168 and out$^-$ derivatives were able to inhibit the growth of *E. carotovora* subsp. *carotovora* in culture and they also reduced the severity of soft rot in potato tubers. The results indicated that uncharacterized antibiotics responsible for antagonism could have been exported through an *out*-independent mechanism (Costa and Loper 1994).

Pseudomonas putida strain BK8661 produced siderophore(s), antibiotic(s) and low levels of hydrogen cyanide (HCN) and suppressed the growth of *Septoria tritici* causing blotch and *Puccinia recondita* f.sp. *tritici,* causing leaf rust disease of wheat. HCN-overproducing derivatives of pleiotropic mutants constructed by the integration of the *hcn ABC* gene cluster from *Pseudomonas fluorescens* CHA0 were significantly more effective in suppressing the wheat blotch and leaf rust diseases, under conditions, when siderophores and antibiotics were not produced (Flaishman et al. 1996). Root colonization by the biocontrol agent did not appear to be essential for suppression of damping-off disease of cucumber caused by *Pythium ultimum.* Strain 17R1, a rifampicin-resistant mutant of *Escherichia coli* strain S17-1

protected cucumber seedlings effectively against the pre- and post-emergence damping-off caused by *P. ultimum*, when applied as seed treatment. The buildup of the mutant population was very slow, reaching 65-fold increase, compared to the 24,000-fold increase in the population of *Enterobacter cloacae* strain 501R3, indicating that extensive root colonization of the host plant roots by the BCA was not required for suppressing the development of damping-off disease, because of the brief period of susceptibility of cucumber to damping-off disease (Roberts et al. 1997). *Enterobacter cloacae* C10, a mutant of *E. cloacae* 501R3 was generated by transposon insertion in *degS* and the mutant was diminished in growth on synthetic cucumber root exudates (SRE), colonization of cucumber seed and roots and control of cucumber damping-off disease caused by *Pythium ultimum*. The strain C10 containing wild-type *degS* from 501R3 or from *Escherichia coli* K12 on pBelo-BAC11 was significantly increased in growth on SRE, colonization of cucumber roots and control of damping-off disease, compared to strain C10. The results indicated a role for *degS* in the spermosphere and rhizosphere during colonization and effectiveness of damping-off disease suppression by *E. cloacae*. Presence of *degS* appeared to enable plant-associated bacteria to cope with the complex nutritional environment found in plant spermosphere and rhizosphere (Roberts et al. 2011).

The role of nitric oxide (NO) production in the biocontrol potential of *Pseudomonas fluorescens* was investigated. The endogenous nitric oxide reductase gene of *P. fluorescens* strain T5 was disrupted by homologous recombination, using a plasmid. Three disruptants generated, exhibited the ability to synthesize higher levels of NO. Tomato plants treated with the NO-overproducing mutants more effectively suppressed infection by *Ralstonia solanacearum* causing bacterial wilt disease, compared with the parent strain. The results suggested that genetic modification of pseudomonads to increase their ability to produce higher level of NO might be an effective approach to enhance the biocontrol potential of the bacterial BCAs (Wang et al. 2005). Actinomycetes isolates (200) were evaluated for their ability to produce chitinase in medium amended with chitin. Seven isolates selected for their greater capacity to produce chitinase were subjected to gamma ray irradiation at 0.4–8 kGy. Among the 173 mutants generated, three mutant strains SJ9I-15, SG4I-17 and SG4I-38 inhibited the mycelial growth of *Fusarium sporotrichoides, Rhizoctonia solani* and *Sclerotium rolfsii* to the maximum extent. The results indicated that selection of efficient actinomycete strains, based on their ability to produce greater amounts of chitinase, followed by generation of mutants from the efficient wild strains, might result in enhancement of the biocontrol potential of BCAs against target pathogens (Rugthaworn et al. 2007).

7.2.2 Protoplast Fusion Technique

Protoplast fusion or genome shuffling technique was applied to improve the biocontrol potential of *Streptomyces melanosporofaciens* EF-76, which produced geldanamycin against *Streptomyces scabies* or *Phytophthora infestans*, causing scab and

late blight diseases of potato respectively. Two rounds of genome shuffling were performed with *S. melanosporofaciens* EF-76. Six fusants that exhibited in vitro antagonistic activity against potato pathogens were selected. All selected fusants retained the capacity to produce geldanamycin. But none of them overproduced this antibiotic. The high performance liquid chromatography (HPLC) profiles showed that parent strains produced seven or eight metabolites, whereas the HPLC profiles of the fusants showed the presence of 12–15 metabolites. Four of the fusants suppressed the development of potato disease more effectively than the parental strain. The combined level of protection provided by the fusants was attributed to their ability to produce a wider range of secondary metabolites (Clermont et al. 2011).

7.2.3 Transformation of Bacterial Biological Control Agents

Genetic modification of bacterial biocontrol agents has been attempted to improve their biocontrol potential only in fewer cases, compared with fungal biocontrol agents. Salicylic acid (SA) has been shown to be an endogenous signal molecule and exogenous application of SA induces systemic acquired resistance (SAR) to diseases affecting various crops. The effect of introducing SA biosynthetic genes *pchBA* from *Pseudomonas aeruginosa* PA01 into *P. fluorescens* strains P3 and CHA0 on induction of SAR was investigated. Transformation of strain P3 which did not produce SA, with *pchBA* rendered this strain capable of SA production. In addition, the ability of strain P3 to induce SAR in tobacco against *Tobacco necrosis virus* (TN) improved significantly. Strain CHA0 was capable of producing SA naturally under conditions of iron limitation. Transformation of CHA0 with *pchBA* enhanced the level of SA production in vitro and in the rhizosphere of tobacco. However, there was no significant improvement in the suppression of black root rot caused by *Thielaviopsis basicola* in plants treated with transformed bacterial strain (Maurhofer et al. 1998).

7.2.4 Nonpathogenic Mutants Derived from Bacterial Pathogens

Tomato bacterial spot disease is caused by several *Xanthomonas* spp., including *X. euvesicatoria, X. perforans* and *X. vesicatoria* (Jones et al. 2004). A bacteriocin-producing strain of *X. perforans* with attenuated pathogenicity was evaluated for its efficacy in suppressing the development of a bacteriocin-sensitive strain of genetically closely related pathogen *X. vesicatoria*. The attenuated mutant (91–118ΔopgHΔbcnB) of *X. perforans* was generated by deletion of the osmoregulated periplasmic glucan gene *opgH*. The mutant had reduced ability to cause disease and it also lacked BcnB activity which negatively affected the competitive ability, resulting in more effective colonization of tomato leaves under field conditions. The mutant inhibited effectively the internal populations of the wild-type *X. euvesicatoria* strain, although to a significantly less extent, compared with the

wild-type 91–118 strain. On the other hand, the mutant strain reduced the epiphytic populations as effectively as the wild-type. The attenuated mutant inhibited the development of sensitive bacterial pathogen more efficiently on the leaf surface than inside the leaf. Under field conditions, weekly application of the mutant strain significantly reduced *X.vesicatoria* populations, compared with the standard control practice of applying copper oxide and mancozeb applied at weekly interval (Hert et al. 2009). *Rahnella aquatilis* HX2, a Gram-negative bacterium produces antibacterial substance with a wide spectrum of inhibitory effect on *Agrobacterium tumefaciens, A. vitis, Pectobacterium carotovorum, Xanthomonas campestris* and *X. oryzae* which cause economically important diseases. *R. aquatilis* HX2 effective against *Agrobacterium vitis*, causing crown gall disease in grapevine was used to generate MH15 and MH16, two Tn5-induced mutants. The mutants lost their abilities to inhibit *A. vitis* and had reduced biocontrol activities. The mutants had single Tn5 insertions. They were also impaired in producing pyrroloquinoline quinine (PQQ) or glucose dehydrogenase (GDH). An in-frame *gdh* deletion mutant had the same phenotypes as the Tn5 mutant of MH16. Complementation of both deletion and Tn5 *gdh* mutants restored the affected phenotypes to wild-type levels. The results suggested that an antibacterial compound might play a role in biocontrol activity of the strain HX2 against *A. vitis* (Guo et al. 2009).

Acidovorax citrulli, a seedborne pathogen of cucurbit seeds causing bacterial fruit blotch (BFB), is a serious threat to cucurbit and fruit production in several countries. A nonpathogenic type III secretion system mutant of *A. citrulli* AACOO-1/ΔhrcC was generated. This mutant lost the pathogenicity to watermelon, but retained its ability to colonize germinating watermelon seed. Treatment of watermelon seed naturally infested with *A. citrulli* with its mutant, reduced the seed to seedling transmission of BFB by 81.8 %, relative to the control seed. Another *A. citrulli* antagonist *A. avenae* strain AAA99-2 was able to reduce BFB seedling transmission by 74.6 % for seed samples from the same lot. Furthermore, when female watermelon blossoms were protected with AACOO-1/ΔhrcC and subsequently challenged with AACOO-1, the resulting seed lots displayed only 8 % BFB seedling transmission. This reduction was not significantly different from the seeds from blossoms protected with AAA99-2 (4 %), but significantly less than those from blossoms protected with 0.1 M phosphate-buffered saline (36 %). The results suggested that nonpathogenic *A. citrulli* could be considered as a potential candidate for seed treatment component in a comprehensive BFB management program (Johnson et al. 2011).

7.3 Genetically Modified Strains of Plant Viruses

7.3.1 Cross-Protection by Engineered Mild Strains of Viruses

Cross-protection involves the use of a mild strain of virus for protecting the plants against infection by severe strains of the same virus or related viruses.

Cross-protection, as a virus disease management strategy, has been successfully applied in the case of *Tobacco mosaic virus* (TMV) (Rast 1972), *Citrus tristeza virus* (CTV) (Costa and Muller 1980), *Papaya ringspot v*irus (PRSV) (Yeh et al. 1988) and *Zucchini yellow mosaic virus* (ZYMV) (Wang et al. 1991). A mild strain HA5-1 of PRSV was artificially produced from a severe Hawaii PRSV type strain (PRSV-P-HA) by treatment with nitrous acid. The mild strain HA5-1 protected the papaya plants effectively (90–100 %) against severe parental strain P-HA, both under greenhouse and field conditions. However, protection was strain-specific, limiting its wider application against other strains of PRSV (Yeh and Gonsalves 1984). The possibility of widening the effectiveness of cross-protection against the type W strains of *Papaya ringspot virus* (PRSV) which can infect cucurbitaceous plants, but not papaya (infected by strain P only) was explored. Chimeric mild strains were constructed from HA5-1 to carry a heterologous coat protein [(CP)-coding region and/a 3′ UTR (untranslatable) region HA5-1 Wep 3 U] of a type W strain that originated from Taiwan (PRSV W-CI). The chimeric mild virus HA5-1 Wep 3U provided much higher level of cross-protection against the type W virus W-CI than the mutant HA5-1, while still providing a high degree of cross-protection against the type P virus P-HA in cucurbits. The results indicated the broad-spectrum protection against two strains differentiated only by their ability to infect [(PRSV-R) or not (PRSV-W)] can be achieved by using a chimeric mild potyvirus carrying a heterologous 3′ genomic region, since the 3′ UTR of the potyvirus is involved in the replication of positive-strand of viral RNA. Sequence of homology-dependent post-transcriptional gene silencing (PTGS) is considered an antiviral defense mechanism in virus-infected plants, playing an important role in cross-protection against the same virus or related viruses (Voinnet 2001). HA5-1Wep3U provided more effective protection against W-CI in horn melon and squash plants than HA5-1 could provide. This may be a consequence of the effect of identical sequences of the CP region and 3′ UTR between HA5-1Wep3U and W-CI through a PTGS mechanism. The high degree of protection provided by all chimeric mild strains against P-HA in horn melon and squash plants could be triggered by PTGS induced by long homologous RNA segments shared by individual chimeric mild strains and the strain P-HA. The results demonstrated that the chimeric attenuated virus strains have great potential for providing high degree of cross-protection of different strains of the same virus varying in their host range (You et al. 2005).

7.3.2 Pathogen-Derived Resistance

Transgenic expression of pathogen sequences was postulated to provide protection to plants against the respective pathogen itself by interfering with its development or multiplication (Sanford and Johnson 1985). By applying this approach, transgenic protection could be offered against several viruses affecting a wide range of crop plants with varying degrees of success and acceptability. Powell-Abel et al. (1986) demonstrated that incorporation of coat protein (CP) gene of *Tobacco mosaic*

virus (TMV) in tobacco protected them against this virus. The pathogen-derived gene interferes with the replication process of viruses in their host plants in different ways. The gene product expressed in the transformed plant may inhibit the normal function(s) of the cognate gene in the infecting viral genome or the gene product may be so modified as to become detrimental to viral replication or maturation. The genomes of several plant viruses have been well characterized. The functions encoded by different viral genes have also been determined. Hence, it has been possible to transform plants with different viral genes encoding structural and nonstructural proteins and the effectiveness of protection provided by viral genes against viruses has been assessed.

7.3.2.1 Protection by Viral Coat Protein Genes

Incorporation of viral coat protein (CP) genes into plants for protection against virus infection has been the most frequently followed strategy based on pathogen-derived resistance (PDR) gene against plant viruses. The possibility of reducing the adverse effects of virus infection has been demonstrated in the case of several host-virus combinations (Narayanasamy 2008). The expression of viral coat protein (CP) gene in transgenic plants was demonstrated to induce protective effects similar to classical cross-protection. Hence, this kind of protection was distinguished as 'coat protein-mediated' protection. The transgenic tobacco plants expressing *CP* gene of *Tobacco mosaic virus* (TMV) either did not develop symptoms or developed symptoms after a longer incubation period. The virus titer was very low or TMV could not be detected in some plants. The resistance of transgenic tobacco plants was only against intact virus, but not against viral RNA, since inoculation of transgenic tobacco plants with TMV-RNA resulted in production of symptoms of infection in as many plants as in non-transgenic control plants (Nelson et al. 1997). The ability of *CP* gene of plant viruses functioning as an elicitor of resistance was revealed in Capsicum-*Pepper mild mottle virus* (PMMoV) pathosystems (de la Cruz et al. 1997). The potato cv. Igor was nearly ruined by NTN isolates of *Potato virus Y* (PVY^{NTN}), causing potato necrotic ringspot disease (PTNRD). This cultivar was transformed with the CP gene of PVY^{NTN} and several transgenic lines were highly resistant, showing no visible symptom, when they were graft-inoculated. This virus could not be detected by either ELISA or infectivity test. Among the 34 transgenic lines, two lines were resistant to PVY^{NTN} and PVY^{O} and one line exhibited resistance to PVT^{O} (Racman et al. 2001).

Coat protein-mediated resistance has been induced against several plant viruses infecting a variety of crops (Table 7.3). However, the success of generating virus-resistant plants has been limited to a stage of proof of principle in the laboratory or at best small-scale field trials in all plant-virus pathosystems. The only case of exception is that of production of transgenic papaya plants expressing the *CP* gene of *Papaya ringspot virus* (PRSV). Transgenic papaya plants expressing the *CP* gene of PRSV have been demonstrated to be highly resistant, not only under greenhouse conditions, but also under natural field conditions. The transformed papaya plants

Table 7.3 Development of transgenic plants expressing viral genes and showing resistance to respective viruses

Plant virus	Transgenic crop plants	References
Beet necrotic yellow vein virus	Sugar beet	Lennefors et al. (2006)
Cucumber fruit mottle mosaic virus	Cucumber	Gal-On et al. (2005)
Cucumber mosaic virus	Cucumber	Chen et al. (2004)
Grapevine fan leaf virus	Grapevine	Maghuly et al. (2006)
Lettuce big vein-associated virus	Lettuce	Kawazu et al. (2006)
Papaya ringspot virus	Papaya	Ferreira et al. (2002), Davis and Ying (2004), Tenant et al. (2005)
Papaya ringspot virus and *Papaya leaf-distortion mosaic virus*	Papaya	Kung et al. (2009)
Passion fruit woodiness virus	Passion fruit	Trevisan et al. (2006)
Plum pox virus	Plum	Malinowski et al. (2006)
Potato leaf roll virus	Potato	Kawachuk et al. (1999)
Potato virus X	Potato	Bazzani et al. (2006)
Potato virus Y	Potato	Racman et al. (2001)
Rice stripe virus	Rice	Hayakawa et al. (1992)
Tobacco mosaic virus	Tobacco	Powell-Abel et al. (1986)
	Eggplant	Dardick and Culver (1997)
Tomato spotted wilt virus	Tomato	Accotto et al. (2005)

were not infected, whereas 91–100 % of the non-transformed plants were infected, indicating the effectiveness of transgenic resistance provided by *CP* gene of PRSV to the cultivars SunUP and Rainbow, homozygous and heterozygous for the *CP* transgene respectively (Ferreira et al. 2002). In another investigation, four transgenic papaya lines expressing *CP* gene of PRSV were evaluated for their usefulness under field conditions. At 3–5 months after planting, some of the transformed plants exhibited mild symptoms of PRSV infection, whereas non-transformed plants showed severe disease symptoms. However, the quality of the fruits was not affected in the transformed plants which yielded 10–56 % more fruits, compared with non-transformed plants (Basu et al. 2004). Resistance to PRSV was demonstrated to be RNA-mediated via post-transcriptional gene silencing (PTGS) mechanism and dependent on plant age and gene dosage (Tenant et al. 2001). Another line 63–1 was derived from the cv. Sunset, transgenic papaya expressing the *CP* gene from a mild mutant of a Hawaiin isolate of PRSV. These plants showed a range of resistance to severe PRSV isolates from different geographical locations. Genetic and molecular analyses confirmed that the line 63–1 had two *CP* transgene insertion sites and the *CP* and *nptII* genes were present at both loci. The number of plants in a 63–1 derived population was directly correlated with the number of plants with multiple transgene copies. The results indicated that transgene dosage had a pivotal role in determining the degree of resistance of the line 63–1 to PRSV isolates from different ecosystems (Souza et al. 2005).

Papaya embryos were transformed with translatable (CP_T) and non-translatable (CP_{NT}) versions of the *CP* gene of *Papaya ringspot virus* (PRSV) along with *nptII*

and *uidA* genes via microprojectile bombardment. Varying levels of CP transcript and protein were detected by northern analysis and enzyme-linked immunosorbent assay (ELISA) with CP_T lines. On the other hand, CP expression was not detected in CP_{NT} lines as expected. Initial greenhouse evaluation identified lines resistant to mechanical inoculations with homologous and heterologous isolates of the virus (Tennant et al. 2002). Transgenic papayas carrying CP_T or CP_{NT} gene constructs were evaluated over two generations for field resistance to PRSV in a commercial papaya growing area in Jamaica. Reactions of R_0CP_T transgenic lines included no symptoms and mild or severe leaf-fruit symptoms. All three reactions were observed in one line and among different lines. Plants of most CP_{NT} lines exhibited severe symptoms of PRSV infection and some showed mild symptoms. The transgenic lines appeared to possess virus disease resistance that could be manipulated in subsequent generations for the development of lines with acceptable commercial performance (Tenant et al. 2005). The transgenic papaya varieties Rainbow and SunUP carrying the *CP* gene of *Papaya ringsopt virus* (PRSV) were released commercially as resistant to PRSV. Papaya transformed with the CP gene of PRSV is widely grown over 70 % acreage in Hawaii and fruits are exported to the mainland USA and Canada. Transgenic papaya has clearly demonstrated a great impact on controlling the destructive virus disease. This is considered as a good model of engineered resistance in papaya for disease control. Further, the transgenic papaya had a great socio-economic impact on the Hawaiian papaya industry. The success was largely attributed to both stability/durability of the transgenic resistance and the desirable horticultural and fruit quality attributes of the transgenic cultivars Rainbow and SunUP. However, resistance breakdown could occur with emergence of new viral strains locally or by the introduction of divergent strains from other regions. Hence, it is important to monitor the PRSV population for its diversity and the arrival or emergence of new or more virulent strains of PRSV (Tripathi et al. 2008; Gonsalves et al. 2010).

7.3.2.2 Protection by Noncoat Viral Genes

The effectiveness of transformation of crop plants with viral genes other than viral coat protein gene has been assessed for providing protection against virus diseases. Transgenic tobacco plants expressing the 54K replicase protein of the U1 strain of *Tobacco mosaic virus* (TMV) were highly resistant to the same strain and the effectiveness of protection was better than that provided by *CP* gene of TMV. The resistance was effective against both TMV virions and TMV-RNA, whereas the CP-mediated resistance was effective only against intact TMV virions (Golemboski et al. 1990). Replicase-mediated resistance to *Pea early browning virus* (PEBV) in pea (MacFarlane and Davies 1992), and to *Cucumber mosaic virus* (CMV) (Gal-On et al. 1998) and *Cucumber fruit mottle mosaic virus* in cucumber (Gal-On et al. 2005) was reported to be effective in protecting the plants against the respective viruses. The effectiveness of movement protein (MP) genes in protecting transgenic plants has been observed to be variable. Tobacco plants expressing 30K MP gene of

TMV did not show any decrease in their susceptibility to TMV infection (Deom et al. 1987). However, tobacco plants expressing a mutated form of the MP gene BC1 of Tomato mottle virus (ToMV, *Geminivirus*) showed resistance to ToMV and also to another geminivirus *Cabbage leaf curl virus* (CabLCV). The presence of ToMV was detected in the inoculated sites in the highly resistant plants (Duan et al. 1997). RNA-mediated resistance in plants against RNA viruses and DNA viruses has been reported. One of the drawbacks of RNA-mediated resistance is that it is ineffective against viruses whose sequence differs from that of the transgene by more than 10 %. In order to create broader resistance, 150-nt fragments of viral sequences of four tospoviruses were fused into a single small chimeric inverted repeat (IR) construct. This strategy resulted in a high frequency of multiple resistant plants (Bucher et al. 2006). Tobacco is seriously damaged by three major virus diseases caused by *Potato virus Y* (PVY), *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV). The cDNA fragments from the 5′ ends of the coat proteins (CP) gene of PVY, TMV and CMV with lengths of 200-, 200- and 250-bp respectively, were subcloned. The three cDNA fragments were cloned into one chimeric cDNA and introduced into the binary vector pROKII in a way of inverted repeat which was supposed to form a hairpin RNA (hpRNA) after transcription. The corresponding construct pRHPTC was introduced into tobacco NC89 via *Agrobacterium tumefaciens*. Transgenic plants (1,018) were generated and expression of the chimeric cDNA was observed. Resistance assay indicated that up to 18 % of the transgenic plants were immune to co-infection with PVY, TMV and CMV. Molecular analysis revealed that multiple virus resistance was RNA-mediated and stably inherited in T_2 progeny (Zhu et al. 2009).

Rice tungro disease (RTD) is caused by the simultaneous infection of rice plants by two viruses, *Rice tungro bacilliform virus* (RTBV with DNA as genome) and *Rice tungro spherical virus* (RTSV with RNA as genome). Most of the RTD symptoms are induced by RTBV, while RTSV assists the transmission of RTBV by green leafhoppers from infected plants to healthy plants. Development of rice plants with transgenic resistance to both RTBV and RRSV has been attempted. Transgenic japonica rice plants expressing RTSV replicase (Rep) gene in the sense or antisense orientation were produced. More than 70 % of transformants contained 1–5 copies of Rep gene with integration occurring at a single locus in most cases. Rice plants producing antisense sequences showed significant, but moderate resistance to RTSV (60 %). Plants expressing the full-length Rep gene, as well as a truncate Rep gene, in the (+)-sense orientation were highly resistant to RRSV, even when challenged with a high concentration of RTSV. Since accumulation of viral RNA was low, it was concluded that RTSV Rep-mediated resistance was not protein-mediated, but was of cosuppression type. Rep gene-mediated RTSV resistance was effective against geographically distinct RTSV isolates. Furthermore, rice plants with RTSV Rep gene did not assist vector transmission of RTBA, indicating that transgenic rice plants could be useful in checking the spread of the RTBV to healthy plants (Huet et al. 1999). The effectiveness of RNA interference (RNAi) as a strategy of controlling RTBV infection was investigated. Transgenic rice plants expressing DNA encoding ORF IV of RTBV both in the sense as well as in anti-sense orientation,

resulting in the formation of ds-RNA were generated. RNA blot analysis of two representative lines indicated specific degradation of the transgene transcripts and the accumulation of small molecular weight RNA, a hallmark of RNA-interference. Transgenic line with antisense orientation (RTBV-O-Ds2) showed extremely mild symptoms, whereas transgenic line with sense orientation (RTBV-O-Ds1) exhibited symptoms similar to that present in untransformed plants (Tyagi et al. 2008, 2012). In another investigation, indica rice cultivar Pusa Basmati-1 was transformed to express the coat protein (CP) gene of the Indian isolate of RTBV. Rice plants, containing the transgene integrated in low copy numbers, showed accumulation of CP in the leaf tissues. When challenged with RTBV, using viruliferous leafhoppers, in two independent transgenic lines low levels of RTBV DNA were detected, towards later stages of infection and consequently reduction in severity of tungro symptoms was observed (Uma et al. 2009).

The potential of mycoviruses for managing plant pathogenic fungal pathogens has been demonstrated. Hypoviruses are transmitted from a hypovirulent strain to a virulent fungal strain by hyphal fusion (anastomosis) that is vegetatively compatible. Vegetative incompatibility among many fungal species in agricultural ecosystems has been shown to be a major barrier to the use of hypoviruses as biocontrol agents (Leslie 1993). The failure of mycovirus transmission due to vegetative incompatibility may be overcome in the laboratory by applying protoplast fusion technique. The FgVI-DK21 virus was isolated from *Fusarium boothi* (earlier known *as F. graminearum* strain DK21) one of the causative agents of Fusarium head blight (FHB) of wheat and other cereals. FgVI-DK21 virus was associated with hypovirulence of *F. boothi*. Protoplast fusion technique was applied to transmit FgVI-DK21 virus to *F. asiaticum, F. oxysporum* f.sp. *lycopersici* and *Cryphonectria parasitica*. The results showed that protoplast fusion could be used to transmit FgVI-DK21 ds-RNA both interspecifically and intergenerically. The hypoviruses transmitted through protoplast fusion was able to replicate in the new hosts, though protoplast generation capacity was different between the strains tested. Hypovirulence induced in *C. parasitica* and measured in apple inoculation was greater with FgVI-DK21 ds-RNA than with CHVI virus. The results showed that FgVI-DK21 had the potential for use as a biocontrol agent against fungal pathogens (Lee et al. 2011).

7.4 Protection by Antibody Expression

Crop plants may be engineered to express resistance to diseases caused by microbial pathogens through development of transgenic plants producing recombinant proteins. Antibody engineering has been developed as a novel approach to produce plants resistant to target pathogen by expressing recombinant antibodies (rAbs) or rAb fragments that are capable of inactivating pathogens or proteins involved in the process of disease development (pathogenesis). This approach has been shown to be highly flexible and powerful, since the cloned antibody genes can be tailored to

inactivate the pathogen, when the recombinant antibody is expressed in infected plants (Fischer et al. 2000). Both *Agrobacterium tumefaciens* (At)- mediated transformation and particle bombardment technique have been employed to introduce the antibody genes into plants (Giddings et al. 2000). Particle bombardment allows the simultaneous introduction of multiple constructs, thereby expediting the recovery of transgenic lines expressing multimeric antibodies, such as secretory immunoglobulin A (Larrick et al. 2001). The recombinant antibodies (proteins) can be deposited throughout the plant or in specific organs. The deposition and storage of antibody molecules in seeds of various crop plants has been achieved (Giddings et al. 2000). The antibodies expressed in plants against specific nucleotide sequences are termed 'plantibodies'. The epitope-specific monoclonal antibodies (MAbs) may be used to interfere directly with the activity of virus gene products, allowing their functions in the virus life cycle to be investigated without introducing mutations into the genome.

With development of hybridoma technology, the usefulness of monoclonal antibodies (MAbs) for rapid detection, identification and differentiation of microbial pathogens and plant disease diagnosis has been demonstrated in many pathosystems. It is possible to clone antibodies and express outside animals. Cloned genes may be modified by replacing or deleting codons using site-directed mutagenesis or polymerase chain reaction (PCR) assay. Functional full-size recombinant antibodies (rAbs) may be expressed in transgenic plants. Antibody expression useful for engineering resistance may be employed due to the possibility of selecting desired antigen-antibody interaction. Animal cells resistant to *Human immunodeficiency virus* (HIV) have been successfully generated by applying this form of immunomodulation (Duan et al. 1997). The possibility of using recombinant antibodies (rAbs) for enhancing resistance to viral diseases has been indicated. MAbs specific to plant viruses such as *Tobacco mosaic virus* (TMV) are commercially available. The antibodies can be cloned from hybridoma cell line for protecting susceptible cultivars against target viruses. The phage display technology provides tools to generate MAbs tailored to specific needs and with the desired specificity to any isolated pathogen antigen. The antibody genes are modified before they are tested for expression in *Escherichia coli*, yeast, mammalian or plant cells (Voss et al. 1995). The antibody engineering and phage display may be exploited to isolate desired antibodies against pathogen proteins involved in pathogenesis. Thus armory for combating plant pathogens may be expanded significantly, beyond the genetic resources of plants. New plant lines expressing antibodies constitute an effective barrier preventing pathogen spread (Fischer et al. 2001).

Plants expressing resistance to *Artichoke mottle crinkle virus* (AMCV) were generated by using single-chain antibody fragment (scFv) specific for ACMV. The scFv was expressed in the plant cytosol. Both transgenic protoplasts and plants were resistant to ACMV (Tavladoraki et al. 1993). The transgenic tobacco plants expressing full-length antibodies cloned from a TMV-specific MAb in the apoplast, were resistant to the virus. When a single-chain antibody fragment of this antibody (scFv) was expressed in the cytosol, the resistance was at a higher level (Voss et al. 1995). A new approach was adopted to enhance resistance, based on the expression of

scFv fragments against a conserved domain in plant viral RNA-dependent RNA polymerase (RdRp), a key enzyme in virus replication in plants against *Tomato bushy stunt virus* (TBSV). The virus has single positive sense RNA genome encoding five genes. The p92 TBSV protein was predicted to contain the highly conserved RdRp subdomains of fingers, palm and thumb. The rAbs specific for the palm domain was found to inhibit the TBSV RdRp activity in vitro and also in planta. The T1 and T2 progenies of transgenic *Nicotiana benthamiana* lines expressing selected scFvs either in cytosol or in the endoplasmic reticulum exhibited high levels of resistance against infection by TBSV and related viruses, *Turnip crinkle virus* (TCV) (*Carmovirus*) and *Red clover necrotic mosaic virus* (RCNMV) (*Dianthovirus*) (Boonrod et al. 2004). The expression of a monoclonal antibody specific for *Potato leafroll virus* (PLRV) P1 protein (one of the four proteins required for virus accumulation) and its impact on the virus life cycle was studied. P1 is translated in at least two forms, the P1 replication protein and the P1/P2 polymerase. The P1 protein of PLRV was chosen as model to develop an antibody-mediated approach to study the formation of virus gene products and provide virus resistance. Much of the P1 coding sequence is found in the putative viral replicase, the P1/P2 transframe protein. To study the relevance of P1 and its autoproteolytic derivate P1-C25 in the viral life cycle, the V_H and V_L domains of monoclonal antibody MAb P1-1, raised against the C-terminus of P1, were used to develop a single-chain variable fragment antibody scFv P1-1 for expression in plants. The transient expression of scFv P1-1 in tobacco (*Nicotiana tabacum*), strongly reduced virus accumulation, while transgenic potato (*Solanum tuberosum*) plants expressing scFv P1-1, showed high levels of resistance, following PLRV inoculation by viruliferous aphids. The results demonstrated that PLRV genome product was essential for the completion of the virus life cycle in vivo without genetic alteration of the viral genome. Further, this investigation indicated the possibility of exploiting plantibody-mediated resistance for effective suppression of development of an important potato virus disease (Nickel et al. 2008).

Appendix 7.1: Interspecies Protoplast Fusion for Improving Biocontrol Activity of *Trichoderma* spp. (Hanson and Howell 2002)

A. Protoplast preparation

i. Introduce a single 4 mm plugs of *Trichoderma koningii* from 48-h old PDA culture or *T. virens* conidia (5×10^3) from 4-day old cultures into 100 ml lots of glucose (15 g/l), yeast extract (3 g/l) and casein enzymatic hydrolysate (5 g/l) medium (GYEC); incubate for 24 h at 28 °C on a rotary shaker at 150 rpm; harvest the hyphae of both fungal species separately by vacuum filtration and wash with sterile distilled water.

ii. Suspend the hyphae (0.6–0.7 g) of each species in 5 ml of sterile 0.1 M potassium phosphate buffer (pH 6.4) with 0.6 M sorbitol as an osmoticum

(KPS) and digest for 3 h at 30 °C with 6 mg/ml of lysing enzymes from *T. harzianum* (Sigma) and 2 mg/ml of cellulase (Sigma).

iii. Harvest the protoplasts by filtration through a 10 μm mesh metal sieve, followed by centrifugation in a microcentrifuge at 100 g for 10 min at 26 °C; wash the protoplasts and resuspend in KPS.

iv. Count the protoplasts using a hemocytometer and use immediately or store at 4 °C up to 24 h prior to use.

B. Fusion of protoplasts

i. Perform fusion of protoplasts using approximately equal numbers of protoplasts of two species (approx. 107/ml) in 30 (w/v) polyethylene glycol (PEG, MW4000) in 0.1 M potassium phosphate buffer (pH 6.4) containing 10 mM CaCl2 and incubate for 30 min at 28 °C.

ii. Collect the protoplasts by centrifugation, as in step A(iii) above and resuspend in KPS.

iii. Incubate the parental strains separately with fusion buffer as in step B (i) above and mix the parent strains with PEG/CaCl2 incubation as controls.

iv. Plate the protoplasts in molten PDA (Difco potato dextrose broth, PDB) with 12 g agar/l, containing 0.6 M sorbitol (SPDA) to determine viability.

v. Use SPDA with selective fungicides (20 μg/ml) propiconazole and either 12 μg/ml fludioxonil or 45 μg/ml gliotoxin to detect the fusants.

vi. Plate the protoplasts of both parental strains and mixed strains on the SPDA with individual fungicides to confirm lack of mycelial growth of parent strain.

vii. Allow the medium in plates to solidify; incubate at 28 °C and examine every 2 days for development of mycelium.

viii. Transfer fungal colonies from selective medium to fresh PDA containing selective fungicides and transfer putative fusants, after 5 days of incubation, by hyphal tipping to fresh selective medium.

ix. Transfer hyphal plugs of putative fusants for five times consecutively on fresh selective medium to test for stability of putative fusants and then transfer to PDA.

x. After 5 days, place agar plugs from the edges of growing colonies on selective media to test for the loss of tolerance to selective agents and maintain controls of parental strains for each step (transfers).

xi. Plate fusants strains and parents on PDA containing the individual fungicides to test sensitivity to the individual fungicides/combinations.

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Chapter 8
Abiotic Biological Control Agents for Crop Disease Management

8.1 Natural Products of Plant and Animal Origin

Naturally-derived products of plant and animal origin have been demonstrated to have potential for suppressing the development of soilborne, seedborne and airborne microbial pathogens infecting various crops.

8.1.1 Soilborne Plant Pathogens

Soils present a complex environment in which the host plants interact with the microbial plant pathogens which have negative effects on plant growth and other microorganisms that may be beneficial or may not have any effect on the plant growth. In addition, the physical and chemical factors do have significant influence on the development of both the host plant and the disease-inducing microorganisms. It is a challenging situation for the researchers attempting to study the host plant-pathogen-soil environment interactions, in order to develop effective disease management strategies.

8.1.1.1 Suppressive Soils

Development of microbial plant pathogens may be favored or hampered by different soils. The soils allowing the pathogen development resulting in incidence of disease and development of characteristic symptoms in infected plants are known as conducive soils, whereas the soils that suppress the pathogen development and consequent failure of disease incidence are called as suppressive soils. The term 'suppressive soil' was applied by Menzies (1959) to describe the phenomenon of soils that suppressed Streptomyces scab disease of potato. In every natural soil, expression of disease occurs at various degrees of incidence and severity. The disease suppression

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occurs along a continuum from highly suppressive to highly conducive, rather than simply being suppressive or conducive (Alabouvette et al. 1996). Baker and Cook (1974) defined "pathogen-suppressive soil" as soil in which the pathogen does not establish or persist, or establishes, but causes little or no damage, or establishes and causes disease for a while, but thereafter the disease is less important, although the pathogen may persist in the soil. Investigations on naturally occurring suppressive soils has provided valuable information, regarding the biological traits that lead to disease control and insight into possible means of managing the disease development. On the other hand, certain suppressive soils are dependent upon physical and chemical attributes of the soil. In some other systems the capacity of a soil to limit disease progression develops over time in response to the specific management systems like crop rotation or sequence. Another phenomenon, associated with suppression of the development of fungi, fungistasis refers to the suppressiveness of natural soils to the germination and growth of fungi. The plant pathogenic fungi appear to be more sensitive to fungistasis than saprophytic fungi (Dobbs and Hinson 1953). Soil fungistasis has been attributed to a form of self-inhibition operative within the pathogen propagule in response to stimuli or signals of many kinds from the external environment (Cook and Baker 1983). Alternatively, soil fungistasis was considered to be due to the presence of high populations of antagonistic microorganisms (Johri et al. 1975). Another aspect of fungistasis is the presence in soil of volatile and/ or nonvolatile compounds that inhibit fungal spore germination. The inhibition of fungal spore germination was shown to be related to soil texture and pH (Liebman and Epstein 1992; Chuankun et al. 2004).

The existence of soils suppressive to pathogens such as *Gaeumannomyces graminis* var. *tritici, Fusarium oxysporum, Phytophthora cinnamomi, Rhizoctonia solani, Phymatotrichum omnivorum* and *Streptomyces scabies* has been reported (Cook and Baker 1983). Various attempts have been made to identify the functional biological entities that contribute to the observed disease suppression. Microbial resources with potential have been recognized so that they can be utilized as agents for the biological control of soilborne plant pathogens. Disease suppression in any given soil may require the interaction among multiple biotic and abiotic factors and the function of a specific agent, at sites external to that specific system, could effectively be diminished. Two modes of biological suppression of soilborne diseases may be differentiated. Specific suppression occurs, when there are exclusive interactions involving one or several agents that suppress a soilborne pathogen. On the other hand, in soils that are generally suppressive, disease suppression is due to the cumulative effects of complex interactions between the pathogen and a multiplicity of biotic and abiotic factors (van Bruggen and Semenov 2000). A well known model for suppressive soils is that of soils suppressive to take-all of wheat incited by *Gaeumannomyces graminis* var. *tritici*. Continuous monoculture of wheat during the initial years, increased the incidence of wheat take-all disease. But a spontaneous decline in disease severity termed take-all decline occurs, after an indeterminate period of time. Then the soil remains suppressive as long as wheat monoculture is not interrupted. This suppression apparently is the resultant of a qualitative change in the soil microbial population, following monoculture of wheat (Gerlagh 1968; Shipton et al. 1973).

Soil suppressiveness of diseases is a property primarily conferred by soil microbial community, as indicated by the following facts: (i) soil pasteurization or sterilization abolishes disease suppressive capacity of soils and (ii) transfer of suppressiveness occurs, when conducive soils is mixed with a small quantity of suppressive soil (Stutz et al. 1986). The rhizosphere microbial community is highly diverse and they may act by protecting the plant directly through release of pathogen inhibitors or indirectly by promoting plant growth or enhancing rhizosphere functioning of antagonistic pseudomonads (Lemanceau and Alabouvette 1991; Bally and Elmerich 2005; Raaijmakers et al. 2008). Community-level assessment of taxa more prevalent in suppressive soil has to be performed, but this is carried out rarely with naturally suppressive soils (Hjort et al. 2007). Soil suppressiveness has been observed across geographic locations and it is elicited by the same biological factor across soils. Further, this phenomenon occurs in response to a specific agronomic practice. These evidences indicate that resident microbial resource management has the potential for application, as an effective strategy for the control of soilborne diseases (Weller et al. 2002).

Soils naturally suppressive to *Thielaviopsis basicola*, causative agent of tobacco black root rot disease at Morens (Switzerland) have been described. Clay mineralogical investigations showed that the only consistent difference between suppressive and conducive soils was the predominance of vermiculative soils. None of the other characteristics correlated with soil suppressiveness (Stutz et al. 1985). Later studies indicated that fluorescent pseudomonads were involved in disease suppressiveness. Tobacco was grown in a suppressive and a conducive soil of similar physicochemical properties. The rhizobacterial community composition was compared using a 16S rRNA taxonomic microarray capable of targeting 19 bacterial phyla using 1,033 probes. The microarray comparison of suppressive and conducive soils revealed previously undetected differences in the predominance of a large number of bacterial taxa between the two soils. In addition to fluorescent *Pseudomonas*, the taxa *Azospirillum, Gluconacetobacter, Burkholderia, Comamonas* and Sphingomonadaceae which are known to have beneficial properties, were more prevalent in suppressive soil. *Mycobacterium, Bradyrhizobium*, Rhodobacteriaceae and *Rhodospirillum* were found predominantly in conducive soil. Microarray results were largely corroborated by quantitative PCR and cloning/sequencing. Novel bacterial taxa were identified and they might serve as indicators of disease suppressiveness for soil quality assessments (Kyselková et al. 2009).

Suppressiveness to *Rhizoctonia solani* of soil samples from undisturbed forest, pasture and fallow ground areas, annual crops, perennial crops and ploughed soil was evaluated. The biotic and abiotic variable factors associated with soil suppressiveness to *R. solani* were examined. The biotic variables examined, included total microbial activity, culturable bacterial, fungal, actinomycetes and protozoa communities, populations of *Fusarium* spp. and fluorescent *Pseudomonas* spp. The respiratory activity and overall enzymatic activity (hydrolysis of fluorescent diacetate, FDA) represented total microbial activity. The contribution and relationships of these variables to suppression of *Rhizoctonia solani* were assessed by path analysis which could generate more information than multiple regression. When all samples

were analyzed together, only abiotic variable correlated with suppression of *R. solani*, but the entire set of variables could explain only 51 % of the total variation. In highly suppressive forest soils and pasture/fallow ground areas, several abiotic variables and FDA hydrolysis correlated with growth suppression of *R. solani* and the set of variables explained more than 98 % of suppressiveness. For less suppressive soils, no correlations of suppressiveness with biotic variables could be seen. The results indicated the importance of the vegetation cover and soil management for suppressiveness. The interactions among soil microorganism as well as abiotic variables may influence both the structure and activities of populations, including those of pathogens and their antagonists. The manipulation of microbial communities to induce a disease suppressive soil environment is considered, as a potential factor in the management of soilborne plant diseases (Ghini and Morandi 2006).

Soils suppressive and conducive to *Rhizoctonia solani* AG2-1 were cropped to cauliflower for five successive cropping cycles or allowed to remain fallow in a greenhouse experiment. Soils were inoculated with the pathogen only once or before every crop. Disease decline occurred in all treatments cropped with cauliflower, either because of a decreased pathogen population or increased suppressiveness of the soil. Suppressiveness was determined in seed germination test for assessing pre-emergence damping-off and also by measuring the spread of disease symptoms in young seedlings. Conducive soil became suppressive, after five subsequent cauliflower crops, inoculated in each cycle with *R. solani*. Suppressiveness was significantly stimulated by successive pathogen inoculations and presence of cauliflower had less effect. Suppressiveness was of biological origin, as sterilization of soil abolished this property. Further, suppressiveness was transferable to sterilized soil by adding 10 % of the suppressive soil. Suppressiveness could be correlated to populations of actinomycetes or pseudomonads or parasitic fungi present in the suppressive soil. A potential role of *Lysobacter* in soil suppressiveness could be confirmed by quantitative TaqMan PCR detection which detected a larger population of *Lysobacter* in suppressive soil, compared to conducive soil. The results showed that successive cauliflower plantings could cause significant decline in disease incidence and *Lysobacter* spp. could be the potential key organisms inducing suppressiveness in soils against *R. solani* (Postma et al. 2010).

Soil metagenomics is defined as the study and exploration of the collective genomes present in a particular soil sample. Soil DNA-based metagenomic libraries in a cloning vector are constructed and applied to bulk soil. This approach mainly addresses genes of microbial community (soil microbiome). Suppressive soils restrict the activity of microbial pathogens. This suppression is often biotic and key mechanisms include production of antibiotics. A great part of this antagonistic activity may reside in the uncultured fraction of soil microbiota. Hence, unlocking this antagonistic potential via metagenomic approach may provide valuable information to have a better understanding of the phenomenon of soil suppressiveness. It was assumed that the microbiota of the suppressive soils would serve as rich reservoirs of anti-phytopathogen loci, such as those involved in the production of antibiotics of the polyketide class and chitinase biosynthesis. Four soils each one, suppressive to *Rhizoctonia solani* AG3 in the Netherlands, to *Plasmodiophora brassicae* in

Sweden, to *Fusarium* in France and to *Fusarium* in UK and one control soil were analyzed for antibiotic functions. Owing to the complexity of soil microbial communities, deciphering the key genetic information remains a formidable challenge (van Elsas et al. 2008). Management of resident microbial communities, in order to maintain the level of natural disease suppression and/ or to induce disease suppression, can take several forms, but from a practical stand point, the options are limited to a few. Application of specific soil amendments and use of cropping systems may be feasible to foster soil suppressiveness through enhancement of the activities of the functional biological community.

Application of Composts as Amendments

Various organic residues have been evaluated for their potential as biological control agents against soilborne crop diseases caused by microbial pathogen. Composts have been the most frequently tested substrate and they have been demonstrated to be effective, especially in controlled environment or container-based production systems. The efficacy has been more commonly attributed to an overall enhancement of biological activity in the soil system. But in other systems, a defined element of the microbial community and an operative mechanism have been established. However, the inability to produce predictable, and reproducible compost composition, both from a substrate and microbiological properties, has hampered a meaningful interpretation of the results obtained from the investigations aimed to standardize the compost treatments (Mazzola 2007). Some of the soilborne diseases that have been suppressed by manipulating the level of soil suppressiveness, using compost/ organic amendments are discussed below:

Gaeumannomyces graminis var. tritici (Wheat take-all disease)

Wheat take-all disease decline occurred, due to a form of specific suppression, resulting from a qualitative change in the soil microbial population following wheat monoculture. Decline followed invariably after the incidence of severe disease on at least one or two wheat crops early in the sequence (Cook and Rovira 1976). Among the different theories proposed to explain the process of take-all decline, antagonism from root-colonizing fluorescent *Pseudomonas* spp. appears to be the most plausible mechanism for wheat take-all suppression. It is known that a certain threshold population of 2,4-diacetylphloroglucinol (2,4 DAPG)-producing fluorescent pseudomonads must be reached to achieve a suppressive soil (Raaijmakers and Weller 1998). Certain genotypes of these bacteria are more effective in suppressing the disease than others. Wheat cultivars have been shown to differ significantly in their ability to support resident populations of 2,4-DAPG producers in the rhizosphere and they seemed to exhibit varying preferences for genotypes of these rhizobacteria. Hence, identification of a wheat genotype with a greater capacity to enhance resident populations of 2,4-DAPG-producing pseudomonads or a more active genotype of these bacteria might allow for more rapid development of take-all decline (Mazzola et al. 2004).

Phytophthora spp.

Phytophthora cinnamomi has a wide host range exceeding 1,000 plant species and it is a destructive pathogen of *Eucalyptus* spp., causing root rot disease. Naturally occurring *P. cinnamomi*-suppressive soil was first reported in an avocado orchard on the east coast of Australia (Broadbent and Baker 1974). A *P. cinnamomi-suppressive* soil is a soil in which disease incidence and severity remain low, despite the presence or introduction of *P. cinnamomi*, a susceptible host plant and favorable environmental conditions. *P. cinnamomi*-suppressive soils were well-drained and had a pH between 5.5 and 7.0, high levels of NH_4, NO_3, Ca cations, cation exchange capacity and organic matter content. These factors might have an indirect role by providing conditions that encourage and maintain a soil microbial community antagonistic toward *P. cinnamomi*. Suppression of *P. cinnamomi* was predominantly of biological origin. Terminating the biological activity through autoclaving, fumigating or γ-irradiating the soil, transferring suppression to a conducive soil and cell degradation of *P. cinnamomi* propagules in soil extracts pointed to the biological activity of the soil microbiota being responsible for suppressiveness (Broadbent and Baker 1974; Ko and Shiroma 1989). Mulching with organic materials stimulates plant root growth, increase nutrient uptake, decreases evaporation from soil, increases soil water-holding capacity, reduces surface run-off, facilitates drainage, regulates soil temperature and provides rich substrate for soil microorganisms. Soils with high levels of organic matter have a more complex and active microflora and fauna associated suppression of the activity of *Phytophthora cinnamomi*. All composts, cow, chick, sheep and horse manure increased organic matter content, total biological activity and populations of actinomycetes, fluorescent pseudomonads and fungi. Only chicken manure stimulated endospore-forming bacteria, a factor that was strongly associated with seedling survival. The survival of *Lupinus albus* seedlings in *P. cinnamomi*-infested potting mix was positively correlated with the level of microbial activity and with populations of endospore-forming bacteria, but negatively correlated with organic matter content and populations of actinomycetes (Aryantha et al. 2000).

Phytophthora cinnamomi is a weak saprophytic competitor, fairing poorly in surface organic layers where large number of saprophytes dominate. Some of the saprophytes might even utilize number of actinomycetes isolated from a suppressive organic mulch inhibited the growth of *P. cinnamomi* in vitro (You et al. 1996). Many antagonistic fungi associated with *P. cinnamomi*-suppressive soils were found to be inhibitory to the pathogen. Organic mulches inoculated with *Trichoderma virens* and *T. harzianum* reduced avocado root rot infection and reduced the viability of sporangia (Costa et al. 2000). High microbial activity and large populations of antagonistic bacteria, actinomycetes and fungi are found in soils with high organic matter content, which is a characteristic feature of some *P. cinnamomi*-suppressive soils. Populations of cellulose- and laminarinase-producing bacteria and fungi increase, when organic matter levels are adequate, leading to an accumulation of these enzymes in the soil. The adverse effects of these enzymes on the propagules of *P. cinnamomi* have been determined. High levels of cellulase and laminarinase activity could result in conditions less favorable for the development of *P. cinnamomi*.

Evidence for the involvement of cellulase and laminarinase was provided by the study in which mulch consisting of eucalyptus trimmings was applied to the suppressive soil beneath avocado trees. The mulch increased the microbial activity and root infection by *P. cinnamomi* was significantly reduced (Downer et al. 2001).

Phytophthora parasitica, a soilborne pathogen causes damping-off disease of horticultural plants. The efficacy of composted swine waste (CSW), used as amendment for potting media for biological suppression of the damping-off pathogen was assessed along with aluminum sulfate [$Al_2(SO_4)_3$] for their ability to suppress the disease development. Potting medium amended with both CSW and aluminum sulfate offered both biological and abiotic suppression of *P. parasitica*. The biological and abiotic factors inhibited sporangium production. These two agents might act at different times on the pathogens. Aluminum (Al) inhibited sporangium production at 2 days after treatment. On the other hand, biologically-mediated suppression by CSW of sporangial production persisted from 8 to 23 days after treatment. It is presumed that Al amendment may be effective at protecting the plant before beneficial microbial populations reach a threshold level required for effective suppression of the pathogen development. Addition of compost to peat-based potting media may enhance its ability to support an introduced biocontrol agent. The combination of biotic and abiotic biocontrol agents present in the substrate may render the medium suppressive to a wide range of soilborne pathogens (Fichtner et al. 2004). Application of composts, in addition to enhancing soil suppressiveness against soilborne diseases, has been shown to be effective against Phytophthora diseases of above ground parts of cucumber. The severity of Phytophthora root and crown rot of cucumber caused by *P. capsici* was suppressed significantly in cucumber transplants produced in a composted cow manure-amended mix, compared with those in a dark sphagnum peat mix. Composts may also induce systemic resistance (ISR) in plants to several diseases, including foliar diseases, although the effectiveness of ISR may be variable. The fungal BCA *Trichoderma hamatum* 382 inoculated into the compost-amended potting mix reduced the severity of Phytophthora root and crown rot of cucumber to a level comparable to soil drench with the synthetic ISR inducer benzothiadiazole (BTH) or the fungicide mefenoxam (Khan et al. 2004).

Pythium spp.

Naturally occurring, as well as, compost-amended soils and container media suppressive to Pythium diseases have been recognized in different geographical locations. Several evidences indicated that disease suppression in these soils and composts was due to the activities of microorganisms. These suppressive soils could serve potentially as models for understanding the mechanisms by which complex plant-associated microorganisms interfere with the process of infection by fungal pathogens. Further, disease suppression is most likely a property of the microbial community as a whole and not the result of any single species, since single antagonist recovered from suppressive soil was unable to suppress the pathogen to the level observed with the suppressive soils or medium. Leaf composts prepared from mixed deciduous trees were evaluated for their efficacy in suppressing sporangial

germination of damping-off pathogen *Pythium ultimum* sporangium germination, cotton seed colonization and severity of damping-off disease of cotton. Communities of fatty acid-metabolizing bacteria, that colonized cotton seeds during the first few hours of seed germination, played a major role in the suppression of sporangium germination and seed colonization which ultimately resulted in the suppression of damping-off in the *Pythium*-suppressive compost. The results showed that suppression was expressed rapidly within the first 8 h after sowing cotton seeds and suppression was expressed on the seed surface and not necessarily in the bulk compost. Microbial consortia, recovered from the cotton seed surface during the first few hours of germination in suppressive compost induced, significant levels of suppression of the damping-off disease, whereas no suppression was induced by microbial consortia recovered from cotton seeds germinated in conducive compost (McKeller and Nelson 2003). Soil suppressive effect of paper mill residuals (PMR) on soilborne and foliar diseases of cucumber and snap bean grown on sandy soil was assessed. Raw PMR, PMR composted without bulking agent (PMRC) or PMR composted with bark (PMRBC) were applied annually in a 3-year rotation of potato, snap bean and pickling cucumber. Soilborne diseases, damping-off (*Pythium* spp.) and root rot (*Aphanomyces euteiches*) were suppressed by raw and composted PMR-amended to sandy field soils. In contrast, only the PMRC was consistently suppressive to foliar brown spot and anthracnose of snap bean and angular leaf spot of cucumber (Stone et al. 2003).

The composts have been shown to encourage the development of microorganisms antagonistic to plant pathogens. In addition, they also carry some antagonists that can suppress plant pathogens. In order to isolate and test microorganisms for biological control of *Pythium ultimum*, causing damping-off of greenhouse-grown cucumber, the microflora of the three composts were investigated. A more diverse bacterial population was present in the compost from paper mill sludge (170 groups) than in composts from plant waste and manure (75 and 88 groups respectively). Selected bacterial and fungal isolates were evaluated using dual culture method in agar plates against *P. ultimum*. A total of ten bacterial and four fungal isolates significantly ($P < 0.0001$) reduced the growth rate of *P. ultimum*. The isolates were evaluated in the greenhouse for their efficacy in suppressing the development of damping-off disease in cucumber. *Zygorhynchus moelleri* and *Bacillus marinus* were the most effective in reducing the incidence of damping-off disease under greenhouse conditions, followed by *Penicillium thomii*, *Pseudomonas fluorescens, P. aeruginosa* and *Graphium putredinis, Z. moelleri* was consistently more effective in a second greenhouse trial. The results showed the possibility of obtaining effective antagonists from different compost forms (Carisse et al. 2003).

Pythium ultimum, causing cucumber damping-off disease, was inhibited by the seed-colonizing microbial communities present in the municipal biosolids compost. Selective microbial inhibitors were applied to inactivate portions of the microbial community associated with seed germinated in a compost medium to evaluate their impact on disease suppression. Rifampicin was the most effective inhibitor for inactivating disease suppression. Bacterial communities that colonized cucumber seed sown in compost medium for 8 h followed by a 3-h treatment with either rifampicin

at 500 ppm or water, were dislodged from seed surfaces and subjected to RNA extraction and reverse transcription to cDNA. Differences in the composition of seed-colonizing bacterial communities were assessed, using terminal restriction fragment length polymorphism (T-RFLP) of PCR-amplified 16S rDNA genes. T-RFLP profiles revealed a diversity of distinct bacterial taxa, a number of which dominate seed surfaces within 8-h of sowing. Limited sequencing of clones associated with water-treated and rifampicin-treated seeds revealed the presence of similar taxa dominated by members of the γ-Proteobacteria. Many species within these taxa such as *Pseudomonas* spp., *Enterobacter* spp. and *Bacillus* spp. are known to be effective antagonists of *Pythium* spp. The results indicated that Pythium disease suppression by municipal biosolids compost was mediated by compost-associated bacteria that colonize seed within hours after sowing. It is possible to determine the mechanism of disease suppression by focusing on actively growing microorganisms in the infection courts during early stages of seed germination (Chen et al. 2012a, b). The mechanism underlying suppression of plant infection by *Pythuim ultimum* by compost-derived seed-colonizing microbial communities and the role of competition for fatty acids in seed exudates by the microbial communities were investigated. The germination behavior of *P. ultimum* sporangia in response to cucumber seeds was determined to assess the impact of seed-colonizing microbial communities from municipal biosolids compost. These microorganisms utilized cucumber seed exudates and linoleic acid in vitro, reducing the respiratory stimulatory activity of these elicitors to *P. ultimum* sporangial germination. However, when sporangia were observed directly in the spermosphere of seeds sown in the compost medium, levels of germination and sporangial emptying did not differ from the responses in sand. The percentage of aborted germ tubes was greater, after incubating sporangia in compost medium for 12 h, than the level of germ tube abortion, when sporangia were incubated in sand. Abortion did not occur, if previously germinated sporangia were supplemented with cucumber seed exudates. Further, removal cucumber seed exudates after various stages of germ tube emergence resulted in an increase in aborted germ tube over time. Addition of increasing levels of glucose directly to the compost medium alleviated germ tube abortion in the spermosphere and also eliminated disease suppression. The results did not support a role for linoleic acid competition in Pythium seedling disease suppression, but provided evidence for general carbon competition mediated by seed-colonizing microbial communities as a mechanism of suppression of Pythium seed infection in municipal biosolid compost (Chen and Nelson 2012).

Compost teas are fermented watery extracts of composted materials that have beneficial effects on plants, including antimicrobial activities. Compost tea may be used after active aeration and with additives to increase microbial population densities, for suppressing development of microbial plant pathogens. Aerated compost tea (ACT) and nonaerated compost tea (NCT) produced with or without additives and applied as soil drenches were evaluated for their efficacy of suppression of development of *Pythium ultimum*, causative agent of damping-off disease in container system. ACT fermented with kelp and humic acid activities was suppressive to the maximum extent. The suppressiveness of the formulation was significantly

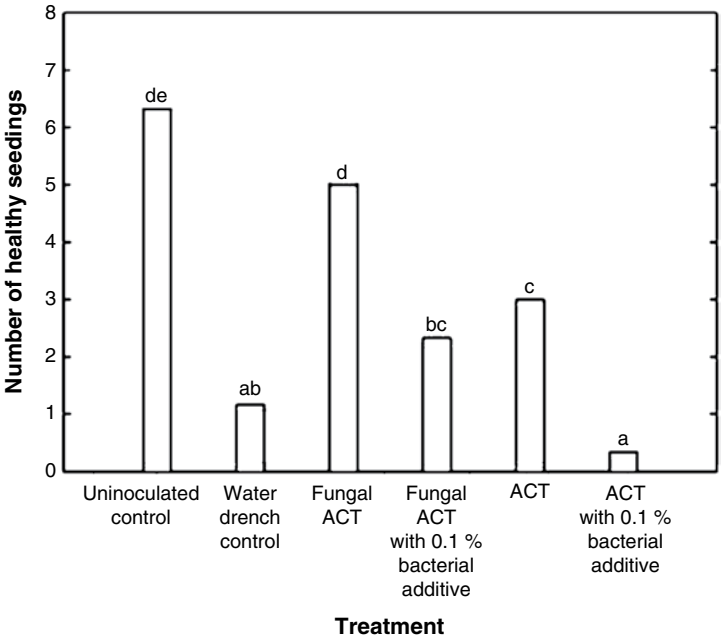

Fig. 8.1 Effect of tank mixing molasses-based Bacterial Nutrient Solution with aerated compost tea (ACT) on cucumber seedling health. Mixing bacterial additive with ACT reduced the number of healthy seedlings, compared with fungal ACT; *bars with same letters* are not significantly different as per Duncan's multiple range test (P = 0.05) (Courtesy of Scheuerell and Mahaffee 2004 and with kind permission of The American Phytopathological Society, MN, USA)

reduced by heat treatment or dilution with tap water. Suppression conferred with the fungal additive was independent of the compost source used in ACT production. The effects of residual nutrients in molasses on disease development were assessed. During production of ACT, increasing the bacterial additive concentration from 0.5 to 1.0 or 1.5 % (v/v) significantly decreased the number of healthy cucumber seedlings, whereas mixing 0.1 % (v/v) bacterial additive with suppressive ACT, just before drenching, reduced the effectiveness of disease suppressiveness (Fig. 8.1). Across all compost tea samples, there was no significant relationship of bacterial populations measured as active cells, total cells or colony forming units (CFUs) with disease suppression (Scheuerell and Mahaffee 2004). Non-aerated compost teas (NCTs) prepared from seaweed compost, shrimp powder compost and chicken, bovine and sheep manure composts were evaluated for their effect on the in vitro growth of tomato root pathogens and for their effect on the development of damping-off diseases caused by *Pythium ultimum* and *Rhizoctonia solani*. The NCT prepared from different types of compost significantly reduced the mycelial growth of *P. ultimum, R. solani, Fusarium oxysporum* f.sp. *radicis-lycopersici* and *Verticillium dahliae* in vitro. Sterilization of NCT resulted in complete or partial loss of their inhibitory effect on the mycelial growth of the pathogens. Application of NCT decreased the percentage of necrotic seedlings in *P. ultimum*-inoculated tomato seedlings from 100 to 42 %, but did not reduce necrosis in *R. solani-inoculated*

seeds. Various NCTs, when applied on tomato seedlings, enhanced plant growth as indicated by fresh and dry weights of treated seedlings, compared with untreated seedlings (Dionne et al. 2012).

A process of wet-sieving biowaste prior to composting was developed to improve the compost quality and disease suppressiveness against *Pythium ultimum* of peat mixes amended with compost prepared as per the new process. The increased organic matter and decreased salt content of the compost allowed for significantly higher substitution rates of peat by compost. Replacement of peat by compost up to 60 % (v/v) did not affect cucumber growth. However, disease suppressiveness of the potting mixes strongly increased from 31 to 94 %, when the compost amendment rate was increased from 20 to 60 %. The general disease suppression for *P. ultimum* could only be effective, when the basal respiration rate was sufficiently high to support microbial activity. Further, organic matter content of the compost should reach the required stability level to turn from disease conducive to disease suppressive status. The results showed that compost from wet-sieved biowaste had high potential to replace peat in growing media for commercial application (Veeken et al. 2005). The disease suppressive potential of five composts from different sources mixed with peat was determined for the control of damping-off diseases caused by *Pythium ultimum, Rhizoctonia solani* and *Sclerotinia minor*, infecting *Lepidium sativum*. Peat amended with composts reduced disease in 60 % of the mixtures. Composts derived from animal manures showed the largest and most consistent disease suppression. Sterilization decreased or eliminated suppressiveness of the mixtures. Several factors were evaluated for their reliability as indicators of disease suppressiveness to predict the efficacy of the composts. The most useful parameters to predict disease suppressiveness were found to be different for each pathogen: extractable carbon, O-aryl C and C/N ratio for *P. ultimum*; alkyl/O-alkyl ratio, N-acetyl-glucosaminidase and chitobiosidase enzymatic activities for *R. solani* and electrical conductivity (EC) for *S. minor*. The results suggested that no single variable could be a reliable and consistent parameter for predicting suppressiveness of all different organic matter amendments versus all soilborne pathogens (Pane et al. 2011). The efficacy of microbe-fortified composts and compost tea preparations in suppressing diseases caused by *Pythium debaryanum, P. aphanidermatum, Rhizoctonia solani* and *Fusarium oxysporum* was assessed. The composts and compost tea amended with *Anabaena oscillarioides* C12 and *Bacillus subtilis* B5 respectively were able to enhance seed germination, seedling length and biomass to the maximum level in pathogen-challenged treatments. In addition, these treatments provided significantly better disease control in terms of reduction in disease severity and reduction in fungal load. The potential of the synergistic activity of the biocontrol agent and the organic amendments like compost may be explored for protecting crops like tomato which are infected by multiple soilborne pathogens (Dukare et al. 2011).

Rhizoctonia spp.

Rhizoctonia solani is an important soilborne pathogen, causing root rot diseases in several agricultural crops. Compost can serve as a food base for endogenous microbes or introduced biocontrol agents to sustain suppression based on the

activities of microbial communities. Composted swine waste (CSW) may be used as a substitute for part of the peat or pine bark in potting mixes. Peat moss-based potting mix was amended with either of the two composted swine wastes CSW1 and CSW2 at rates from 4 to 20 % (v/v) to evaluate suppression of pre-emergence damping-off of impatiens caused by *R. solani*. Pre-emergence damping-off was reduced to a great extent in impatiens grown in potting mix amended with 20 % CSW1, compared to CSW2-amended and non-amended potting mixes. Higher rates of microbial activity were observed with increasing rates of CSW1 amendment than with CSW2 amendments. Mixes amended with CSW1 (20 %), after 35 weeks or more of curing, were consistently suppressive to Rhizoctonia- and Pythium- damping off diseases (Diab et al. 2003). The suppressive potential of six composted substrates viz., farmyard manure, poultry manure, vermicompost, spent mushroom compost, *Lantana camara* and *Urtica* sp. was assessed for the suppression of root rot disease of bean caused by *Rhizoctonia solani* under field conditions. There was a high level of root rot suppression (>33 %) in plots treated with poultry manure, *Lantana* and *Urtica* composts in both experimental years and these treatments were equally effective as the seed treatment with chemical. These compost treatments significantly reduced the disease severity of angular leaf spot disease caused by *Phaeoisariopsis griseola* also. The compost treatments exerted a significant effect on the population of *Trichoderma* spp. and fluorescent pseudomonads in the rhizosphere. The highest population of these antagonists was recovered from poultry manure and *Utrica* compost treatments, indicating that it may be a major factor contributing towards the higher disease reduction observed, following compost application. Higher yields were also realized in poultry manure and Urtica compost treatments (Joshi et al. 2009).

The biocontrol potential of fish emulsion in suppressing radish seedling damping-off disease caused by *Rhizoctonia solani* and *Pythium aphanidermatum* was assessed, using peat mix and soil. Fish emulsion (1–4 %) or equivalent inorganic fertilizer (N-P-K) was incorporated in pathogen-infested peat mix and incubated in plastic bags for different periods prior to planting radish or cucumber seeds. After 7 days of incubation, 70–80 % of the seedlings remained disease-free in peat mix amended with 4 % fish emulsion. The inorganic N-P-K treatment, adjusted to reflect N-P-K levels in fish emulsion, did not have any effect on disease incidence. Hence, it was considered that disease suppression was not, due to increased plant nutrients supplied by fish emulsion. Incorporation of 0.5 % fish emulsion into the soil 5 days before planting, protected the radish seedlings effectively against damping-off disease. Fish emulsion (2 and 4 %) in naturally infested muck soil effectively and consistently suppressed damping-off of cucumber seedlings. Pasteurization of peat mix followed by re-infestation with *R. solani* increased the disease incidence, while addition of fish emulsion restored disease suppressive effect within 7 days, indicating that fish emulsion might create a biological climate in peat substrate or soil, leading to disease suppression. In addition, fish emulsion promoted the growth of the plants in soils and also in peat substrate. Fish emulsion might be a desirable organic amendment for use in organic or conventional transplant production (Abbasi et al. 2004). The effect of incorporation of kraft pine lignin, a side product of the

paper industry on the sclerotial viability of *Rhizoctonia solani* AG1-1b was assessed in different types of soil, after incubation for 4 weeks. Lignin incorporation showed soil-dependent effect. In silt loam soil, the addition of lignin resulted in significant reduction in sclerotial viability, together with enhanced mycoparasitism by *Trichoderma* spp. present in the soil. In sandy loam soil, on the other hand, only a slight and insignificant reduction in sclerotial viability occurred. The phospholipid fatty acid analysis indicated that different kinds of changes in microbial communities structure were induced by lignin incorporation in two soils. A significant increase in Gram-negative bacteria was observed in both soils. Manganese peroxidase produced by lignin-degrading basidiomycetes degraded melanin which protected sclerotia against biotic and abiotic stress. It was hypothesized that lignin-degrading fungi increased the susceptibility of the sclerotia to sclerotial antagonists such as *Trichoderma* spp., Gram negative bacteria and actinomycetes. It appeared that reduction in sclerotial viability could be due to an interaction between different groups of microorganisms in the soil (Van Beneden et al. 2010).

Fusarium oxysporum

Composted green wastes, as organic amendments, are considered to be preferable over other organic wastes, as they have low risk of toxicity due to the presence of heavy metals and pollutants (Witter and Lopez Real 1998). In addition to the positive effect on soil productivity, the green composts exhibit significant suppressive effect on soilborne plant pathogens. The green composts consisting of pine bark pruning wastes with or without coffee wastes or urea were evaluated for their suppressive effect on Fusarium wilt disease of melon caused by *Fusarium oxysporum* f.sp. *melonis*. The ability of green composts to alter both microbial activity of the soil and its physicochemical properties, may favor or hinder the adaptation of certain microorganisms over others. This may be an important factor in the influence of green composts on the effect of pathogenic microorganisms like *F. oxysporum*. Both the fungi and bacteria isolated from the green composts suppressed the development of the pathogen. The growth of melon plants in the soils not infested with the pathogen and amended with green composts were significantly better than those growing in non-amended soils. Addition of green composts to the soil improved its biological and biochemical parameters like ATP and hydrolases involved in the P (phosphatase), C (ß-glucosidase) and N (urease) cycles (Ros et al. 2005). The comparative efficacy of seven compost amendments and the rhizobacterium *Paenibacillus alvei* K165 (with known antagonistic activity against *Verticillium dahliae*) was assessed for the suppression of vascular wilt pathogens *Fusarium oxysporum* f.sp. *melonis* (FOM), infecting melon and *V. dahliae* infecting eggplant (brinjal). The BCA strain K165 showed suppressive effect on both pathogens, whereas the composts had a narrow spectrum of effectiveness against the pathogens. Two composts were effective against *V. dahliae* and one was suppressive to FOM. Under field conditions, one compost fortified with K165 significantly reduced the Verticillium disease, whereas treatment only with compost did not protect the plants satisfactorily (Markakis et al. 2008). *Fusarium*

oxysporum f.sp. *cepae* (FOC) and *Aspergillus niger* cause basal rot and black mold diseases of onion respectively. The efficacy of volatile compounds present in 27 soil samples from onion fields, in inhibiting the pathogen development, was assessed. Volatile compounds in 12 soil samples strongly inhibited germination of spores of *A. niger* only. The fungi isolated from six soil samples were antagonistic to FOC. Volatile compounds from soil did not have a significant role in soil suppression/fungistasis toward all fungal pathogens. Specific antagonistic strains of soil fungus rather than the general population may be involved in suppression of pathogenic fungi. The results suggested that the pathogenic fungi might differentially exhibit responses to the volatiles and fungal antagonists present in the soil (Özer et al. 2009).

The composts consisting of dairy and greenhouse wastes were found to significantly reduce the severity of cucumber Fusarium root rot and stem rot disease caused by *Fusarium oxysporum* f.sp. *radicis-cucumerinum* (FORC). The number of total culturable microbes in the composts showed a positive relationship with disease suppressive ability. The strains of *Pseudomonas aeruginosa* isolated from the composts exhibited the greatest antagonism against FORC. In addition, internal stem colonization of FORC was significantly reduced by *P. aeruginosa*. The locus for 2,4-diacetylphloroglucinol (2,4-DAPG) production was detected by Southern blot analysis and confirmed by PCR analysis. The production of the antibiotic 2,4-DAPG in liquid culture by *P. aeruginosa* was confirmed by thin layer chromatography (TLC) technique. The results indicated that the suppressiveness of the composts against FORC predominantly depended on the production of the antibiotic producing *P. aeruginosa* (Bradley and Punja 2010). Disease suppressiveness of composts is influenced by several factors. In the case of *Fusarium oxysporum* f.sp. *melonis* (FOM), effects of the raw materials, aeration conditions during composting process and compost maturity were investigated to determine their influence on disease suppressiveness and also for isolation of biocontrol agents present in the compost. Microbial strains belonging to bacteria (245), fungi (175) and actinomycetes (73) were isolated from compost samples at different maturation phases. Cell-free extracts of 10 fungi selected from the microorganisms isolated from composts were found to be antagonistic to FOM. Assays under greenhouse conditions showed that *Aspergillus* spp. was the most effective against the Fusarium wilt pathogen (Suarez-Estrella et al. 2007). The effect of storage conditions on compost suppressiveness against FOM was assessed in relation to the dynamics of compost microbial activity and biodegradability. Mature suppressive composts from tomato plants and separated cow manure were stored for 1 year under cool/warm (12 or 28 °C) or dry/wet (15–35 or 55–65 % moisture content) conditions. All composts retained their suppressive potential during storage. Compost microbial populations might compete and interfere with saprophytic stage of FOM conidia, between germination and infection of host roots. The results showed that compost suppressiveness against Fusarium wilt of melon could be maintained for at least 1 year, under a wide range of storage conditions, without any loss of suppressive capacity (Saadi et al. 2010).

Table 8.1 Concentration of individual volatile fatty acids (VFAs) required to reduce the germination of *Verticillium dahliae* microscelerotia by 95 % (EC_{95}) (Tenuta et al. 2002)

		Confidence interval	
Volatile fatty acid	EC95 (mM)	95 % (±)	99 % (±)
Formic acid	3.6	0.7	1.0
Acetic acid	26.2	2.2	2.8
Propionic acid	27.0	7.0	9.3
n-Butyric acid	29.0	4.1	5.3
Isobutyric acid	32.8	6.5	8.6
n-Valeric acid	9.2	1.4	1.8
Isovaleric acid	16.1	1.5	1.9
n-Caproic acid	4.1	0.5	0.7

The values are the means of two experiments with three replicates for each treatment (n = 6)

Verticillium spp.

Liquid swine manure (LSM) has been reported to provide inconsistent protective effect against Verticillium wilt disease of potato caused by *Verticillium dahliae*. Toxicity to microsclerotia of *V. dahliae* was due to volatile fatty acids (VFAs) in LSM. Short-chain VFAs such as acetic, propionic, butyric, valeric and caproic acids are metabolic products of bacterial anaerobic fermentation and they have been detected in liquid manures including LSM, when stored anaerobically (Cooper and Cornforth 1978). Liquid swine manure added to acidic soils killed microsclerotia of *V. dahliae*. The survival of microsclerotia was assessed after exposure to various dilutions to LSM or VFA components: acetic, propionic and butyric acids (constituting major content of LSM). The individual VFAs were more toxic to microsclerotia as the solution pH was decreased, indicating that the protonated forms of the VFAs (acetic acid but not acetate) were toxic (Table 8.1). The toxicity of acetic acid and likely all the others, was directly related to the duration of exposure. The results showed that the nonionized forms of VFAs were toxic to *V. dahliae* microsclerotia, resulting in the suppression of pathogen development (Tenuta et al. 2002). In a further study, the toxicity of the liquid swine manure (LSM) and volatile fatty acid (VFA) mixture equivalent to that in the manure was found to be the same and to occur within 1 day, when added to the acid soil (Fig. 8.2). The VFAs were shown to be the main toxic component in 19 LSMs. About 60 % of the manures tested, reduced germination of microsclerotia of *Verticillium dahliae* to near zero after 1 week exposure. The quantity and composition of VFAs in the manure determined the potential of the manure concerned to be effective in killing the microsclerotia. The total VFA concentration varied from 9.3 to 400 mM. The type of VFA in manure also affected the pathogen suppressiveness. The VFAs such as *n*-valeric, isovaleric and *n*-caproic acids were more toxic to microsclerotia than acetic, propionic, *n*-butyric acid and isobutyric acid. The pH, buffering capacity and amount of LSM added to the soil significantly affected its ability to kill the microsclerotia. The moisture content and temperature of the soil affected the efficacy of LSM to kill the

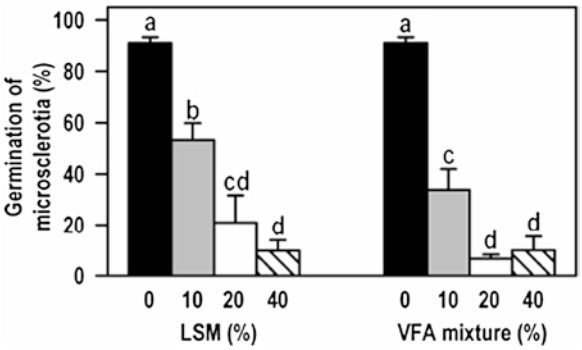

Fig. 8.2 Effect of liquid swine manure (*LSM*) and mixture of volatile fatty acids (*VFAs*) on the germination of microsclerotia of *Verticillium dahliae* in soil from a commercial potato field. Error bars represent ± standard error of mean of two experiments (n = 6). *Means with the same letter* are not significantly different (P = 0.05) as per Student-Newman Keuls method (Courtesy of Conn et al. 2005 and with kind permission of The American Phytopathological Society, MN, USA)

microsclerotia. Addition of LSM to a dry and warm soil increased the effectiveness by increasing the concentration of VFAs in the soil solution. The results indicated that toxicity to microsclerotia of *V. dahliae* might be attained by three mechanisms, namely VFA (in acid soils), nitrous acid (in moderately acidic soils) and ammonia toxicity (in alkaline soils) (Conn et al. 2005).

The efficacy of household waste compost and composted cow manure, in addition to fresh *Brassica* tissue was assessed for the control of *Verticillium albo-atrum*, infecting tomato. Disease severity, as determined by increase in the growth and yield parameters, was significantly reduced by the composts. The biological activity also registered an increase with increasing organic matter input levels. Chitin/chitosan products also reduced the wilt disease incidence and increased tomato fruit yield. However, these products had no effect on soil biological activity. The organic matter suppressive to soilborne pathogens either alone or in combination with chitin/chitosan soil amendments may be effective to achieve disease control through direct effect on the soilborne pathogens and/ or through indirect effect on host plant's defense mechanisms (Giotis et al. 2009). The efficacy of mixtures of wet vegetables (*Brassica*, carrot or onion) and dry onion waste composted at 50 °C for 7 days was assessed for suppression of Allium white rot disease caused by *Sclerotium cepivorum*. The viability of sclerotia was reduced by incorporation of raw or composted vegetable mixtures into sandy loam, silt and peat soils in glasshouse tests. Onion waste was the most effective in reducing viability of sclerotia in all three soils. Reduction in sclerotial viability was at its maximum level with 50 % (w/w) incorporation rate of the wastes. Efficiency of onion waste in reducing viability was less under field conditions, compared with that under glasshouse conditions. The most consistent control was observed in peat soil, while the treatment was completely ineffective in silt soil indicating the effect of soil type on the effectiveness of the organic amendments against the onion white rot disease (Coventry et al. 2005). As the efficacy of onion waste compost (OWC) was variable, the fungal BCA

Trichoderma viride S17A was used as OWC amendment. The effects of OWC and spent mushroom waste (SMC), with and without *T. viride* on sclerotial viability of *S. cepivorum* were investigated. Incorporation of OWC into the soil reduced viability of sclerotia and the incidence of Allium white rot (AWR) on onion plants in the glasshouse pot bioassays. On the other hand, SMC or *T. viride* only reduced incidence of AWR. In the field trials, OWC reduced sclerotial viability and disease incidence as effectively as the fungicide. Addition of *T. viride* to SMC facilitated proliferation of *T. viride* in the soil and increased healthy onion bulb yield. The suppressive activity of OWC was attributed to the presence of sulfur compounds in the compost (Coventry et al. 2006).

The suppressive potential of composted cotton-gin trash (CGT), swine manure and rye-vetch green manure was assessed for the control of southern blight disease caused by *Sclerotium rolfsii* infecting processing tomato. CGT was highly suppressive to the disease in both years (1997 and 1998) of study and it could be used as an alternative to or in combination with conventional soil fumigation. Propagule densities of *Trichoderma* spp. were higher in soils amended with CGT or swine manure than in soils containing synthetic fertilizers or rye-vetch. The disease incidence was higher in plots receiving synthetic fertilizers (61 %) than the plots amended with CGT (23 %), swine manure (44 %) and rye-vetch green manure (53 %). Organic amendments, in addition to enhancing soil fertility, could control soilborne diseases also. The cost of purchase of off-farm inputs might be reduced, if local sources of organic amendments are exploited (Bulluck III and Ristaino 2002). The potential of organic amendments for suppressing *Ralstonia solanacearum* race 3 biovar 2, causing potato brown rot disease was assessed in Dutch sandy and clay soils as well as in sandy soils from Egypt. Organic management significantly increased disease incidence and pathogen survival in Dutch sand and clay soils which correlated with high organic carbon (DOC) contents in the organic Dutch soils. Cow manure amendment significantly reduced disease incidence in organic Dutch sandy soils, but did not affect the bacterial population. However, cow manure reduced the bacterial pathogen densities in Egyptian sandy soils, most probably due to microbial competition as a clear shift in population was detected by denaturing gradient gel electrophoresis (DGGE) technique and Dutch sandy soils after application of cow manure. Suppressiveness of soil towards *R. solanacearum* may be related to soil characteristics and microbial communities which exhibit wide variations, depending on the soil type and organic matter content. Disease suppression was lost, following treatment with methyl bromide fumigation or heat, indicating that a biological principle could be the main factor in enhancing soil suppressiveness towards soilborne bacterial pathogens (Messiha et al. 2007a, b).

Application of Plant Residues as Amendments

Among the various kinds of crop residues used as amendments to soil, cruciferous plants in particular, have been found to be effective in suppressing the development of soilborne plant pathogens, since they contain glucosinolates, sulfur-containing

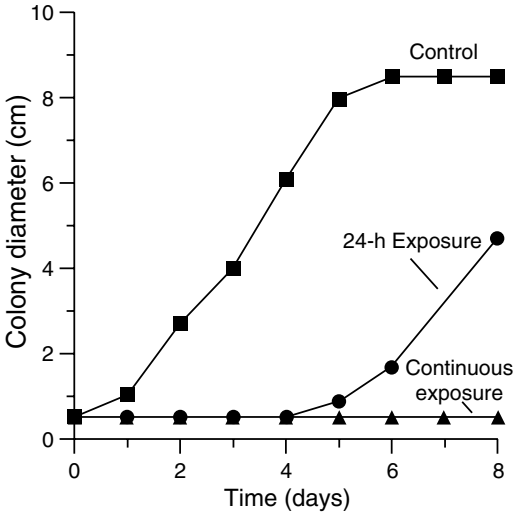

Fig. 8.3 Effect of propenyl isothiocyanate vapor on mycelial growth of *Fusarium oxysporum* 9312F kept in closed containers for 24 h or 8 days. Controls were not exposed to propenyl isothiocyanate (Courtesy of Smolinska et al. 2003 and with kind permission of The American Phytopathological Society, MN, USA)

secondary metabolites. The glucosinolates are hydrolyzed by the enzyme myrosinase to produce isothiocyanates (ITCs) (Brown et al. 1991). Many ITCs are volatile and exhibit inhibitory effects on a wide range of soilborne pathogens (Rosa and Rodrigues 1999). Inhibition of *Rhizoctonia solani* and *Pythium* spp., two major components of fungal complex that incites apple replant disease, could be effectively achieved through exposure to glucosinolates-derived volatiles, suggesting that application of Brassicaceae plant residues could be a suitable approach for tackling apple replant disease (Manici et al. 2000). Since cultivation of *Brassica napus* for incorporating the plant residues into the soil may be impracticable, use of *B. napus* seed meal (a by-product of soil extraction) was suggested as an alternative. The impact of *B. napus* seed meal on pathogens causing apple replant disease was assessed under greenhouse conditions. Seed meal amendment, irrespective of glucosinolate content, significantly suppressed apple root infection by *Rhizoctonia* spp. and the nematode *Pratylenchus penetrans*. On the other hand, seed meal amendment of the cv. Dwarf Essex with high glucosinolate content did not consistently suppress soil populations of *Pythium* spp. Seed meal amendments enhanced *Pseudomonas* spp. and total bacterial populations. Dwarf Essex seed meal amendments were phytotoxic to apple, when applied at the rate of 2 % (v/v) (Mazzola et al. 2001). Individual isothiocyanates (ITCs) released from *Brassica* spp. were evaluated for their efficacy in inhibiting *Fusarium oxysporum* pathogens, infecting conifer seedlings. Propenyl- and ethyl-isothiocyanates were the most fungistatic of the ITCs tested (Fig. 8.3). These ITCs inhibited mycelial growth and completely suppressed conidial and chlamydospore germination. The efficacy of the ITCs was not assessed in the nursery soil to exploit the usefulness of the glucosinolates against *F. oxysporum* infection (Smolinska et al. 2003).

Suppressiveness of rapeseed (*Brassica napus*) seed meal (RSM) applied to orchard soil was assessed against *Rhizoctonia* spp. infecting apple roots. RSM amendment reduced infection by native and introduced isolates of *Rhizoctonia* spp. Root infection by *R. solani* AG-5 was also suppressed in split-root assays, using RSM-amended soil. The hyphal growth of *R. solani* was not inhibited by RSM amendment. Glucosinolate hydrolysis products did not appear to have a significant role in the suppression of *Rhizoctonia* spp. obtained via RSM amendment. The results suggested that transformations in the bacterial community structure may be associated with the observed suppression of Rhizoctonia root rot, with nitric oxide (NO) production by soil bacteria potentially having a role in the induction of plant systemic resistance (Cohen et al. 2005). Soil amendment with *Brassica napus* seed meal was reported to suppress apple root rot caused by *Rhizoctonia solani*, regardless of the glucosinolate content, suggesting that proliferation of soil microbes, after seed meal amendment was responsible for disease suppression in apple. Residues of 12 cultivars of *Brassica rapa* were evaluated for the suppressive capacity against damping-off disease of sugar beet caused by *R. solani*, using soils infested with the pathogen. Residues of clover and peanut were also included in the assessment of suppressiveness of damping-off disease. The plant residues suppressed the development of damping-off to comparable levels in two soils with different schemes of fertilization. All cruciferous plants tested in this investigation belonged to *B. rapa* (AA genome), but the suppressive effect was different, depending on the cultivar from which the residues were obtained. Residues of clover and peanut also induced soil suppressiveness consistently, indicating that disease suppression was not specific to plant genome. Disease suppression in residue-amended soils was reduced or abolished entirely, when antibacterial antibiotics were applied to soils, suggesting that proliferation of antagonistic bacteria resident to the soils were the inducers of disease suppression. When the seed (pericarps) colonized by *R. solani* in the infested soil without residues were replanted into the soils amended with such residues, development of damping-off was suppressed in all treatments with residues. There was no evidence, indicating that glucosinolates or ITCs could be a significant factor in soil suppressiveness (Kasuya et al. 2006).

The potential of several crop residues from broccoli, cauliflower, Indian mustard, ryegrass and corn was evaluated for reducing the viability of microsclerotia of *Verticillium dahliae* present in the soil. The crop residues were incorporated in naturally infested soil samples collected from two cauliflower fields (Is1 and S3). The effectiveness of crop residues in reducing viability of microsclerotia depended on the soil sample and on the type of residue. Incorporation of ryegrass and corn was more effective than incorporation of cruciferous residues. The fungicidal volatile compounds did not appear to play an important role in *V. dahliae* microsclerotia reduction in soil. Volatiles from broccoli and cauliflower did not reduce microsclerotia viability in an in vitro bioassay, whereas the volatiles from Indian mustard killed the microsclerotia in vitro, but when incorporated in soil, it had only a minor effect. Kraft pine lignin (1 % w/w), a waste product of the paper industry, had to be added in S3 soil to obtain a significant reduction in microsclerotial viability, whereas kraft pine lignin was effective even at low concentration in the Is1 soil. Crop residues with high lignin content seemed to be more effective than crops residue with low

lignin content. The effect on microsclerotial germination depended on lignin type and crop structure, since lignin extracted from cauliflower leaves was more effective than that from cauliflower stems and leaves or corn roots (Debode et al. 2005). A native soil microbial community was found to be necessary to elicit suppressiveness of disease development following, application of *Brassica napus* seed meal. Pasteurization of *B. napus* seed meal did not influence the level of disease suppression attained, but pasteurization of soil prior to seed meal amendment and infestation with inoculum of *Rhizoctonia solani*, abolished the capacity of seed meal to suppress apple seedling root infection by *R. solani* (Cohen et al. 2005). Similar response to soil pasteurization was reported for application of multiple brassicaceae seed meals, including *B. juncea* seed meal which yielded allylisothiocyanate (AITC) as hydrolysis products inhibitory to *R. solani* (Mazzola et al. 2007). The pattern of disease suppression by the *B. juncea* seed meal amendment corresponded with the pattern of AITC generation was completed with 24 h after application. Hence, no suppressive effect of seed meal was observed, when the pathogen inoculation was delayed until 48 h post-seed meal amendment. Establishment of disease suppression was associated with the elevation of resident *Streptomyces* spp. population (Mazzola et al. 2007). The potential of several crop residues from broccoli, cauliflower, Indian mustard, ryegrass and corn was evaluated for reducing the viability of microsclerotia of *Verticillium dahliae* present in the soil. The crop residues were incorporated in naturally infested soil samples collected from two cauliflower fields (Is1 and S3). The effectiveness of crop residues in reducing viability of microsclerotia depended on the soil sample and on the type of residue. Incorporation of ryegrass and corn was more effective than incorporation of cruciferous residues. The fungicidal volatile compounds did not appear to play an important role in *V. dahliae* microsclerotia reduction in soil. Volatiles from broccoli and cauliflower did not reduce microsclerotia viability in an in vitro bioassay, whereas the volatiles from Indian mustard killed the microsclerotia in vitro, but when incorporated in soil, it had only a minor effect. Kraft pine lignin (1 % w/w), a waste product of the paper industry, had to be added in S3 soil to obtain a significant reduction in microsclerotial viability, whereas kraft pine lignin was effective even at low concentration in the Is1 soil. Crop residues with high lignin content seemed to be more effective than crops residue with low lignin content. The effect on microsclerotial germination depended on lignin type and crop structure, since lignin extracted from cauliflower leaves was more effective than that from cauliflower stems and leaves or corn roots (Debode et al. 2005).

Lettuce incorporation into the pathogen-inoculated soil significantly reduced the incidence of root and stem rot disease of cucumber caused by *Fusarium oxysporum* f.sp. *radicis-cucumerinum*. In addition, total cucumber yield was also increased. Lettuce residue incorporation may be adapted into the integrated disease management system (Pavlou and Vakalounakis 2005). Residues of various plant species *Diplotaxis tenuifolia* (Wildrocket, WR), *Artemisia dracunculus* (tarragon), *Salvia officinalis* (sage) and *Brassica oleracea* var. *italica* (broccoli) were evaluated for their efficacy in suppressing the development of cucumber crown and root rot disease caused by *Fusarium oxysporum* f.sp. *radicis-cucumerinum* (FORC). Disease incidence

and severity of the disease in cucumber plants inoculated with the pathogen were reduced by 20–80 %, when seedlings were planted in soils incorporated with residues of different plant. Effective soil suppressiveness persisted, after repeated inoculation and plantings in the same soil without additional treatment between inoculations. Furthermore, residues of WR induced soil suppressiveness in two additional tested soils, differing in their physical and chemical properties. Soil suppressiveness to Fusarium crown and root disease was induced, when cucumber seeds were sown in soils which were initially amended with WR residues and later infested with FORC chlamydospores. The results revealed the possibility of containing the incidence of soilborne diseases caused by *Fusarium oxysporum* by incorporating suitable plant residues that contribute to development of soil suppressiveness (Klein et al. 2011).

Cultivation and incorporation of specific green crops into soil, as a means to manage specific biologically active resident soil microbial communities, has been evaluated against certain microbial plant pathogens. The effects of green manures (buckwheat and canola) and crop sequences on potato scab (*Streptomyces scabies*) and Vertcillium wilt (*V. dahliae*) were assessed in 2-year field trial. Densities of indigenous streptomycetes and in vitro pathogen inhibitory activity were also determined. Tubers grown in buckwheat-treated soil had significantly lower Verticillium wilt ratings, while tuber yield was increased significantly. Potatoes grown in soil planted to corn or alfalfa in the previous year had lower Verticillium wilt and potato scab ratings significantly, as well as higher yields than potatoes grown in soil previously planted to potato. *Streptomyces* from soils collected from green manure (GM)-treated plots tended to have greater in vitro pathogen inhibitory activity than streptomycetes from fallow-treated plots. In addition, pathogen inhibitory activity of streptomycetes was frequently negatively correlated with plant disease and positively correlated with potato yield. The streptomycete communities in GM-treated soils were in consistently greater proportion of pathogen antagonists than communities in fallow-treated plots in the second season of the investigation. GM crops may selectively enrich the abundance or activity of antibiotic producers within the soil microbial community (Wiggins and Kinkel 2005a). The relative increase in inhibitory activity of the streptomycetes community was frequently associated with a decrease in disease development and an increase in potato yields. Similar increases in the proportion of antagonistic streptomycetes and reduction in alfalfa root rot were observed in buckwheat or sorghum-sudangrass-treated soils (Wiggins and Kinkel 2005b). Two single-year field experiments were conducted for comparing the effects of Austrian winter pea (*Pisum sativum*) cv. Melrose, broccoli cv. Excelsior and Sudangrass amendment on soil populations and root infection by *Verticillium dahliae* wilt severity and yield of Russet Burbank potato. The results showed that potato Verticillium wilt disease was consistently reduced by all three green manure types applied at the highest amendment rate. A positive correlation between amendment rate and degree of disease reduction was also observed. Austrian winter pea was found to be more effective than the other two green manure types even at a reduced rate of amendment. Although several green manure treatments reduced soil populations of *V. dahliae*, reduction of disease severity was not

always linked to reduced inoculum density, indicating different mechanisms other than inoculum reduction might contribute to green manure-mediated reduction of Verticillium wilt severity (Ochiai et al. 2007). The inconsistent results obtained may be due to the varying conditions, use of diverse green manure species, different soil types, potato cultivars, varying pathogen inoculum densities and the use of naturally versus artificially infested soils.

Fungal pathogens produce different kinds of propagules which show variations in their sensitivity to volatile compounds produced in soils amended with green manures. The impact of volatiles from residues of five species of Brassicaceae and *Avena sativa* and volatiles of pure allylisothiocyanate (AITC) or 2-phenylethyl isothiocyanate (2-PEITC) on the hyphal growth of *Rhizoctonia solani* produced from different propagules was assessed. The amendment of a sandy soil with green manures at high concentration (100 g/kg, 10 %) suppressed the growth of the pathogen, but low concentration (10 g/kg, 1 %) did not inhibit the radial growth of *R. solani*, but increased the density of the hyphae through more branching. Inhibition by volatiles from the residues of Brassicaceae species at 10 % concentration was stronger (82–86 %) than that from oat amendment (64 %) at 10 % concentration. Pure AITC and 2-PEITC (0.5–2.0 mM) inhibited the hyphae initiated from all forms of propagules. Hyphae produced from agar plugs were the most sensitive to the pure volatiles tested. It appeared that hyphae formed from the medulla of the sclerotia might be comparatively tolerant to volatile compounds arising from the decomposing *Brassica* green manure amendments in the field. In vitro test results on inhibition of hyphal growth may not be reliable as indicators of the pathogen response to the green manure amendments incorporated into the field (Yulianti et al. 2006a). The performance of green manure amendments was found to be inconsistent in providing effective control of soilborne pathogens including *Rhizoctonia solani*. In order to identify the factors contributing to inconsistent effectiveness, the saprophytic competence of *R. solani* AG2-1 (ZG5) in sandy soil amended with various green manures was assessed. The volatiles emanating from green manures (100 g/kg) of Brassicaceae and non-Brassicaceae species (Arena) or lupin reduced the hyphal growth, except for *B. napus* B1, *D. tenuifolia* (100 g/kg) inhibited the growth and sclerotial formation of *R. solani*. Most green manures at 10 g/kg and at 40 % water holding capacity, stimulated growth of *R. solani* for up to 3 months and also increased the activity of other microbes. Further, *R. solani* infected the brassicaceous plants and colonized the residues mixed with soil at 10 g/kg. This inoculum increased the severity of damping-off in canola by 27 %. Disease severity reached the peak, when the green manure species, except *D. tenuifolia* and oat, were grown in situ and residues returned to the pot from which they came, before sowing canola. The results revealed the potential hazard in applying green manures of *Brassica* spp., as their residues might under certain conditions, support the saprophytic activity of *R. solani* which could increase the disease incidence in canola sown in amended soils (Yulianti et al. 2006b).

Hairy vetch (*Vicia villosa*) as a soil amendment was investigated for its ability to suppress the development of water melon Fusarium wilt disease and to reduce the soil populations of the causative agent *Fusarium oxysporum* f.sp. *niveum* (FON)

under greenhouse, microplot and field conditions. Pulverized dry hairy vetch reduced the wilt incidence by 53–87 %, compared with nonamended controls. The suppressive effect was comparable to the level obtained with commercially used preplant soil fumigant methyl bromide. Furthermore, the hairy vetch winter cover crop, when incorporated as a green manure, increased the marketable fruit yields as well as fruit sugar content. Suppression of Fusarium wilt in watermelon by hairy vetch at 5 % (w/w) could be due, in part to, the generation of ammonia (≤140 ppm) that resulted in reductions (66–78 %) in soil propagules of FON comparable to the level of reduction (66–100 %) obtained with soil amendment of 0.1 or 0.5 % urea. In microplot and field experiments, incorporation of hairy vetch at rates below 0.5 % suppressed wilt disease incidence, but not soil populations of the pathogen. The hairy vetch soil amendment was considered to have few human and environmental risks and it was also found to be compatible with crop production systems practised (Zhou and Everts 2004). Hairy vetch-induced suppression of watermelon Fusarium wilt was evaluated in the greenhouse on 12 watermelon cultivars with different levels of genetic resistance to the disease and also in 16 naturally infested soil samples collected from commercial watermelon fields. Application of hairy vetch induced suppression of Fusarium wilt in all cultivars except two highly susceptible watermelon cultivars. There appeared to be positive correlation between resistance level of the cultivars and the magnitude of disease suppression by hairy vetch. Fusarium wilt suppression was 22, 53 and 63 % respectively in susceptible, moderately resistant and highly resistant cultivars grown in hairy vetch-amended soil. The pathogen populations in the soil were found to be an important factor that adversely affected the effectiveness of hairy vetch-induced disease suppression. If the pathogen population was below 1,100 CFU/g of soil, hairy vetch was effective in suppressing the wilt disease development. The magnitude of wilt suppression decreased with increase in pathogen inoculum in the soil. Hairy vetch soil amendment did not significantly reduce soil populations of *Fusarium oxysporum* f.sp. *niveum*. But the populations of bacteria, fungi and actinomycetes that were culturable significantly increased, following incorporation of hairy vetch into the soil. The results indicated that hairy vetch-induced wilt suppression was dependent on the level of genetic resistance of watermelon cultivars and the disease suppression could be overcome by the high level of pathogen inoculum in the soil (Zhou and Everts 2007).

Use of *Brassica* crops, as green manure, to function as a biofumigant has been demonstrated to be successful in suppressing development of some soilborne diseases. An investigation to assess the possible negative side-effects of biofumigation was taken up to prevent damage caused by wilt pathogens that could attack both plants used for fumigation, as well as agricultural crops. Four *Brassica* crops selected for their resistance or susceptibility to *Fusarium oxysporum* f.sp. *conglutinans* (*Foc*) and *F. oxysporum* f.sp. *raphani* (*For*) were evaluated. Many of the *Brassica* crops used for biofumigation tested, were susceptible to *Foc* and/or *For*. Green manure treatment, carried out by growing nine cycles of biocidal plants, with a short crop cycle of 30–35 days, did not reduce Fusarium wilts on susceptible *Brassica* crops. The population of the pathogen was partially increased as a result of incorporation of tissues of susceptible plants. In contrast, *Brassica* crops resistant to both *Foc* and *For* proved to

be biocidal to both pathogens. The results indicated clearly that biofumigation with *Brassica* spp. may not be desirable for soil disinfestation on crops susceptible to the pathogen concerned. *Brassica* resistant to Fusarium yellows disease have to be raised for biofumigation for suppressing disease development (Lu et al. 2010).

Efficiency of disease suppressiveness of organic amendments from several plant species was assessed against the microsclerotia (MS) of defoliating (D) and non-defoliating (ND) isolates of *Verticillium dahliae* in infested soil. The efficacy of suppression depended on factors such as particular species of plants, incubation time, pathogen isolate and whether the soil was sterilized. The ND isolate was more sensitive than the D isolate to amendment treatments. Debris of *Diplotaxis virgata* provided the most consistent reduction of MS viability among the plant species tested. No viable inoculum of either isolate (D or ND) could be detected after 6 weeks of incubation with *D. virgata* debris in sterilized or non-sterilized soils. The eradicative effect of organic amendments on *V. dahliae* MS was confirmed by observations on disease incidence and severity of symptoms of Verticillium wilt on cotton plants grown in infested, amended soil. All organic materials reduced the disease incidence and severity in soils infested with both isolates to different extent. No diseased cotton plant in soil infested with ND isolate and treated with amendments, could be seen indicating that the organic amendments strongly reduced the final disease incidence levels (López-Escudero et al. 2007). The efficacy of sweet corn (cv. Jubilee Sweet corn and Jubilee Supersweet corn) as green manure was assessed for suppressing Verticillium wilt disease of potato. The sweet corn varieties suppressed the disease incidence by 60–70 %. These treatments did not affect the pathogen populations directly, but the colonization of *Verticillium dahliae* on potato feeder-roots and in potato tissues of stem apices were reduced. Feeder-root colonization was positively correlated with Verticillium wilt incidence ($P \leq 0.05$) and negatively correlated with yield. In addition, corn green manures increased populations of several fungi like *Ulocladium* and *Fusarium equiseti*, when potato was grown consecutively for 2 years, the beneficial effects of sweet corn green manures was almost entirely lost. But following two consecutive years of potato, a single sweet corn crop was enough to restore the original benefit of disease suppression and increased yield by fourfolds. The study indicated the possibility of reducing disease incidence and enhancing potato yields by growing sweet corn as green manure crops (Davis et al. 2010a). Green manures of Austrian pea, Sudangrass, rape, oats and rye also provided similar effects of suppression of Verticillium wilt and enhancement of potato yields. Sudangrass treatment exerted lasting effects on *Fusarium avenaceum* populations with quantitative changes extending for about 5 years, after soil incorporation of Sudangrass (Davis et al. 2010b).

8.1.1.2 Biological Soil Disinfestation

Biological soil disinfestation (BSD) involves induction of anaerobic soil condition by increasing microbial respiration through incorporation of fresh organic amendments such as grass or potato haulms and by reducing re-supply of oxygen by

covering with airtight plastic sheets. BSD is different from solarization, because the pathogens are not killed by high temperatures attained due to solar heating. Soil disinfestation by either chemical or nonchemical method, allows the potential risk of lowering the general soil suppressiveness toward pathogens by disturbing the composition of soil microflora. A general soil anerobiosis was established by increasing microbial respiration through incorporation of readily decomposable organic amendments into moist soil and by reducing the re-supply of oxygen by covering with ensilage plastic with low oxygen-permeability characteristics. The effects of amendments with broccoli (*Brassica oleracea* convar. *botrytis*) and perennial ryegrass (*Lolium perenne*) incorporated into the soil and covered with airtight plastic sheet on the suppression of *Fusarium oxysporum* f.sp. *asparagi, Rhizoctonia solani* and *Verticillium dahliae* were assessed. In plots amended with broccoli or grass and covered with plastic sheets, anaerobic and strongly reducing soil conditions developed rapidly, as indicated by rapid depletion of oxygen and a decrease in redox potential values. After 15 weeks, the inoculum of the pathogenic fungi was reduced in amended and covered plots. In contrast, the pathogens were hardly inactivated in amended and noncovered or nonamended and covered soil. The results indicated that thermal inactivation, due to enhancement of soil temperatures under plastic cover was not responsible for pathogen inactivation. The biological soil disinfestation approach to suppress soilborne plant pathogens might serve as an alternative to chemical soil disinfestation for high value crops under conditions, where other alternatives like solarization or soil flooding are not effective or feasible (Blok et al. 2000).

Biological soil disinfestation (BSD) approach was verified for its effectiveness for suppression of development of *Verticillium dahliae*, causative agent of Verticillium wilt diseases of *Acer platanoides* and *Catalpa bignonioides* trees. BSD was compared at two locations with a non-treated control, Italian ryegrass amendment and plastic mulch alone. Soil inoculum levels of *V. dahliae* were reduced by 85 %, after BSD, compared with nontreated control. *V. dahliae* population did not increase for the next 4 years. *V. dahliae* infection was reduced by 80–90 %. Disease severity was significantly reduced in *A. platanoides* in all the 4 years of experimentation at one location. Application of Italian ryegrass or plastic covering alone did not reduce pathogen population. The market value of the plants in BSD plots was significantly greater, because of larger trunk and shoot length of the trees. The results showed that BSD could be an effective, economically profitable and environmentally safe control method for tree nurseries (Goud et al. 2004).

The efficacy of the BSD in suppressing potato brown rot disease caused by *Ralstonia solanacearum* race 3 biovar 2 was assessed. The effect of BSD on the survival of *R. solanacearum* was determined at three different scales: (i) glass mesocosms, (ii) microplots and (iii) natural infested commercial field. BSD process was found to strongly reduce soil population densities of *R. solanacearum* both under in vitro and microplot conditions. In the field experiments, anaerobic conditions could not be maintained for long periods, due to bird damage to plastic tarp. Nevertheless, pathogen populations were reduced significantly by more than 95 %. After BSD treatment, potato tubers which could be major reservoirs of the pathogen,

left over in the field after harvest of previous potato crops were completely decomposed. It was presumed that toxic compounds produced during fermentation of organic matter under anaerobic conditions may probably play a major role in the decline of *R. solanacearum* populations. This investigation indicated that BSD could reduce the soil infestation by *R. solanacearum* as effectively as chemical soil disinfectants (Messiha et al. 2007a, b).

8.1.1.3 Use of Steam-Exploded Biomass of Plants

Disease suppressive effect of the plant biomass waste obtained in a steam explosion plant was assessed against soilborne plant pathogens. The steam-exploded biomass (SEB) of *Miscanthus sinensis* var. *giganteus*, a herbaceous perennial energy crop is a multifunctional renewable energy source. The suppressive nature of the SEB was tested against *Phytophthora nicotianae, Pythium ultimum, Fusarium oxysporum* f.sp. *melonis, F. oxysporum* f.sp. *lactucae* and *Rhizoctonia solani*. By using the high performance liquid chromatography technique, the microbial inhibitors, furfurals, organic acids and lignosulfonates were isolated from the SEB and tested under in vitro and in potting soil in the greenhouse. *P. nicotianae, P. ultimum* and *R. solani* were drastically inhibited by furfuraldehyde (3.2 g/l) and 5-hydroxymethyl furfural (0.48 g/l). These compounds were not inhibitory to *Fusarium* at the concentrations tested. The SEB increased the suppressiveness level of peat substrate significantly against *P. ultimum* in cucumber and *R. solani* in bean in all experiments. Addition of SEB significantly suppressed *P. nicotianae*, infecting tomato at 20 and 30 % doses. Incorporation of SEB did not have any suppressive effect on the development of *F. oxysporum* f.sp. *lactucae*, infecting lettuce and *F. oxysporum* f.sp. *melonis*, infecting melon. The results indicated the potential of SEB as an organic soil amendment in place of compost for suppressing the development of diseases induced by oomycetes and *R. solani* in different crops (De Corato et al. 2011).

8.1.2 Airborne Plant Pathogens

8.1.2.1 Application of Compost Amendments

Composts incorporated into soil or planting mixes may provide protection, not only to soilborne plant pathogens, but also to airborne plant pathogens, infecting above ground organs of plants. The efficacy of composted cannery wastes and yard wastes in suppressing the anthracnose fruit rot disease caused by *Colletotrichum coccodes* and bacterial spot disease caused by *Xanthomonas vesicatoria* and *X. axonopodis* pv. *vesicatoria* was determined. The incidence of anthracnose fruit rot was reduced in organic tomato plots amended with a high rate of cannery wastes, but not by yard wastes. On the other hand, composted yard wastes reduced bacterial spot incidence, when disease pressure was high in the conventional tomato production system.

Table 8.2 Effect of composted cannery wastes on incidence of anthracnose disease and fruit yield in organic tomato production system (Abbasi et al. 2002)

Effect on disease incidence/yield	Quantity of composted cannery wastes (t/ha)[a]		
	0	12–15	24–30
Anthracnose (%)			
1997	2.0a	1.8a	2.4a
1998	15.5a	2.4a	9.0b
Yield			
Marketable (t/ha)	38.6b	51.0a	51.4a
Total (t/ha)	61.5b	70.8a	72.4a
Ripe fruit (%)	80.3b	89.1b	91.6a

[a]Means followed by the same letter with each year are not significantly different ($P < 0.05$)

Marketable yield was increased by 33 % in compost-amended organic plots, whereas no increase in marketable yield in compost-amended conventional plots could be seen (Table 8.2; Abbasi et al. 2002). The suppressive potential of composts from olive marc- cotton gin trash, grape marc, cork, spent mushroom and municipal organic and yard wastes was assessed, as growth media against *Botrytis cinerea*, infecting cucumber. The possible relationship of different biotic and abiotic factors involved in disease suppression was considered. Correlations were made between the occurrence of disease and leaf nutrient status, as well as electrical conductivity (EC) and microbial activity (as reflected by ß-glucosidase activity) in growth media. Disease severity in plants grown in the composts was significantly less than in those grown on peats. The minerals Mo, Ca and Si contents in leaves were negatively correlated with the disease severity. Likewise, reduction in severity of the disease in plants grown in composts was related to the supply of specific chemical elements and high microbial activity of composts (Segarra et al. 2007).

Botrytis cinerea causes the Botrytis blight disease of begonia and other greenhouse crops. Incorporation of composted cow manure (5 %, v/v) into the light peat mix significantly reduced blight disease of begonia ($P = 0.05$) and the shoot dry weight and salability of the plants in compost treatment were also increased. Blight severity on plants in the compost mix and the light peat mix inoculated with *Trichoderma hamatum* 38 (T38) was on par, indicating that addition of compost was a desirable strategy. The suppressive effect of compost mix against Botrytis blight compared with the control light peat mix was found to be unusual, since composts typically do not induce systemic resistance in plants without inoculation with a biological control agent capable of inducing this effect (Horst et al. 2005). The efficacy of tomato or pepper residue composts combined with cattle or chicken manure was determined in suppressing bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*). The composts reduced the disease incidence by 79–100 %, under both natural infection of mature plants and artificial inoculation conditions. Populations of *Cmm* in composts declined to undetectable levels within 15–20 days, while those in peat remained high for 35–40 days.

Likewise, colonization of compost-grown tomato plant tissues by the pathogen was reduced to 0–20 %, compared to plants growing on peat or perlite which showed 53–90 and 30–90 % colonization respectively. The results indicated that compost application could be an effective component of integrated *Cmm* management programs (Yogev et al. 2009).

Disease suppressive potential of the leachates of composts prepared from alfalfa and sunflower stalks at concentrations of 10 and 20 % respectively was determined for the control of onion black mold disease caused by *Aspergillus niger*. Onion seeds were treated with the leachates of composts and their ability to induce production of antifungal compounds and to suppress the black mold disease was assessed at seedling and set stages. The leachates reduced the disease incidence in sets, but not disease severity in onion seedlings. Fractionation by thin layer chromatography of extracts from treated plants showed that there was no difference in their antifungal activity against *A. niger*. The presence of fluorescent pseudomonads and *Pantoea agglomerans* was detected in both leachates. The sunflower compost leachate had higher population of *P. agglomerans*, compared with alfalfa leachate. Both leachates inhibited the mycelial growth of the pathogen in agar tests, indicating that the bacterial BCAs might have a role in the induction of antifungal compounds effective against onion black mold disease (Özer and Köycü 2006). The efficacy of an aerated compost tea prepared from composted market and garden wastes in suppressing the development of powdery mildew pathogen *Erysiphe polygoni*, infecting tomato plants grown in perlite in unheated greenhouse, was assessed. Compost tea treatment reduced the disease incidence by 19 %, compared with unrelated plants. Yellow spots without fungal growth in compost tea-treated plants were observed, while untreated plants exhibited whitish patches of powdery growth of the pathogen. Compost tea eradicated the pathogen, when applied as curative treatment. The mechanism of the biocontrol activity of the compost tea was not known. Hence, the compost tea was characterized chemically, physico-chemically and microbiologically. The effects of the compost tea could be, due to the presence of bacteria and fungi which might act as antagonists to the pathogen. The compost tea is rich in organic salts, organic carbon and phenols which might have deleterious effect both on the pathogen and phyllosphere microorganisms. The chemical and/ or microbiological composition of compost tea might interfere with the development of *E. polygoni*, with the curative effect (100 %), being more efficient than preventive effect (19 %). Thus compost tea might exert negative effect on the pathogen directly or indirectly (Segarra et al. 2009). A compost extractor in liquid phase, with forced air blowing system and assembled using farm facilities, was employed to produce “on farm” aerated compost teas (CTs) from five types of composts, in a 14-day fermentation cycle. Solid feed stocks, including one biowaste compost and four composted tomato residues, were extracted in water (waCTs) and whey (whCTs), separately. Ten teas showed high biocontrol potential both in vitro and in vivo against three tomato pathogens viz., *Alternaria alternata, Botrytis cinerea* and *Pyrenochaeta lycopersici*. Disease suppressive activity of CTs was lost after sterilization, suggesting involvement of antibiotic-like antagonism effect due to active microorganisms in suspension. Direct application of teas on tomato plants significantly reduced disease

severity induced by the three pathogens. In vivo suppressiveness was more significant, when liquid phase fermentation (whey or water) was applied, compared with that of compost. Whey could be a viable extractant for suppressive compost-tea production. However, further dilution in dechlorinated water at a ratio of at least 1:5 was required to avoid foliar or root phytotoxicity, probably due to high salt concentrations and sub-acid pH of the relative teas (Pane et al. 2012).

8.1.3 Products from Plant Sources

Secondary metabolites of plants such as essential oils and extracts of plant tissues have been shown to have antifungal, antibacterial, insecticidal, acaricidal and cytotoxic activities. Hence, various plant species have been screened for the presence of antimicrobial compounds and the possibility of applying them for the control of microbial plant pathogens has been explored.

8.1.3.1 Plant Extracts

Soilborne Diseases

Formulated products containing plant extracts or oils were able to reduce the soil populations of *Fusarium oxysporum* and *Verticillium dahliae*. In addition, incidence of pepper Fusarium wilt in the greenhouse was reduced, when the soil was treated with formulated plant extracts (Bowers and Locke 2000). Formulated plant extracts were effective in inhibiting the germination of chlamydospores and conidia of *F. oxysporum* and microsclerotia of *V. dahliae* (Bowers and Locke 1998, 2000). Formulated plant extracts and oils were used to treat the soil infested with chlamydospores of *Phytophthora nicotianae*. Formulations containing 1, 5 and 10 % aqueous emulsions of clove oil, neem oil, mustard oil, synthetic cinnamon oil, pepper extract and cassia extract were evaluated for their efficacy in pathogen suppression. Treatment of soil with 5 and 10 % aqueous emulsions reduced the pathogen populations significantly. The population densities were reduced at 1 day after treatment to the level below the limit of detection (<0.04 CFU/cm^3). Soil was treated with 10 % aqueous emulsions of two pepper extract-mustard oil formulations and two cassia extract formulation. Populations of *P. nicotianae* in soil treated with one of the pepper-mustard oil formulations, were still not detectable at 21 days after application (Fig. 8.4). The neem oil formulation and metalaxyl did not reduce pathogen populations at any of the rates tested. In the greenhouse assays, 10 % aqueous emulsions of a pepper extract-mustard oil formulation, cassia extract and cinnamon oil formulation at 35 days after treatment, suppressed disease development in periwinkle by 93–87 % of the disease incidence compared with untreated, infested soil (control) (Table 8.3) (Bowers and Locke 2004). The effectiveness of leaf extracts of *Arbus precatorius* alone or in combination with *Pseudomonas*

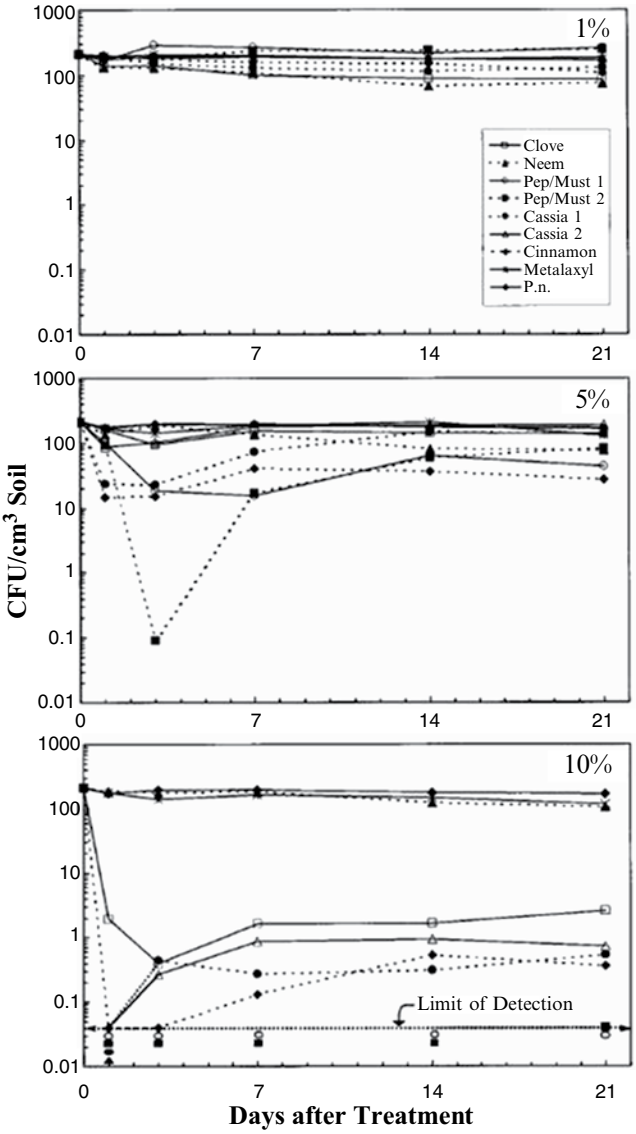

Fig. 8.4 Effect of soil treatment with different concentrations of formulated plant extracts and oils or metalaxyl on population density of *Phytophthora nicotianae* (CFU/cm^3) Aqueous emulsion of formulated plant extracts, oils or metalaxyl were applied at 0.364, 0.728 and 1.46 µl a.i./150 cm^3 of soils respectively; *symbols below the limit of detection* represent populations of *P. nicotianae* that were not detectable in the dilution plate assay for those treatments at the times indicated (Courtesy of Bowers and Locke 2004 and with kind permission of The American Phytopathological Society, MN, USA)

Table 8.3 Effect of soil treatment with oils and plant extracts on populations of *Phytophthora nicotianae* and healthy plant stand (Bowers and Locke 2004)

Treatment (10 % concentration)	Population density	Healthy plant stand (%)
Untreated (control)	167.5a	0
Clove	2.6c	41.0b
Neem	105.3b	3.8c
Pep/Must 1	<LD[a]	93.3a
Pep/Must 2	0.04d	5.0c
Cassia 1	0.5c	93.0a
Cassia 2	0.7c	–
Cinnamon	0.4c	96.7a
Metalaxyl (1.46 µl/150 cm^3 of soil)	117.0ab	62.5b

[a]<LD Populations of *P. nicotianae* were below the limit of detection in soil dilution assays (0.04 CFU/cm^3 of soil)

Mean values in the same column followed by the same letter are not significantly different at P = 0.05 for the population density experiment and P = 0.01 for the disease control experiment, based on multiple comparisons of least-square means using the Bonferroni method

fluorescens Md1 for suppressing the development of *Colletotrichum falcatum*, the causative agent of sugarcane red rot disease was assessed. Sugarcane planting materials (setts) were treated with the plant extract or the bacterial suspension and then planted in pots under greenhouse conditions. Infection by red rot pathogen was the lowest with 24.2 and 20 % respectively in setts treated with plant extract and bacterial BCA. Under field conditions treatment with plant extract was not effective. However, when combined with *P. fluorescens* which was applied as spray or soil drench, the disease incidence was the lowest at 3 %. The combination of plant extract and bacterial BCA could be adopted where disease pressure was less (Jayakumar et al. 2007).

Sclerotium rolfsii causes the destructive damping-off and stem rot diseases in many crops. The biocontrol potential of leaf extracts of *Moringa oleifera* either alone or combined with *Trichoderma* Kd63, *Trichoderma* IITA508 or *Bacillus subtilis* was evaluated in vitro, greenhouse and field tests, when applied as seed treatment, soil drench or sprinkle. In the in vitro assays, the concentration of the extract was negatively correlated and no mycelial growth was seen with extract from 15- or 20-g leaves/ml of water (w/v). In the greenhouse, highest disease control was achieved, when the leaf extract at 15 kg/10 l of water was applied. Under field conditions, Moringa seed treatment combined with *Trichoderma* soil sprinkle, reduced the disease incidence and severity by more than 70 % with significant yield increase in cowpea. The results indicated the effectiveness of combination of leaf extract and a fungal BCA for effective disease management (Adandonon et al. 2006). In a similar approach of combining plant extract with fungal and bacterial biocontrol agents, their effectiveness was assessed for the management of pre- and post-emergence damping-off disease of pepper (chilli) caused by *Pythium aphanidermatum*. Zimmu leaf extract (*Allium sativum* × *A. cepa*) was found to be the most effective among 66 medicinal plant species tested in inhibiting the mycelial growth of the pathogen in vitro. The presence of antifungal compounds (22)

was detected and identified through gas chromatography-mass spectroscopy (GC-MS) technique. Seed treatment with a combination of *Trichoderma viride, Pseudomonas fluorescens* and zimmu leaf extract provided protection to the seedlings to the maximum extent and increased the plant growth and yield as well (Muthukumar et al. 2010). The efficacy of water- and organic solvent extracts of seven plant species and fruits extracts of three plant species was determined for the control of damping-off and wilt diseases of lupine, caused by *Fusarium oxysporum* f.sp. *lupini*. Organic solvent extracts were more effective in reducing the damping-off and wilt diseases. Under field conditions, ethereal and butanolic extracts of *Nerium oleander* and *Eugenia jambolina* leaves and *Citrullus colocynthis* fruits significantly reduced the percentage of wilt severity, in addition to improving the growth parameters and seed yield of lupine (Abdel-Monanini et al. 2011). The inhibitory effects of Chinese leek (*Allium tuberosum*) on banana Fusarium wilt pathogen *F. oxysporum* f.sp. *cubense* (*Foc*) were assessed. In the greenhouse, treatment of soil with Chinese leek reduced the disease incidence and disease severity index by 58 and 63 % respectively in the cv. Baxi (AAA) and by 79 and 81 % respectively in the cv. Guangfen No. 1 (ABB). Crude extracts of Chinese leek completely inhibited the growth of *Foc* race 4 in the in vitro assays, suppressing the proliferation of the spores by 91 % and caused 87 % spore mortality. Under field conditions, adopting a rotation system of Chinese leek-banana reduced the Fusarium wilt disease incidence and severity index by 88–97 and 91–96 % respectively. Further, the crop rotation with Chinese leek improved the crop value by 36–86 % in an area heavily infested by *Foc* during 2007–2009. The results indicated the potential of Chinese leek for suppression of development of Fusarium wilt disease which seriously affected the production system (Huang et al. 2012).

Leaf extracts of *Pistacia* spp. have been reported to have medicinal properties. Crude extracts of *P. vera*, *P. terebinthus* and *P. lentiscus* significantly inhibited the growth of *Pythium ultimum* and *Rhizoctonia solani*, causative agents of damping-off and root rot diseases (Kordali et al. 2003). The antimicrobial effects of leaf extracts of *Pistacia vera, P. atlantica, Schinus terebenthifolus* and *S. molle* against *Agrobacterium tumefaciens, Pseudomonas savastanoi* pv. *savastanoi, Fusarium solani* and *Rhizoctonia solani* were assessed. Aqueous and methanolic extracts of the leaves of different plant species tested, were highly inhibitory to both bacterial and fungal pathogens. Leaf extracts from *P. atlantica* exhibited maximum antimicrobial activity in in vitro assays. Polyphenol contents were at higher levels in *P. atlantica* than in other plant species. Presence of tannins, flavanoids and alkaloids in the extracts of *Pistacia* spp. and *Schinus* spp. was indicated by phytochemical screening and these compounds are likely to have a role in the suppression of the growth of the fungal and bacterial plant pathogens (Rhouma et al. 2009). The efficacy of extracts from 37 plant species in suppressing the development of cucumber root rot and damping-off caused by *Phytophthora melonis* and *Pythium aphanidermatum* was assessed. The ethanolic extract of *Acroptilon repens* showed high inhibitory effect on the mycelial growth of the pathogens. After fractionation of the ethanolic extract of *A. repens* using thin layer chromatography (TLC), each fraction was assessed for its antifungal activity. The fraction with Rf value equal to 0.89 was the

most effective against the pathogens causing root rot and damping-off diseases of cucumber under greenhouse conditions. Soil treatment with the ethanolic extract of *A. repens* was able to significantly reduce the infection by *P. melonis*, compared to control. On the other hand, seed treatment with 10 % of ethanolic extract of *A. repens* effectively suppressed damping-off symptoms induced by *Pythium aphanidermatum*, compared with control (Ghasemi et al. 2012).

The effectiveness of essential oils for suppressing development of fungal diseases has been established. The potential of the essential oils in suppressing the development of soilborne bacterial pathogens like *Ralstonia solanacearum* was examined by a few studies. Thymol and palmarosa oils are antibacterial agents produced by thyme (*Thymus vulgaris*) and palmarosa (*Cymbopogon maritinii*) and they have been used as a general antiseptic. The antifungal activity of thyme against several fungal pathogens such as *Botrytis cinerea, Rhizoctonia solani, Pythium ultimum* and *Colletotrichum lindemuthianum* was demonstrated (Zambonelli et al. 1996; Wilson et al. 1997). Thymol and palmarosa, thyme and oregano (*Oregano vulgare*) oils were shown to effectively reduce the population of *R. solanacearum* in in vitro tests and to reduce the bacterial wilt incidence in tomato in the greenhouse assays (Momol et al. 1999). Thymol, palmarosa and lemon grass oil were evaluated for their ability to suppress the tomato bacterial wilt pathogen *Ralstonia solanacearum* and incidence of the disease in greenhouse assays, when applied as soil fumigant. The soil infested with *R. solanacearum* and then treated with the essential oils at 400 mg or µl and 700 mg or µl/l of soil. Assessment of *R. solanacearum* at 7 days after treatment showed that populations declined to undetectable levels in thymol, palmarosa oil and lemongrass oil at both concentrations. Tomato seedlings planted in soils treated with higher dose (700 mg) of essential oils were not infected by the pathogen. Furthermore, all plants (100 %) in thymol treatments were free of *R. solanacearum* (Table 8.4). Incorporation of fresh leaves of essential oil-producing plants had no effect on bacterial wilt incidence. The thyme + oil-producing plants such as thyme, creeping thyme and Greek oregano were found to be symptomless carrier of the bacterial wilt pathogen. Hence, they should not be considered for crop rotation as a strategy for management of tomato bacterial wilt disease (Pradhanang et al. 2003).

Field evaluation of the efficacy of biocontrol agents has been successfully carried out only in a few cases. Such a demonstration of the effectiveness of the volatile plant essential oils thymol and palmarosa oil at a concentration of 0.7 % was achieved against the bacterial wilt pathogen *Ralstonia solanacearum* (*Rs*). The experimental plots were infested with *Rs* and after 2 h, the essential oils were applied, followed by sealing off the soils with plastic mulch for 3 or 6 days. Tomato seedlings were planted 7 days later. Both thymol and palmarosa oil treatments reduced bacterial wilt incidence significantly. Thymol application significantly reduced bacterial wilt incidence in susceptible cv. SolarSet to 12 % as against 65.5 % incidence in untreated plots. Thymol can be artificially synthesized and it is available commercially. This can reduce the cost of the compound, compared with the cost of producing the antibacterial compounds from plants. Further, thymol can be applied through drip irrigation system and this is likely to reduce the cost of

Table 8.4 Effect of essential oils on the population of *Ralstonia solanacearum* and incidence of bacterial wilt disease (Pradhanang et al. 2003)

Treatments/dose/liter of soil	Population density (10^6)*		Bacterial wilt (%)	Plants with pathogen (%)
	Pretreatment	Posttreatment		
Thymol (0.7 g)	5.0	0	0	0
Palmarosa (0.7 ml)	3.7	0	0	10
Tea tree oil (0.7 ml)	3.7	1.9×10^7	75	10
Lemongrass oil (0.7 ml)	6.2	0	0	10
Thymol (0.4 g)	2.0	0	10c	0
Palmarosa oil (0.4 ml)	4.1	0	40cb	10
Tea tree oil (0.4 ml)	2.9	4.5×10^7	100a	–
Lemongrass oil (0.4 ml)	6.5	0	65b	0
Untreated control	4.5×10^7	5.7×10^7	100a	–

*Population densities of *R. solanacearum* estimated by plating on SMSA medium, confirmed by whole cell fatty acid methyl esters and PCR assay and expressed in 10^6 for all treatments except control for pretreatment and posttreatment counts and for tea tree oil posttreatment counts

application. As thymol has a wide spectrum of activity against bacteria, fungi and nematodes, it has several attributes for selection as a desirable candidate against the serious bacterial wilt disease of tomato (Ji et al. 2005). The potential of essential oils (EOs) in suppressing the development of *Phytophthora capsici*, infecting zucchini (*Cucurbita pepo*) fruit was assessed by in vitro and in vivo tests. Effect on mycelial growth of *P. capsici* was investigated by amending the media with EO or exposing to EO vapors. The efficacy of EOs was determined by estimating the effective concentration for 50 % inhibition of *P. capsici* mycelial growth (EC_{50}). Among 14 commercial products tested, oregano, palmarosa and red thyme EOs had the lowest EC_{50} values (<0.15 μg/ml) for inhibiting the production and germination of sporangia and zoospores and mycelial growth of *P. capsici*. The EOs were also effective in inhibiting some mutants of *P. capsici*, exhibiting resistance to fluopicolide and zoxamide. Populations of *P. capsici* in soil were significantly reduced by the three EOs. Zucchini fruits sprayed with red thyme (0.1 μg/ml) or oregano and palmarosa (0.2 μg/ml) were effectively protected from infection by *P. capsici*. Zucchini seedling emergence was affected by oregano, but not by red thyme. Zucchini seedlings survived in pathogen-infested soil treated with red thyme at 0.1 μg/ml, whereas all seedlings collapsed in untreated control soil. The results indicated that red thyme oil could offer effective protection to zucchini seedlings and fruits against infection by *P. capsici* (Bi et al. 2012).

Diseases of Aerial Plant Parts

The antifungal activity of extracts of *Rheum rhabarbarum* and *Solidago canadensis* against *Phytophthora infestans* was assessed using detached leaf assay and potted potato plants. Leaf infection by *P. infestans* was significantly reduced by the leaf extracts. Preventive application of leaf extracts was more effective in reducing

Table 8.5 Effect of Osthol applied as protective or curative treatment on suppression of powdery mildew of pumpkin leaves (Shi et al. 2007)

Treatments (μg/ml)	Mean disease index[a]	Control efficacy
Osthol Protective – 100	15.56c	77.36
50	42.22b	41.64
Osthol Curative – 100	22.63c	68.72
50	50.78b	29.81
Control (water treated)	72.35a	–

[a]Mean values followed by the same letter in the same column are not statistically different as per LSD tests (P = 0.05)

disease incidence than the post-inoculation treatment. The plant extracts at 50 % concentration were effective, when applied up to 3 days before and immediately after inoculation with the pathogen (Stephan et al. 2005). Efficacy of leaf extracts of *Datura metel*, a plant species growing in wasteland was assessed for suppressing the development of pearl millet downy mildew caused by *Sclerospora graminicola*. Treatment of seeds with 2 % extract for 3 h enhanced the seed germination and seedling vigor. The mechanism of action of *D. metel* extract appeared to be through induction of systemic acquired resistance (SAR) to the downy mildew disease. An association between induction of resistance and salicylic acid (SA) content was observed. The defense-related enzymes, peroxidase, ß-1,3-glucanase and chitinase were appreciably stimulated. The leaf extract also promoted the growth of plants developing from treated pearl millet seeds (Deviah et al. 2009). Hexane, ethyl acetate and methanol extracts of the pericarp of *Areca catechu* were evaluated for their efficacy in inhibiting the development of anthracnose pathogen *Colletotrichum gloeosporioides* infecting mango fruits. The triterpenes, fernol, arundoin and a mixture of stigmasterol and ß-sitosterol and the fatty acid, lauric acid present in the extract inhibited the conidial germination and germ tube elongation. These antifungal compounds suppressed the disease development in mangoes inoculated with the pathogen at 100–200 mg/l concentrations (Yenjit et al. 2010).

Osthol, a coumarinic compound [7-methoxy-8-(3-methylpent-2-enyl) coumarin] has been used as external medicine for human skin ailments. The antifungal activity of osthol against plant pathogens like *Rhizoctonia solani, Colletotrichum musae* and *Phytophthora capsici* was reported by Shi et al. (2004). The efficacy of Osthol in suppressing the development of pumpkin powdery mildew caused by *Sphaerotheca fuliginea* was assessed. Osthol applied prior to or after inoculation with *S. fuliginea* at 100 μg and 50 μg/ml significantly reduced disease index, compared with control treated with water. Osthol applied as protective or curative spray was equally effective in reducing the disease development (Table 8.5). The effect of pretreatment with Osthol on the activation of PR-proteins was determined. Osthol application induced the accumulation of chitinase and peroxidase (PO), indicating that the coumarinic compound could stimulate a resistance response against the obligate fungal pathogen. The induction period required by the agent is the duration between the application of the agent and challenge inoculation of the pathogen. The potentiation

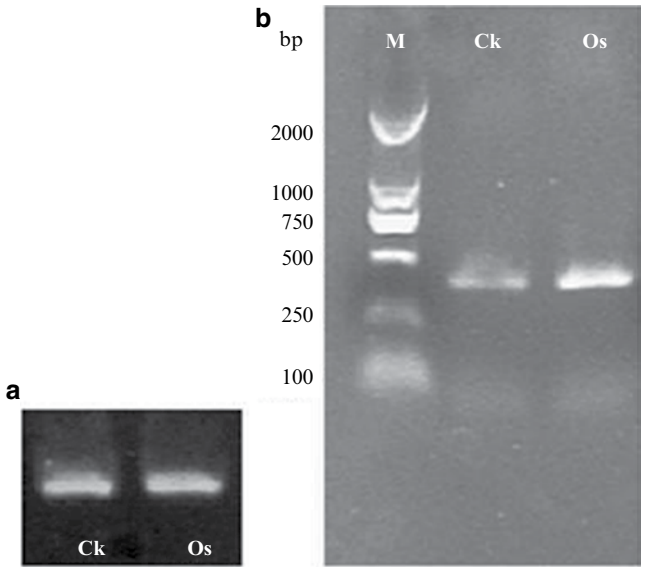

Fig. 8.5 Expression of chitin in Osthol-treated pumpkin as detected by RT-PCR assay using total RNA derived from Osthol-treated pumpkin (*Os*) compared with water as control (*CK*). As an internal control, the actin transcript was also amplified by RT-PCR (**a**) and molecular standards were placed at *left* (*M*) (**b**) (Courtesy of Shi et al. 2007 and with kind permission of International Journal of Molecular Sciences)

of defense in plants by Osthol was found to be 5 days, after inoculation with the pathogen. Application of Osthol induced a progressive and significant increase in the activities of chitinase and PO. This response was more dramatic in Osthol-treated and pathogen-challenged pumpkin plants. Expression of chitinase gene was enhanced in Osthol-treated plants, compared with control, after challenge inoculation with *S. fuliginea* (Fig. 8.5; Shi et al. 2007). *Fusarium mangiferae* is a component of the mango malformation disease complex which causes considerable losses to the grower. Concoctions brewed from *Datura stramomium, Calotropis gigantea, Azadirachta indica* (neem) and cow manure (t1) and methanol extracts of these plant species (T2) were evaluated for their efficacy in suppressing the development of mango malformation disease. Satisfactory level of disease suppression was observed in mango trees sprayed with T1 treatment and more effective than T2 treatment, applied at bud break stage and again at fruit set stage. The treatments did not eliminate the pathogen completely, but the disease incidence was reduced, because of the direct inhibition of the pathogen development. The compost concoction had the potential for disease suppression and feasibility for application, because of the ease and inexpensive nature of the preparation (Usha et al. 2009).

The ability of the crude extract from the green macroalga *Ulva armoricana* to induce plant defence responses in three crop plants, in common bean against *Erysiphe polygoni*, in grapevine against *Erysiphe necator* and in cucumber against *Sphaerotheca fuliginea* was assessed. The crude extract contained high concentrations

of ulvans which are green algae polysaccharides essentially composed of uronic acid and sulfated rhamnose. Different dilutions of the extract were sprayed on the leaves of bean, grapevine and cucumber at weekly intervals. The dilution (3 g/l) provided protection to treated plants resulting, in significant reduction in disease incidence by 50 %, while disease severity was reduced to the maximum extent (90 %) by the highest concentration of the extract (6 g/l). The polysaccharide composition of different batches of the extract of the alga, harvested at different year/periods, varied considerably. However, all extracts elicited a reporter gene regulated by a defense-gene promoter in a transgenic tobacco line and protected cucumber plants against the powdery mildew infection. The results indicated that *U. armoricana* could be used as a reproducible source of active compounds that are capable of enhancing the resistance of different crops against powdery mildew diseases (Jaulneau et al. 2011). Application of leaf extracts and oil cake extracts has been reported to be effective against rice brown spot diseases caused by *Bipolaris oryzae*. Mycelial growth and spore germination were inhibited in in vitro assays. Spray application of *Nerium oleander* leaf extract and neem cake extract, as post-inoculation treatments, reduced the incidence of brown spot disease, under greenhouse conditions. Two sprays of these extracts at 15 days interval, commencing from the initial disease appearance, reduced the disease incidence by 70 and 53 % respectively, in addition to enhancement of grain yield (Harish et al. 2008). The potential of *Yucca schidigera* extract, in reducing disease incidence and severity of apple scab disease caused by *Venturia inaequalis*, was assessed. Yucca extract inhibited conidial germination and penetration of leaf surfaces. However, expression studies of two genes, encoding PR-proteins PR1 and PR8 in apple seedlings, indicated that yucca extract might also affect host defence systems, since expression of both genes was up-regulated, following application of yucca extract to a level similar to that observed after treatment with the chemical resistance inducer acibenzolar-S-methyl (ASM) (Bengtsson et al. 2009). The methanolic extracts and essential oils of *Erigeron ramosus* exhibited antifungal activity by inhibiting the mycelial growth of *Fusarium oxysporum, Phytophthora capsici, Rhizoctonia solani, Sclerotinia sclerotiorum* and *Botrytis cinerea*. The inhibitory effect was, due to monoterpenes and sesquiterpenes, present in the essential oil from *E. ramosus* (Rahman et al. 2010). The antimicrobial potential of chestnut extract was assessed. Extracts from chestnut pellicle had the highest concentration of antimicrobial compound, compared with leaf and shell. The active fraction contained several substances with molecular masses consistent with one flavonol glycoside and several terpenoid compounds. Extracts of pellicle and shell tissue reduced radish scab disease caused by *Streptomyces scabies* under greenhouse conditions (Hao et al. 2012).

The efficacy of two microbial chitinases Chi I (from *Streptomyces* sp.) and Chi II (from *Serratia marcescens*) in inhibiting the development of *Didymella applanata*, causing raspberry spur blight disease was assessed. Chi I had an effective concentration of inhibition of pathogen mycelial growth was 0.4 U/ml, while Chi II did not have inhibitory effect on the mycelial growth of the pathogen. On the other hand, both chitinases reduced pathogen development at 0.5 U/ml concentration in inoculated raspberry canes. In plantations where canes were inoculated after spraying

chitinase, fruiting bodies of the pathogen failed to form in all enzyme treatments, whereas numerous fruiting bodies (12.8/cm^2) were produced in untreated control canes. The chitinases reduced the size of the lesions and limited the infection of internal tissues of canes. Under field conditions, significant suppression of incidence of spur blight was observed. The results indicated the usefulness of chitinases from bacterial species in suppressing fungal diseases of plantation crops like raspberry (Shternshis et al. 2006). In another study, commercial cellulases from the antagonistic fungi *Trichoderma reesei* and *T. viride* were shown to have antiviral activity against *Pepper mild mottle virus* (PMMoV), as shown by significant reduction in the number of local lesions formed on *Nicotiana glutinosa*. Pretreatment of sweet pepper leaves (*Capsicum annuum*) with cellulose solutions before inoculation with PMMoV, greatly reduced the number of plants infected (Oka et al. 2008).

The ability of hyaluronic acid (HA) isolated from the culture filtrates of *Streptomyces* sp. strain KLO1888 to induce systemic resistance against *Colletotrichum orbiculare* (anthracnose) and *Pseudomonas syringae* pv. *lachrymans* (angular leaf spot) in cucumber, *P. syringae* pv. *tomato* (tomato speck disease) in tomato and *Cucumber mosaic virus* (CMV) in pepper was assessed. HA was applied as sprays, injection and soil drenching. The experiments showed that HA provided higher levels of protection in all the tested pathosystems, irrespective of the mode of application. The fact that drenching of planting medium with HA suppressed development of diseases effectively suggested that the mechanism of protection provided by HA was through induction of systemic resistance to all diseases investigated and not through direct antagonism. Specifically HA induced activation of *PR1-a* and *PDF1.2* genes in tobacco, demonstrating the potential of HA. Both SA-mediated and JA-mediated defense mechanisms are likely to be activated in different plant hosts, following application of HA (Park et al. 2008).

Components of fungal pathogens have been shown to induce resistance in plants against microbial pathogens. Plant defense responses were induced in four different varieties of *Arachis hypogaea* (peanut), using the fungal components of *Sclerotium rolfsii* in the form of fungal culture filtrate (FCF) and mycelial cell wall (MCW). The levels of defense-related signal molecule salicylic acid (SA), marker enzymes such as peroxidase (PO), phenylalanine ammonia lyase (PAL), ß-1,3-glucanase and lignin were determined. Substantial increases in PO, PAL, SA, ß1,3-glucanase and lignin content in FCF- and MCW- treated plants of all peanut cultivars were observed, compared with untreated control plants. The enzyme activities were much higher in FCF-treated plants than in MCW-treated plants. The extent of increases in enzyme activities and signal molecules varied with different cultivars. The results indicated the possibility of using fungal components for enhancing the resistance levels to fungal plant pathogens infecting peanut (Nandini et al. 2010). The biocontrol agent *Trichoderma harzianum* SQR-T037 could effectively suppress the development of Fusarium wilt disease of cucumber caused by *Fusarium oxysporum* f.sp. *cucumerinum* (*Foc*) in continuously cropped soil. One of the antifungal compounds purified from the BCA strain was identified as 6-pentyl-α-pyrone (6PAP), using both mass spectrometry and nuclear resonance spectroscopy. The antifungal activity of 6PAP increased proportionately with increase in its concentration. At 350 mg/l of 6PAP, the mycelial growth and conidial germination of *Foc* were inhibited by 73.7

and 79.6 % respectively. In addition, at a concentration of 6PAP of 150 mg/l, sporulation and fusaric acid production of *Foc* decreased by 88 and 52.68 % respectively. Addition of 6PAP (350 mg/kg of soil) in soil continuously cropped with cucumber, could decrease the population of indigenous *F. oxysporum* by 41.7 % and suppress the disease development by 78.1–89.6 %, resulting in enhancement of dry weight of cucumber seedlings by 60.0–92.6 % in greenhouse assays. The results indicated the antifungal property of the purified 6PAP from *T. harzianum* with high biocontrol potential against the *Fusarium oxysporum* f.sp. *cucumerinum* (Chen et al. 2012a, b).

The type I plant lipid transfer proteins (LTPs) are small, basic, cystein-rich proteins involved in plant defense mechanisms. Five type I LTPs isoforms were isolated from *Vitis vinifera* (LTP 1–5) and purified to homogeneity from the grapevine cell suspension cultures. The LTP VvLTP4 was found to be the most efficient to interact with jasmonic acid. Exogenous application of VvLTP4-JA complex of grapevine plantlets induced a high level of tolerance (80.3 ± 10.05 %) towards the gray mold pathogen *Botrytis cinerea*, as compared with control plants (18.65 ± 12.3 %). The results indicated that naturally existing plant constituents may possibly be used to combat the fungal pathogens (Girault et al. 2008). Plants produce antimicrobial compounds known as phytoanticipins. Aqueous infusions of healthy banana (*Musa acuminata* cv. Grande Naine) leaves contained a phytoanticipin with antifungal activity against *Mycosphaerella fijiensis*, causing black Sigatoka leaf spot disease. The phytoanticipin was purified by the bioassay guided VLC procedure from the lyophilized infusion of the leaves of 4-month old healthy banana plants. The purified compound showed strong antifungal activity against *M. fijiensis*. The LC-MS analyses of the purified phytoanticipin suggested a steroidal saponin structure with four sugar units attached to the C3-position of a diosgenin-like aglycone (Cruz-Cruz et al. 2010). Burdock fructooligosaccharide (BFO), a type of linear BFO extracted and isolated from the roots of *Arctium lappa* was evaluated for its ability to induce systemic acquired resistance (SAR) in cucumber seedlings against the anthracnose pathogen *Colletotrichum orbiculare*. The BFO strongly induced changes in salicylic acid (SA) and SA-glucoside (SAG) in BFO-treated leaves. Similar changes in SA and SAG contents were observed in the untreated leaves of the same seedling. The patterns of expression of SA and SAG in the untreated leaves were similar to that of treated leaf of the same seedlings. Pretreatment with BFO reduced the lesions caused by *C. orbiculare* by 57 %. In addition, the amount of lignin and the activities of defense-related enzymes such as peroxidase, superoxide dismutase, polyphenol oxidase and ß-1,3-glucanase significantly increased in the first leaves pretreated with BFO, followed by challenge inoculation with *C. orbiculare*. The results indicated that BFO had the potential to induce SAR in cucumber seedlings, resulting in reduction of severity of anthracnose disease symptoms (Zhang et al. 2009).

The antiviral effect of CAP-34, a protein obtained from *Clerodendron aculeatum* was assessed on the infection of *Papaya ringspot virus* (PRSV) in papaya (*Carica papaya*). The treated papaya plants did not show any symptoms of infection by PRSV up to 30 days during which period 56 % of the untreated plants exhibited clear and intensified symptoms of virus infection. During next 30 days, 95 % of the control plants exhibited a full range of symptoms from mosaic to filiformity of

leaves, whereas only 10 % of the plants treated with CAP-34 protein showed restricted mild mosaic patterns. The presence of PRSV was determined by bioassay, plate ELISA, immunoblot and RT-PCR techniques in the experimental plants. PRSV-RNA was not detectable in treated plants which were asymptomatic. The results suggested that suppression of virus replication may be the possible mode of the antiviral activity of CAP-34 protein (Srivastava et al. 2009). The antiviral effect of the oligo-sulfated galactan poly-Ga from a marine algal species was assessed against *Tobacco mosaic virus* (TMV), using tobacco (*Nicotiana tabacum*) cv. Xanthi NN plants as assay hosts. The plants were sprayed with Poly-Ga at different concentrations and control plants were sprayed with water. The number of necrotic lesions decreased with increasing concentrations of Poly-Ga, with increase in number of applications and with increasing time after treatment. The concentrations of TMV-capsid protein (CP) transcripts were quantified in apical leaves of tobacco plants treated with Poly-Ga and untreated plants. The TMV-CP transcripts decreased in distant leaves, indicating that Poly-Ga induced systemic protection against TMV. The defense-related enzymes phenylalanine ammonia lyase (PAL) showed progressive increase with increasing time after treatment with Poly-Ga, whereas lipoxygenase (LOX) activity remained unchanged. In addition, the increase in PAL activity was negatively correlated with the number of necrotic local lesions formed and the decrease in TMV-CP transcript level in plants treated with Poly-Ga. The results showed that Poly-Ga had the potential for effective protection of tobacco plants against TMV (Vera et al. 2011).

Products extracted from plants have been examined for their ability to suppress development of plant diseases caused by microbial pathogens. Volatile antimicrobial compound allicin (diallyl-thiosulfinate) is produced in garlic, when the tissues are damaged and the substrate allin (S-allyl-L-cysteine sulfoxide) mixes with enzyme allin-lyase. Allicin in garlic juice inhibited the germination of sporangia, encysted zoospores and subsequent germ tube elongation in *Phytophthora infestans* both in vitro and in vivo on tomato leaf surface. Disease severity in *P. infestans-infected* tomato seedlings was reduced by spraying garlic juice containing allicin (55–110 µg/ml) with an effectiveness of about 45–100 % mortality. In growth room experiments, the severity of cucumber could be reduced by spraying garlic juice by 50–100 % on cucumber plants (Portz et al. 2008). The efficacy of garlic juice was assessed for its antimicrobial action against a range of phytopathogens. Allicin effectively controlled seedborne pathogens *Alternaria* spp. in carrot, *Phytophthora infestans* in tomato and tuber blight in potato and *Magnaporthe grisea* in rice. Reduction in diseases was due to a direct action of allicin apparently on the pathogens tested (Slusarenko et al. 2008). The effect of application of xanthan gum on the development of *Bipolaris sorokiniana*, infecting barley was assessed. Barley plants cvs. 127, 128 and 129 were treated with xanthan gum and after different periods, the plants were challenged with the pathogen. The mechanism of suppression of disease development was due to induction of local and systemic resistance by xanthan gum. Barley plants treated with xanthun gum exhibited an increase in the concentration of proteins, as well as in the activity of ß-1,3-glucanase, when compared with the extract from healthy plants. The most important macromolecules in the defense demonstrated to be PR-proteins which accumulated, following the application of

the resistance inducer (Castro and Bach 2004). Application of saccharin on hydroponically grown soybean plants was shown to induce systemic acquired resistance (SAR) to the rust pathogen *Phakopsora pachyrhizi*. Saccharin (3 mM) was applied either as a foliar spray or root drench at the second trifoliate and early reproductive stages. Saccharin applied as root drench was more effective than foliar spray in inducing SAR with increase in resistance at 1 day after application of saccharin. Foliar application induced perceptible resistance only at 15 days after treatment. No adverse effect of saccharin on the growth of soybean was observed. The results indicated the potential of saccharin as the resistance inducer against the foliar fungal pathogen (Srivastava et al. 2011).

Milsana, a commercial product made from the leaves of the giant knot weed plant (*Reynoutria sachalinensis*) is an eliciting mixture containing proteins, glycoproteins, peptides, carbohydrates and lipids that recognize and signal the presence of the pathogen, subsequently triggering defense responses in a non-cultivar-specific manner (Daayf et al. 1997, 2000). Resistance to *Podosphaera xanthii* (= *Sphaerotheca fuliginea*), causing powdery mildew disease was induced in cucumber by the treatment with Milsana. This resistance was correlated with increased mRNA accumulation and greater extractable enzymatic activity for chalcone synthase (CHS) and chalcone isomerase, two key enzymes of the flavonoid pathway (Fofana et al. 2002). In a later study, accumulation of readily synthesized C-glycosyl flavonoid phytoalexins (cucumerins) was observed at sites of pathogen penetration within Milsana-elicited cucumber plants (McNally et al. 2004). The role of flavonoid phytoalexin production, lignification and chitinases in powdery mildew disease resistance in cucumber was studied. After elicitation of resistance by Milsana, the cucumber plants were infiltrated with inhibitors of cinnamate 4-hydroxylase, 4-coumarate:CoA ligase (4CL) and CHS. The elicited cucumber plants exhibited a high level of induced resistance. In contrast, down-regulation of CHS, a key enzyme of flavonoid pathway resulted in nearly complete suppression of induced resistance and the development of healthy haustoria within these plants was revealed by electron microscopy. Inhibition of 4 CL had little effect on induced resistance. The results suggested that induced resistance in cucumber was largely correlated with de novo synthesis of flavonoid phytoalexin compounds rapidly (Fofana et al. 2005). The efficacy of Milsana for the control of powdery mildew of tomato caused by *Leveillula taurica* in the greenhouse crops was determined. Application rates and disease pressure were found to be important factors, affecting the effectiveness of Milsana. The powdery mildew disease incidence was reduced by 42–65 %. The efficiency of Milsana in protecting the tomato plants was less than that of fungicides (Konstantinidou-Doltsinis et al. 2006). The formulations, consisting of a dispersion of Brassicaceae meal in vegetable or mineral oils were evaluated for their efficacy against powdery mildews infecting sugar beet (*Erysiphe betae*) and cucumber (*E. cichoracearum*). Both formulations reduced the leaf area infected by powdery mildews to 3–10 %, as against 56 % in untreated control plants. The formulations appeared to have direct adverse effects on the conidia which showed distortion. The vegetable oil alone or with Brassicaceae meal did not induce any phytotoxicity, whereas the mineral oil-based formulation reduced the fresh and dry weight of tomato plants (Rongai et al. 2009).

The blue green algae *Laminaria digitata* is the source of a laminarin, ß-1,2-glucan laminarin which elicits defense responses in grapevine cells and plants against *Botrytis cinerea* and *Plasmopara viticola*. The development of these pathogens in infected grapevine plants was effectively arrested by laminarin. The defense responses induced, include calcium influx, alkalinization of the extracellular medium, an oxidative burst, activation of two nitrogen-activated protein kinases, expression of ten defense-related genes with different kinetics and intensities, increases in chitinase and ß-1,3-glucanase activities and production of two phytoalexins, resveratrol and epsilon-viniferin. Application of laminarin on grapevine plants reduced infection by *B. cinerea* and *P. viticola* which could infect berries later, by about 55 and 75 % respectively. By reducing the pathogen population on grapevine plants, the incidence of the disease on berries was reduced considerably. The results indicated that activation of natural defense response using elicitors could be advantageously exploited as an effective strategy to protect grapevine plants against pathogens occurring at pre- and post-harvest stages (Aziz et al. 2003).

Essential oils, a group of plant-derived compound, contain a mixture of different terpenoid compounds and their oxygenated derivatives. These oils have a wide-spectrum of antifungal activity against plant and human pathogens. Clove oil, cinnamon oil and five essential oil components viz., citral, eugenol, geramol, limonene and linalool were evaluated for their antifungal activity against 14 plant pathogenic fungi. Cinnamon oil, citral and clove oil inhibited the spore germination of peanut (groundnut) pathogens, *Cercospora arachidicola, Phaeoisariopsis personata* (both causing leaf spot diseases) and *Puccinia arachidis* (causing rust disease) by >90 % at a concentration of 0.01 % (v/v). Clove oil (1 %) applied as foliar spray at 10 min before inoculation with *P. personata* reduced the severity of late leaf spot disease up to 58 %, when challenge inoculated with 10^4 conidia/ml. Seed treatment with the essential oils had no influence on the incidence of crown rot disease caused by *Aspergillus flavus*. However, soil amendment with 0.25 % clove oil and cinnamon oil reduced the pre-emergence rotting by 71 and 67 % and post-emergence wilting by 58 and 55 % respectively, compared with untreated controls. None of the treatments was effective, as compared with the fungicide thiram against the crown rot disease (Kishore et al. 2007). *Fusarium mangiferae* is a component of the mango malformation disease complex which causes considerable production losses. A concoction brewed from *Datura stramonium, Calotropis gigantea, Azadirachta indica* (neem) and cow manure (T1) and methanol extracts of these plant species (T2) were evaluated for their efficacy in suppressing the development of mango malformation disease. Satisfactory level of disease suppression was observed in mango trees sprayed with T1 treatment and it was more effective than T2 treatment, applied at bud break stage and again at fruit set stage. The treatments did not eliminate the pathogen completely, but the disease incidence was reduced, because of the direct inhibition of the pathogen development. The compost concoction had the potential for disease suppression and feasible to apply, because of the ease of preparation and inexpensive nature of the extracts (Usha et al. 2009).

The effectiveness of plant extracts in suppressing the development of bacterial plant pathogens has been assessed. The potential of the extracts of cloves of *Allium sativum* and fruits of *Ficus carica* was evaluated against *Pseudomonas syringae* pv. *tomato* (bacterial speck), *Xanthomonas vesicatoria* (bacterial spot) and *Clavibacter michiganensis* subsp. *michinganensis* infecting tomato. The extracts were effective in inhibiting the development of the bacterial pathogens at a population of 10^6 CFU/ml. Under in vivo conditions, the disease incidence was reduced by 58 and 30 % respectively by *Allium* and *Ficus* extracts. However, the plant extracts were less effective, compared with standard copper treatment (Balestra et al. 2009). The extracts and essential oils of the plant *Metasequoia glyptostroboides* were evaluated for their ability to suppress the development of the bacterial pathogens *Xanthomonas campestris* pv. *vesicatoria* (*Xcv*) and *X. oryzae* pv. *oryzae* (*Xoo*). The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values of oil and extracts ranged from 125–250 and 125–500 µg/ml and 250–1,000 and 250–2,000 µg/ml respectively. The essential oil was more effective in reducing the viability of the bacterial cells than the extracts. Furthermore, the oil displayed remarkable antibacterial activity, resulting in up to 65–100 % disease suppression under greenhouse conditions, using oriental melon (*Cucumis melo* var. *makuwa*) as assay host. The results indicated the potential of the oils of *M. glyptostroboides* as a source of natural antibacterials effective against bacterial plant pathogens (Bajpai et al. 2010). The effectiveness of the essential oil thymol from thyme and acibenzolar-S-methyl (ASM; Actigard 50WG), a systemic acquired resistance (SAR) inducer, was assessed by applying them alone or in combination against tomato bacterial wilt disease. Thymol was applied as a soil fumigant at 7 days before transplanting tomato seedlings in pathogen infested field. ASM was applied as foliar spray once in the greenhouse and five times after transplantation in the field. The combination of thymol and ASM significantly reduced the bacterial wilt disease in wilt-tolerant genotype 7514, compared with thymol alone in the year 2006. The combination of thymol and ASM not only reduced the disease incidence more effectively, but also increased the yield significantly over thymol or ASM alone and the untreated controls. The results showed that bacterial wilt disease could be effectively controlled in moderately resistant tomato cultivars using the combination of thymol and ASM (Hong et al. 2011).

The ability of bio-organic fertilizer product (BIO) containing organic fertilizer and *Paenibacillus polymyxa* (3×10^7 CFU/g) and *Trichoderma harzianum* (5×10^7 CFU/g) to suppress the development of Fusarium wilt disease of watermelon, caused by *Fusarium oxysporum* f.sp. *niveum* was determined under growth chamber and greenhouse conditions. The incidence of Fusarium wilt disease, at 27 and 63 days after treatment with bio-organic fertilizer at 0.5 %, was reduced respectively by 84.9 and 75.0 % in growth chamber and greenhouse experiments. The activities of antioxidases (catalase, superoxide dismutase and peroxidase) in treated watermelon leaves were enhanced by 39, 150 and 250 % respectively. In the roots, stems and leaves, the activity of ß-1,3-glucanase increased by 80, 1,140 and 100 % and that of chitinase increased by 240, 80 and 20 % respectively. The results

suggested that the elevated levels of defense-related enzymes consistent with induction of SAR may contribute to the suppression of watermelon Fusarium wilt disease (Wu et al. 2009). In a further investigation conducted under greenhouse and field conditions, best control was achieved by application of the bio-organic fertilizer product BIO into soil, during the nursery phase of watermelon seedlings, followed by a second application to Fusarium-infested soil at the time of transplantation of watermelon seedlings. The incidence of the disease was reduced by 60–100 % in pot experiment and by 59–73 % in the field experiment. Nursery application of BIO reduced the pathogen population significantly. The bacterial antagonist *P. polymyxa* effectively colonized the rhizosphere of watermelon and proliferated along the extending plant roots. Application of BIO might prove to be an effective strategy for the management of watermelon wilt disease (Ling et al. 2010).

Postharvest Diseases

Natural plant- and animal-derived compounds with antimicrobial properties have been shown to offer safe alternatives to synthetic fungicides applied for the control of postharvest diseases of fruits and vegetables. Furthermore, these naturally existing compounds are able to induce resistance to some of the postharvest diseases, as the biotic agents do.

A wide range of secondary metabolites produced by plants such as essential oils possess antimicrobial, allelopathic, antioxidant and bioregulatory properties (French 1985; Elkovich 1988). The presence of essential oils in plants belonging to the genera *Ocimum, Thymus, Origanum, Anethum, Eucalyptus, Foeniculum* and *Citrus* is well known. The fungicidal property of the essential oils was attributed to carvacrol present in thyme and origanum oil and to *p*-anisaldehyde formed due to oxidation of anethole present in anise oil (Caccioni and Guizzardi 1994). During ripening of fruits, volatile aromatic compounds with antifungal property are produced. Acetaldehyde formed during fruit ripening provided protection to apples, stone fruits and cherries against postharvest pathogens (Mattheis and Roberts 1993; Caccioni et al. 1994). The fungitoxicity of 12 essential oils (EOs) distilled from medicinal plants was assessed in vitro. The oil from *Thymus capitatus* showed strong antifungal activity against *Penicillium digitatum, P. italicum, B. cinerea* and *Alternaria citri* at 250 ppm. The fungitoxicity of *T. capitatus* EOs sprayed on healthy orange inoculated with *P. digitatum* was weak at atmospheric pressure, but in vacuum conditions conidial mortality on fruit exocarp was high (90–97 %). The efficacy of the EO was comparable to that of thiabendazole (TBZ) at 2,000 ppm concentration. The morphology of *P. digitatum* hyphae and conidia was markedly affected by the exposure to *T. capitatus* EO vapors as revealed by observations under the scanning electron microscope (SEM). Carvacrol was found to be the predominant compound, accounting for 81–83 % of EO vapors produced by *T. capitatus*, exhibiting antifungal activity (Arras and Usai 2001). The fungistatic/ fungicidal activity of hexanal and benzaldehyde, produced during etheric metabolism in stone fruits against *Monilinia laxa* and *Rhizopus stolonifer* was reported (Caccioni et al. 1995). The components of essential oils were

assessed for their efficacy in inhibiting the mycelial growth and conidial germination of *Neofabrae alba*, causing lenticels rot of apples. Carvacrol inhibited the mycelial growth of the pathogen to the maximum extent with ED_{95} of inhibition at a concentration of 17 ul/l. Carvacrol (25 µl/l) could reduce the fungal infection on artificially inoculated apples by 11.4 % only and it was less effective, compared with hot water treatment at 45 °C for 10 min (Neri et al. 2009).

The potential of essential oils (EOs) from thyme (*Thymus vulgaris*) and clove (*Syzygium aromaticum*) and massoilactone extracted from the bark of *Cryptocarya massoia* in inhibiting the mycelial growth of *Botrytis cinerea*, causing gray mold diseases of grapes and other fruits was determined. The sporulation in artificially induced necrotic lesion was markedly arrested by thyme (Thyme R) and massoilactone oils at 0.33 % which was not phytotoxic. The bunch rot and leaf colonization by *B. cinerea* was significantly suppressed by a single application of either EO at veraison. Spray applications of Thyme R oil at 0.33 % from flowering to harvest effectively controlled bunch rot disease. Senescence of floral tissues following treatments with Thyme R was also noticed (Walter et al. 2001). Fumigation of apricots (*Prunus armeniaca*) with 2 mg/l of thymol vapor reduced conidial germination of *Monilinia fructicola* to 2 %, as against 98 % germination on untreated fruits. Shrinkage and protoplast collapse of conidia due to treatment were revealed by observations under the light microscope. Disease incidence also was reduced significantly by thymol application. Fumigation with thymol resulted in increased fruit firmness, but phytotoxicity on fruits was also noted (Liu et al. 2002). The efficacy of other essential oils of plant origin has been assessed for the control of postharvest pathogens. Treatment of Embul banana with *Ocimum basilicum* oil (0.16 %, v/v) controlled crown rot and anthracnose caused by *Colletotrichum musae* effectively enabling bananas to be stored for up to 21 days at 13 ± 1 °C with no adverse effect on organoleptic properties. The effectiveness of this treatment compared well with the application of the fungicide benomyl. Hence, spraying emulsions of essential oil of *O. basilicum* prior to cool storage was recommended as a safe, cost-effective method with commercial potential for controlling postharvest diseases and extending storage life (Anthony et al. 2003). Cassia oil alone or in combination with magnesium sulfate were evaluated for the suppressive effect on the development of postharvest storage rots of cherry tomatoes caused by *Alternaria alternata*. Magnesium sulfate (0.25 %) enhanced the antifungal effect of cassia oil (200 µl/l) in vitro. Cassia oil at 500 µl/l in combination with $MgSO_4$ (0.25 %) significantly inhibited the development of *A. alternata* on cherry tomatoes stored at 20 °C for 3 days. These treatments did not affect quality parameters of cherry tomatoes. The results indicated that combination of cassia oil and $MgSO_4$ might provide more effective control of the storage rots of cherry tomatoes (Feng et al. 2008).

Fumigation with thymol (30 mg/l) of sweet cherries for 25 min before sealing in modified atmosphere packages, prior to cold storage, reduced the incidence of gray mold disease caused by *Botrytis cinerea* to 0.5 %, as against 36 % in untreated control. The treated fruits had lower total soluble solids, higher titrable acidity and greater stem browning than in untreated control fruits (Chu et al. 1999). Sweet cherries inoculated with conidia of *Monilinia fructicola*, causative agent of brown rot disease and *Penicillium expansum*, causing blue mold disease fumigated with thymol

for 10 min were stored at 10 °C. After storage for 13 days, sweet cherries were fumigated with thymol (10 mg/l). Brown rot disease incidence was significantly reduced, but there was no perceptible effect on blue mold disease development (Chu et al. 2001). Antifungal effect of the thyme oil on the germination of arthroconidia of sour rot pathogen *Geotrichum citri-auranti*, infecting citrus and germ tube elongation was determined. Thyme oil effectively arrested the mycelial growth of the pathogen, conidial germination and germ tube elongation. Ultra-structural changes, as revealed by light microscope, scanning electron microscope (SEM) and transmission electron microscope (TEM), included marked shriveling, crinkling of hyphae, plasma membrane disruption and mitochondrial disruption. Treatment with thyme oil of Satsuma mandarin oranges wounded and inoculated with *G. citri-auranti* reduced the infection from 78.1 % (in untreated citrus fruits) to 14 % after 5 days at 20 °C. No visible phytotoxic symptom was evident on citrus fruits, due to treatment with thymol. The results indicated the potential of thymol for protecting the citrus fruits under greenhouse and storage conditions (Liu et al. 2009). Another investigation evaluated the efficacy of thyme along with Mexican lime essential oils in suppressing the development of *Colletotrichum gloeosporioides* and *Rhizopus stolonifer*. Thyme was more effective than Mexican lime essential oils (MLEOs) in inhibiting the mycelial growth of the pathogens. The decay due to *C. gloeosporioides* and *R. stolonifer* was reduced by the thyme and MLEOs up to 50 and 40 % respectively, compared to 100 % infection in untreated control papaya fruits. In papaya immersed in mesquite gum emulsion formulated with both essential oils, the development of anthracnose disease induced by *C. gloeosporioides* was entirely suppressed (100 %) by both essential oils (Bosquez-Molina et al. 2010).

Harpins are proteins encoded by *hrp* genes present in the type III secretion system (TTSS) of plant bacterial pathogens and these proteins can induce resistance in susceptible plants. Application of harpin on two melon cultivars induced resistance to *Trichothecium roseum*. Harpin significantly reduced lesion diameter in inoculated fruit and did not cause any phytotoxicity to melons. Harpin did not show any fungicidal activity in vitro, but suppressed lesion diameter in treated and untreated halves of the same fruit, suggesting induction of local and systemic resistance. Efficacy of disease suppression persisted for 3–8 days, depending on the melon cultivar. Protection offered by harpin was associated with the activation of the enzymes peroxidase and chitinase (Yang et al. 2005). Polygalacturonases (PGs) are produced by fungal pathogens and they have an important role in the process of disease development (pathogenesis). A PG-inhibiting protein (PGIP) was extracted from healthy stored 'Cripps Pink' apple and its activity against the postharvest pathogen *Colletotrichum acutatum*, causing soft rot of apple fruits was determined in vitro and in vivo. In the in vitro tests, the inhibition of the endo-PG (EC 3.2.1.15) of *C. acutatum* determined by radial diffusion assay was over 62 % after 24 h, while in inoculated fruit the inhibition of endo-PG ranged from 33.9 to 54.4 % after 4 days at 20 °C. The PG inhibitor isolated from healthy skin of apple was a protein that could be denatured by boiling. The results indicated the possibility of using PGIPs for suppressing the development of storage rots caused by fungal pathogens that depend on polygalacturonases for invading host tissues (Gregori et al. 2008). Infection of vineyard

grapes by various fungal pathogens results in loss in yield and sensory properties of wines. The efficacy of a mixture of rhamnolipids (RLs) and lipodepsinonapeptide syringomycin E (SRE) resulted in 50 % killing of germinating conidia of *Aspergillus japonicas, Cladosporium cladosporioides, Curvularia brachyspora, Greeneria uvicola, Nigrospora sphaerica, Penicillium sclerotiorum, P. thomii* and *Trichoderma* sp. at concentrations between 0.75 and 3 μM. The SRE + RL mixture was more effective than SRE alone. The RLs alone did not inhibit the growth of germinating conidia of these fungi. This investigation indicated the broad fungicidal properties of SRE which when mixed with RLs could be more lethal to a wide range of grape-associated fungi at conidial germination stage (Takemoto et al. 2010).

Plant-derived substances have been used to protect the fruits against infection by fungal pathogens. The efficacy of *Aloe vera* gel applied at 250 ml/l, as a post-harvest treatment to table grape vineyards at 1 or 1 and 7 days before harvesting was assessed. The in vitro assays showed that inhibition of mycelial growth rate of *Penicillium digitatum* and *Botrytis cinerea*, causing fruit decay, increased with the concentration of *Aloe*, indicating direct adverse action of the gel against the pathogens. The percentage of rotted berries was significantly reduced in treated berries, compared with untreated control treatment. The respiration rate and weight loss, after storage for 35 days were significantly reduced in treated grape samples, while ripening parameters such as color and fruit firmness were significantly delayed. The results indicated that *A. vera* gel could be a promising candidate for preharvest treatment of grapes to maintain table quality, in addition to protection against fungal pathogens during postharvest storage (Castillo et al. 2010). The ability of carnauba wax (from *Copernicia cerifera*) to inhibit mycelial growth and spore germination of *Monilinia fructicola* and *Rhizopus stolonifer*, causing brown rot and Rhizopus rot diseases respectively in nectarines and plums was determined at different concentrations (1–4.5 %). The mycelial growth of both pathogens was completely arrested at concentrations above 2 %. There was no germination of spores for both fungi at all concentrations of carnauba wax. The germination of spores of *M. fructicola* was reduced by 50 % and that of *R. stolonifer* by 90 % on the surface of nectarines covered with 9 % carnauba wax. Protective application of 4.5 and 9.0 % carnauba wax significantly reduced incidence of both diseases in nectarines and plums. Application of carnauba wax was ineffective as a curative treatment (Gonçlaves et al. 2010). The efficacy of nine wild edible herbaceous species in suppressing the development of seven postharvest fungal pathogens was assessed. Extracts from *Sanguisorba minor* and *Orobanche crenata* showed highest antifungal activity in all experiments in vitro. Extract of *S. minor* entirely inhibited conidial germination *of Monilinia laxa, Penicillium digitatum, P. italicum* and *Aspergillus niger* and strongly inhibited the germination of spores of *Botrytis cincerea*. By employing the high performance liquid chromatography (HPLC) technique, phenolic composition of extracts of all plant species were determined. Several derivatives of caffeic acid, of flavones apigenin and luteolin and of flavonols kaempferol and quercetin were detected in the crude extracts of different species. A dose effect on conidial germination was evident with increase of antifungal activity, as the phenolic concentration increased. *S. minor* extract applied on

wounded apricots and nectarines completely inhibited the development of brown rot disease. *O. crenata* extract strongly reduced the infection by gray mold, brown rot and green mold on table grapes, apricots and nectarines respectively. Derivatives of caffeic acid and/ or flavonoids were considered to be responsible for the suppression of the postharvest diseases (Gatto et al. 2011).

The effectiveness of volatiles from grapevine cv. Isabella (*Vitis labrusca*) on the development of the gray mold pathogen *Botrytis cinerea* was assessed in vitro as well as in situ. The biological activities were quantified by using the closed Mariotte system. The Isabella volatiles inhibited the sporulation, whereas volatiles from Roditis grapes (*V. vinifera*) stimulated the sporulation. The in situ determination showed that the Isabella volatiles had antifungal properties against *B. cinerea* as reflected by the reduction in the amount of inoculum produced and pathogenicity. The antibiotic activity of volatiles was more pronounced at 21 °C indicating the possibility of using Isabella volatiles as biocontrol agents of *B. cinerea* (Kulakiotu et al. 2004a). The effects of volatiles from 'Isabella' grapes on the development of *B. cinerea* and gray mold disease incidence on kiwifruit cv. Hayward (*Actinidia deliciosa*) were determined. Reduction in both the inoculum density and the activity of the pathogen, following exposure to 'Isabella' volatiles resulted in the lower level of disease incidence. The inhibitory action of volatiles reached the peak at 21 °C, irrespective of the interval between wounding and inoculation of the kiwifruits with the pathogens (Kulakiotu et al. 2004b). The results indicated that the potential of the 'Isabella' volatiles could be exploited for effective control of gray mold disease of other fruit crops. Vapor-rich atmosphere is created for exerting negative influence on the development of postharvest pathogens. The effect of ethanol vapors on the development of gray mold disease caused by *Botrytis cinerea* on 'Chasselas' table grapes was assessed. At a concentration of 2 ml/kg of grapes, ethanol vapor was as effective as sulfur dioxide pads. No significant difference in sensory perception between control and treated grapes could be detected by consumer panels (Chervin et al. 2005). In a later study, preharvest application of a 16 % ethanol (EtOH) solution, containing 1 % calcium chloride reduced gray mold development in 'Chasselas' table grapes picked at a late harvest date. The losses due to rotten clusters were reduced from 15 % in controls to 5 % in treated grapes. Over 6 weeks of cold storage, the losses due to gray mold rots were reduced by 50 % in EtOH + $CaCl_2$ treated clusters, compared to untreated controls. Ethanol (2 %) alone was able to reduce gray mold growth significantly. As the reduction in disease incidence, without loss of fruit quality could be achieved by ethanol vapor, treatment with ethanol could be easily applied by the industry, since this technology is similar in effectiveness to sulfur dioxide treatment (Chervin et al. 2009).

The glucosinolates from the members of the family Cruciferaceae have been demonstrated to suppress the development of soilborne pathogens. The antifungal effect of six glucosinolates was evaluated against postharvest pathogen *Monilinia laxa*. Glucoraphenine isothiocyanate inhibited the development of *M. laxa* in inoculated pear cvs. Conference and Kasier to the maximum extent. Allyl-isothiocyanate as a volatile compound provided effective control of green mold disease in Conference pear inoculated with a TBZ-resistant strain of *M. laxa* (Mari and Guizzardi 1998). Kiwifruit cv. Hayward were inoculated with *B. cinerea*, causing gray mold disease

and treated with δ-decalactone, δ-dodecalactone or ß-ionone at 5 µl/fruit. The percentage decay due to *B. cinerea* was less in most of the treated fruits, compared with untreated controls, after storage for 18 weeks. Treatment with δ-dodecalactone provided a higher level of control and was comparable with that of the fungicide vinclozolin (Ward et al. 1998). Glucosinolates (GLs) are a large group of ß-thioglucoside N-hydroxysulfates found in many plants, particularly in cruciferous crops. Some products of enzymatic hydrolysis of glucosinolates exhibited high levels of antifungal activity against postharvest pathogens. The fungitoxicity of allyl-isothiocyanate (AITC) vapor against *Penicillium expansum*, causing blue mold of pear was assessed. Blue mold disease was suppressed by exposing the inoculated pear cvs. Conference and Kaiser for 24 h to an AITC-enriched atmosphere. At 5 mg/l, AITC showed high antifungicidal activity on pears inoculated with *P. expansum* (10^4 conidia/ml). No phytotoxic symptoms could be observed on treated pear fruits. Disease incidence was influenced by inoculum concentration and AITC treatment concentration. AITC treatments controlled a thiabendazole-resistant strain of *P. expansum*, reducing the incidence of blue mold by 90 % in both pear cultivars tested. Use of AITC produced from pure sinigrin or from *Brassica juncea* defatted meal might be an economically viable alternative to synthetic fungicides against *P. expanusm*. It would be desirable to apply sinigrin where TBZ-resistant srains are prevalent, as it is entirely harmless (Mari et al. 2002). The effectiveness of allyl isothiocyanate (AITC) and ethyl isothiocyanate (EITC) either alone or in combination was assessed for the control of *Penicillium expansum* and *Botrytis cinerea* infecting apples, as fumigants. A 3:1 ratio of AITC:EITC was more effective in reducing spore germination of both pathogens, than other combinations or fumigant alone. In in vivo trials, artificially inoculated apples exposed for 4 days to AITC, EITC and their combinations reduced the disease incidence by >85 %, after 3–4 days of apple incubation at 20 °C. The results showed the usefulness of isothiocyanates ITCs as fumigants for the effective control of postharvest mold diseases in apples (Wu et al. 2011).

Penicillium expansum, causing blue mold disease produces the mycotoxin patulin in apple fruits. The flavonoid quercetin was evaluated for its ability to suppress the development of blue mold disease. Quercetin was scarcely effective in inhibiting the growth of the pathogen in in vitro assays. However, when quercetin was applied on Golden Delicious apple fruits, significant reduction in blue mold rots was observed, suggesting the involvement of induction of resistance by quercetin. To verify the possibility of induced resistance, being the mechanism of biocontrol activity of quercetin, genes differentially expressed in quercetin-treated apples were identified by suppression subtractive hybridization (SSH). A pool of 88 unique gene transcripts was obtained. Several sequences revealed high similarities with different classes of PR-proteins (PR 8 and PR 10) or with proteins expressed under stress conditions. Other transcripts were similar to genes coding for proteins having a role in pathogen recognition and in signaling pathways. Quantitative real-time PCR assay indicated that 11 genes were up-regulated in freshly harvested apples. The results showed that quercetin might provide protection primarily as inducer of resistance in apples against the blue mold disease (Sanzani et al. 2010).

A wide range of plant species has been tested for the presence of compounds with antimicrobial property. However, the effectiveness of the plant-derived

compounds has been tested only under in vitro conditions for the inhibition of mycelial growth and/ or conidial germination. Only a few investigations indicated the effectiveness of natural products in controlling postharvest diseases in vivo. Aqueous extracts of *Vitex negundo* var. *purpurescens* (10 %) offered protection to carrots against Cladosporium rot (*Cladosporium oxysporum*), Fusarium rot (*Fusarium solani* f.sp. *radicicola*) and Geotrichum rot (*Geotrichum candidum*) (Prakasam et al. 2001). Extracts of *Solanum torvum* was found to be more effective in suppressing the development of banana anthracnose disease caused by *Colletotrichum musae*, compared with the fungicide benomyl (0.1 %). In addition, an increase in shelf life by 16–20 days over control was an added advantage of applying the extract of *S. torvum* for disease management (Thangavelu et al. 2004). The antifungal potential of cinnamon extract (CE), piper extract (PE) and garlic extract (GE) was assessed for the control of banana crown rot fungi *Colletotrichum musae*, *Fusarium* sp. and *Lasidiplodia theobromae*. CE completely inhibited conidial germination and mycelial growth of all three pathogens. Combinations of different extracts were evaluated for the efficacy in suppressing disease development. Crown rot disease intensity was scored during storage at 13 °C for 7 weeks. Disease development was the least (25 %) on CE-treated bananas after inoculation, compared to treatment with carbendazim (CBZ), but was higher when CE was applied before inoculation. Chitosan application significantly delayed fruit ripening as in terms of peel color, firmness, soluble solids and disease severity. CE exhibited no negative effects on fruit quality (Win et al. 2007).

Seed extracts and powders of *Pithecellobium dulce* (huamuchil) exhibited fungicidal and fungistatic activities against *Botrytis cinerea, Penicillium digitatum* and *Rhizopus stolonifer*. Partial purification indicated that triglycerol and triterpene saponins could be the compounds that were effective against these postharvest pathogens (Bautist-Banos et al. 200; Barrera-Necha et al. 2003). Ethanol extracts of garlic cloves (0.1 %) alone or in combination with sunflower cooking oil or fruit wax, were evaluated for their efficacy in controlling green and blue molds of grapefruit cvs. Valencia and Shamouti caused by *P. digitatum* and *P. italicum* respectively. Clove extracts, when combined with oil provided 100 % control of both green and blue mold diseases. The treatment (1 % extract + oil) was equally effective as the fungicide application (imazalil 500 ppm + quazatine 1,000 ppm) on Valencia oranges (Obagwu and Korsten 2003). The antifungal activity of grapefruit seed extract (GSE) was assessed against *Botrytis cinerea* infecting Redglobe table grapes. The GSE inhibited the spore germination and radial growth of *B. cinerea* effectively. The GSE and chitosan (an inducer of disease resistance) alone or in combination, significantly reduced postharvest fungal rot of the table grapes, compared with control, when challenged with *B. cinerea*. Differences in weight loss, color change, ripening, sensory quality and microorganism index between grapes treated with GSE and control fruit suggested that GSE had both antifungal and antioxidative activity. In addition, the sensory analyses showed that GSE treatment offered beneficial effects in terms of delaying rachis browning and dehydration and maintenance of the visual aspect of the berry without any detrimental effect on taste or flavor. GSE and chitosan appeared to have synergistic

effect in reducing postharvest fungal rot and maintaining the keeping quality of 'Redglobe' grapes (Xu et al. 2007a, b).

8.1.4 Products from Animal Sources

Among the products from animal sources, chitosan has been evaluated for its efficacy in controlling a wide range of crop diseases under field conditions and also diseases affecting harvested produce during storage. Chitosan, a deacetylated form of chitin is a natural biodegradable fiber (polymer) derived from crustaceous shells such as crabs and shrimps, whose main attributes correspond to its polycationic nature. Chitosan should be dissolved in an acid solution to activate its antimicrobial properties. Chitosan has fungicidal properties, resulting in direct suppression of fungal pathogens and also it can function as an inducer of resistance in plants against the pathogens by stimulating the host's defense systems. Chitosan has been reported to be effective against soilborne and airborne pathogens some of which may be seedborne also.

8.1.4.1 Soilborne Diseases

The biocontrol potential of different organic amendments applied to soil was assessed for the control of cotton Verticillium wilt disease caused by *Veticillium dahliae*. Among the organic amendments, crab shell (chitin) was the most effective in reducing the disease severity by 72 % in pots, whereas soybean stalk and alfalfa reduced the disease severity by 60 and 56 % respectively. Crab shell amendment stimulated the proliferation of antagonists to *V. dahliae* in the rhizosphere. The extracts of organic amendments including crab shell chitin were slightly inhibitory to *V. dahliae*. The changes in rhizosphere population of microorganisms induced by crab shells may contribute to the suppression of cotton Veriticillium wilt disease (Huang et al. 2006). Chitosan has been used as a soil amendment either alone or in combination with other treatments. In soilless tomato, root rot caused by *Fusarium oxysporum* f.sp. *lycopersici* (FORL) was suppressed by applying chitosan amendment (Lafontaine and Benhamou 1996). Chitosan has been applied as seed treatment for the control of *Fusarium oxysporum* in many host plant species (Rabea et al. 2003). Infections of plants in forest nurseries by *Fusarium acuminatum* and *Cylindrocladium floridanum* were drastically reduced by applying chitosan as soil amendment (Laflamme et al. 1999). The toxicity of chitosan to fungal root pathogen FORL was assessed by its effect on colony growth and spore germination. The tomato wilt pathogen was sensitive to treatment with chitosan. Ultrastructural studies using transmission electron microscopy (TEM) revealed marked alterations in the sensitive fungal cells. Confocal laser microscopy showed that Rhodamine-labeled chitosan entered rapidly into the conidia in an energy-dependent process. The results suggested that chitosan application might be combined

with biotic biocontrol agents to enhance the level of effectiveness of disease suppression (Palma-Guerrero et al. 2008).

Chitosan (CHN) oligomers induced defense responses in grapevine leaves, as evidenced by an accumulation of stilbene phytoalexins, trans- and cis- resveratrol, ε-viniferins and piceids and stimulation of chitinase and ß-1,3-glucanase activities. CHN applied in combination with copper sulfate ($CuSO_4$) strongly induced phytoalexins (resveratrol and their metabolites) accumulation. Induction of cis-resveratrol and cis-ε-viniferin was very responsive to $CuSO_4$. This activity of $CuSO_4$ in grapevine appears to have been recorded for the first time in this investigation. These two phytoalexins were considered to be important markers of induced resistance in grapevine. The eliciting ability of chitosan and $CuSO_4$ was associated with an induced resistance to gray mold disease caused by *B. cinerea* and downy mildew caused by *Plasmopara viticola* (Aziz et al. 2006). Pearl millet downy mildew pathogen *Sclerospora graminicola* is both soilborne and airborne. It is difficult to achieve satisfactory level of control of the disease, although several different kinds of strategies have been suggested. The efficacy of seed priming with chitosan in reducing the incidence of downy mildew disease was assessed. The combination of chitosan at 2.5 g/kg of seeds was found to be optimum and at this concentration, the seed germination percentage and seedling vigor were greater than that of untreated seeds. Chitosan did not have any adverse effect on sporulation and release of zoospores from sporangia. Seedlings grown from the treated seeds showed stimulation of activities of defense-related enzymes chitosanase and peroxidase, following challenge inoculation with the pathogen, compared with untreated controls. Assessment of effect of chitosan treatments under greenhouse and field conditions showed 79 and 76 % protection respectively against downy mildew disease (Manjunatha et al. 2008).

Oligochitosan, obtained by hydrolysis or degradation of chitosan, is not only water soluble, but also shown to be more effective than chitosan in eliciting multiple plant defense responses such as production of H_2O_2 and stimulation of defense-related enzymes. Antifungal activity of oligochitosan against several plant pathogens has been reported (Zhang and Tan 2003). The sensitivity of nine fungal pathogens to oligochitosan was assessed, based on the effects on mycelial growth. *Phytophthora capsici*, with no chitosan in cell wall, was the most sensitive to oligochitosan with EC_{50} and MIC values of 100 and 580 μg/ml respectively. Difference in the sensitivity of *Botrytis cinerea* to oligochitosan depended on the concentration. Oligochitosan at low concentrations inhibited different stages in the life cycle of *P. capsici*, including zoosporangia production, zoospore release, cystospore germination and induced rupture of released zoospores. Addition of ATP did not reduce the inhibition of zoospore release and zoospore rupture by oligochitosan. Treatment with oligochitosan also induced leakage of electrolytes from the mycelium of *P. capsici*, indicating oligochitosan also may act on the cell membrane by upsetting osmotic pressure, like chitosan. Observations with transmission electron microscope revealed only slight distortion of some hyphal cell walls of *P. capsici* in the presence of oligochitosan (10 μg/ml). The most profound structural alteration of the hyphae treated with oligochitosan was the disruption of endomembrane system, especially vacuole and secretory vesicles, such as plasmalemmasomes in hyphal tips. The

polycationic nature of oligochitosan might contribute to its antifungal and multiple modes of action, including induction of disease resistance in treated plants (Xu et al. 2007a, b). Application of oligochitosan was shown to induce resistance to *Tobacco mosaic virus* (TMV) in transgenic tobacco var. Samsun NN. Protein kinase (OPIK) gene induced by oligochitosan in the transgenic tobacco plants was inhibited by antisense transformation. Wild-type (WT) tobacco showed longer lesion appearance time, higher lesion inhibition ratio and smaller average final lesion, compared to the transgenic (TG) line. The phenylalanine ammonia lyase (PAL) and peroxidase (PO) activities were positively related to OPIK, but not to polyphenol oxidase (PPO) activity. The results suggested that OPIK could function in the development of resistance to biotic stresses, possibly via PR proteins (Yafei et al. 2009).

8.1.4.2 Airborne Diseases

Chitosan has been applied as foliar spray to suppress the development and spread of airborne microbial plant pathogens infecting aerial plant parts. Increase in yield and tuber quality of micro-propagated greenhouse-grown potatoes has been reported (Rabea et al. 2003; Kowalski et al. 2006). Application of chitosan as foliar spray on barley resulted in significant reduction in local and systemic infection of barley powdery mildew pathogen *Blumeria graminis* f.sp. *hordei* (Faoro et al. 2008). Chitosan application on tomato enhanced the resistance to *Cladosporium fulvum*. The fungal effectors, cysteine-rich proteins, secreted by the pathogen have a role in its virulence. The proteins Avr2 and Avr4 were found to be inhibitors of plant cysteine proteases and help protect chitin and the integrity of fungal cell walls against plant chitinases. It may be possible that application of chitosan against this fungal pathogen might interfere with the process of recognition of the effectors and their cognate counterparts (Stergiopoulos and de Wit 2009).

Foliar application of mixtures of cow's milk and water were found to be effective against powdery mildew infecting greenhouse-grown zucchini plants caused by *Podosphaera xanthii*. On the other hand, under field conditions the efficacy of these sprays against powdery mildew on field-grown pumpkins was highly variable. Milk-based treatments were about 50–70 % effective in reducing foliar symptoms and postharvest fruit rot and 40–50 % as effective in increasing marketable yield as the conventional chemical control. Skim milk was not effective as the whole milk, especially during rainy seasons. The milk treatments were consistently more effective than baking soda (1 % $NaHCO_3$), indicating that milk treatment's potential was not just based on its ability to buffer the pH of leaf surfaces of pumpkins (Ferrandino and Smith 2007).

8.1.4.3 Postharvest Diseases

Chitosan forms films that can be used to coat the surface of fruits and vegetables. Chitosan has been applied to suppress the development of many pre- and postharvest diseases on various horticultural commodities. In addition, it has fungicidal

properties and when applied as coating, fruit ripening may be delayed, consequently increasing the shelf life of fruits and vegetables. Furthermore, chitosan has been shown to be an inducer of resistance to postharvest diseases.

Strawberry cv. Seascape plants were sprayed twice at 10 days interval with chitosan (6 g/l) and harvested fruits were challenged with gray mold pathogen *Botrytis cinerea* and stored at 3 or 13 °C. Chitosan reduced postharvest fungal rot significantly and maintained the keeping quality of fruits, compared with water-sprayed controls. The incidence of decay was reduced in proportion to the concentration of chitosan applied and increased with increased storage time and temperature (Reddy et al. 2000b). Preharvest sprays of chitosan three times, reduced rots due to *B. cinerea* and *Rhizopus stolonifer* with greatest reductions observed at 1 % concentration. Likewise, dipping strawberries in chitosan (1 %) for 3–4 s resulted in maximum protection against *B. cinerea*, where incidence of *R. stolonifer* was negligible (Romanazzi et al. 2000). Different acids were evaluated for their ability to activate the antimicrobial properties and resistance-inducing capability of chitosan which was found to be soluble in 1 % solutions of acetic, L-ascorbic, formic, L-glutamic, hydrocholoric, maleic, malic, phosphoric and succinic acid. Chitosan dissolved in acetic acid had similar effectiveness as the water-soluble glycol-chitosan in controlling postharvest gray mold of table grapes caused by *B. cinerea* and it was found to be less expensive. Table grape berries were immersed in chitosan solutions at pH 5.6 for 10 s. Chitosan acetate or formate were the most effective to control decay on grape berries at 15 ± 1 °C, whereas on those stored at 0.5 ± 1 °C, the best performance was observed with chitosan lactate or acetate. The activity of chitosan film was based on its antifungal and eliciting properties rather than acting as a mechanical barrier. Chitosan acetate had the lowest viscosity, compared with lactate or formate. Low viscosity is preferred, when the applications are done by spraying. Chitosan acetate was the most effective treatment reducing gray mold at cold and ambient storage temperature decreased CO_2 and O_2 exchange and did not cause injury to grape berries (Romanazzi et al. 2009).

The effectiveness of chitosan with different molecular weights on the suppression of *Botrytis cinerea*, infecting tomato fruits was determined in vitro and in vivo. The antifungal activity of chitosan increased with decrease in its molecular weight in in vitro assays. Chitosan treatments significantly reduced fungal decay in the greenhouse and all compounds, at concentrations of 2,000 and 4,000 mg/l, completely controlled the pathogen development in wound-inoculated fruit. Chitosan with MW of 5.7×10^4 g/mol was the most effective compound among those tested. Further, high chitosan concentrations correlated with low disease incidence, regardless of storage conditions. In addition to direct antifungal activity, chitosan also had the potential for eliciting defense markers, including total soluble phenolic compounds, polyphenol oxidase (PPO) activity and total protein content. Chitosan application decreased the PPO activity and enhanced the total protein and phenolic compounds in wounded tomato fruit (Badawy and Rabea 2009). The effects of chitosan applied as preharvest treatment were determined on the development of *Botrytis cinerea*. Chitosan inhibited the mycelial growth of *B. cinerea* in liquid

culture and suppressed gray mold development on detached grapevine leaves and bunch rot in commercial winegrapes. However, conidia of *B. cinerea* treated with chitosan were able to infect detached leaves of Chardonnay grapes, indicating that the pathogenicity was not affected, even after incubation for 24 h in chitosan at 10 g/l. But when added after conidial germination, chitosan inhibited further growth of *B. cinerea* and induced morphological changes, suggestive of possible eradicative effect of chitosan. As a foliar treatment, chitosan protected detached Chardonnay leaves against *B. cinerea*. In vivo experiments in vineyards showed that Chardonnay winegrapes exhibited 7.4 % Botrytis bunch rot severity, after treatment with an integrated program that included chitosan sprays. The results indicated that suppression of *B. cinerea* infection in winegrapes might involve direct and indirect modes of action (Reglinski et al. 2010). In another investigation, effect of chitosan treatment of grapes on *B. cinerea* was assessed. Aqueous solution of chitosan and acibenzolar-S-methyl (ASM) inhibited the mycelial growth of *B. cinerea*. Single grapes were treated with different concentrations of these compounds and stored at 4 and 24 °C. After 4 days at 24 °C, all concentrations of chitosan and ASM significantly reduced the growth of *B. cinerea*. ASM was more effective than chitosan. The results indicated that chitosan and ASM could act on the pathogen directly and also confer resistance in grapes against gray mold disease (Muñoz and Moret 2010).

An antifungal peptide designated CgPep33 was isolated from the enzymatic hydrolysates from the Pacific Oyster (*Crassostrea gigas*) and purified by DEAE Sephadex A-25 ion exchange, Sephadex G-25 gel filtration and high-performance liquid chromatography technique. This peptide was highly inhibitory to the mycelial growth of *Botrytis cinerea* and it inhibited the growth by 50 % at a concentration of 20–40 μg/ml and by 100 % at 120 μg/ml. In CgPep33-treated strawberries, the disease incidence and lesion diameter were 76 % and 6.3 mm respectively lower than that of the untreated fruits on the 3rd day after challenge inoculation. The results indicated that the peptide from oyster might provide an alternative to fungicides for managing the postharvest diseases of strawberry (Zeng et al. 2007). Treatment of fruits with chitosan has been shown to be effective in reducing the incidence of postharvest diseases caused by other fungal pathogens. Stem scar application of chitosan (400 μl of a 10 g/l solution) to tomatoes stored at 20 °C provided protection against black mold rot caused by *Alternaria alternata* (Reddy et al. 2000a). Chitosan treatment (5 and 10 mg/ml) reduced the incidence of brown rot disease of peaches caused by *Monilinia fructicola* significantly and also delayed the disease development, compared with untreated controls. Treated peaches were firmer and had higher titrable acidity and vitamin C content than the fungicide procloraz-treated or control peaches. Chitosan was considered to have the potential for the control of brown rot, preservation of the valuable attributes and prolonging the shelf-life of the postharvest peaches (Li and Yu 2001). Chitosan treatment reduced the infection of anthracnose disease caused by *Colletotrichum* sp. on papaya. Chitosan exhibited antifungal activity and also stimulated the defense response on papaya peel by enhancing the chitinase and ß-1,3-glucanase activities. The antifungal activity could be attributed to the induction of elicitation activity due to the defense

enzymes. Further, chitosan formed a semipermeable coating around the fruit and extended the storage life of papaya by reducing the rate of respiration and delaying ripening (Hewajulige et al. 2009).

Rhizopus stolonifer causes destructive storage rots of tomato accounting for high economic losses, whereas *Escherichia coli* may be responsible for life-threatening diseases. Chitosan-based formulations (1 %) mixed with beeswax (0.1 %), oleic acid (1.0 %) and lime or thyme essential oil (0.1 %) were evaluated for their efficacy in suppressing the development of the fungal and bacterial pathogens. Tomatoes at three different maturity stages and at two storage temperatures (12 and 25 °C) were used for the experiments. Control fruits were dipped in water only. Fruits were wounded, coated and inoculated for all treatments. After application of chitosan-based edible coatings, 20 µl of *R. stolonifer* spore suspension at 10^5 spores/ml and 35 µl of bacterial suspension of *E. coli* DH5α at 10^5 CFU/µl were dispensed over the wounded surface in in vitro and semi-commercial level. Overall, the protective effect of coating applications was better against *E. coli* DH5α than against *R. stolonifer*. The most effective treatment was chitosan (1 %) + bees wax (1 %) + lime essential oil (0.1 %) and no growth of *R. stolonifer* and *E. coli* occurred in this treatment under in vitro conditions. Observations under scanning electron microscope (SEM) showed distorted mycelia and absence of sporangia-spore production. Further, the development of *E. coli* was also arrested. In the semi-commercial level, the combination completely controlled *E. coli* at both storage temperatures. The results indicated that application of chitosan-based edible coating containing beeswax and lime essential oil could be an environment-friendly alternative for the control of the important pathogens (Ramos-García et al. 2012).

The efficacy of a bio-elicitor (FES) formulated from *Fusarium oxysporum* grown on potato dextrose agar (PDA) amended with netted melon skin was assessed for suppressing the development Fusarium rot disease in netted melon fruit. The bio-elicitor was applied on netted melon and inoculated with *F. oxysporum* (FES + IN). Control (C), untreated and inoculated with *F. oxysporum* (IN) and treated with FES were the other treatments maintained. All fruits of different treatments were stored for 8 days at 20 °C with 90–92 % RH. Samples from different treatments taken at every 2 days interval were used to determine disease index percentage (DI), changes in phenolic compounds, changes in PAL activity, chitinase activity (ChA) and ß-1,3-glucanase activity (GA). The FES-treated fruits had significantly lower level of disease severity, when inoculated with the pathogen. Further, fruits treated with FES, whether inoculated with the pathogen or not, showed highest increase in the contents of phenolic acids, in addition to enhancement of activities of PAL and CHA, after 4–6 days of storage. No variations in ß-1,3-glucanase activity could be observed, following treatment with FES and challenge inoculation with the pathogen. The results indicated that the bio-elicitor FES had the potential for suppressing the Fusarium rot of netted melon by inducing resistance to the disease (Sánchez-Estrada et al. 2009).

The efficacy of chitosan, *Streptomyces melanosporofaciens* strain EF76 and talc alone or in combination was assessed for suppressing the development of potato common scab disease. Combination of the geldanamycin-producing *S.*

melanosporofaciens strain EF76 and chitosan was the most effective in reducing the common scab disease incidence in the greenhouse assessment. But field application of EF76 did not allow the selection of geldanamycin-resistant actinomycetes (GRA) in the bulk soil during the potato growing season. The number of GRA on harvested potato tubers was, however, significantly higher in treatments that contained chitosan than in other treatments, suggesting that chitosan might promote the establishment of the antagonistic actinomycetes on progeny tubers. The results revealed the effectiveness of the combined application of the strain EF76 and chitosan for the suppression of development of potato common scab disease (Prévost et al. 2006). Chitosans available as commercial products of low molecular weight (Clmw) and medium molecular weight (Cmmw) were evaluated for their efficacy in suppressing the development of tomato bacterial spot disease caused by *Xanthomonas gardneri*. Chitosans provided protection of up to 56 % with best results from Clmw at 3 mg/ml applied at 3 days prior to challenge inoculation with the pathogen. The spectrophotometric profile of tomato plants treated with Clmw showed an increase of absorbance between wavelengths of 280 and 300 nm, indicating that the polysaccharide may be involved in the synthesis of different compounds as a response to *X. gardneri*. Analysis of total phenolic compounds and flavonoids corroborated the results of spectrophotometric scanning, revealing a significant increase in those metabolites at 3 days after inoculation with *X. gardneri*. The results indicated that chitosan could induce defense mechanisms of treated plants resulting in suppression of bacterial spot disease (Coqueiro et al. 2011).

Chitosan (CHT) application has been shown to ameliorate the adverse effects of virus infection in plants. The antiviral activity of chitosan was assessed against *Tobacco necrosis virus* (TNV), infecting French bean (*Phaseolus vulgaris*). CHT treatment elicited both callose apposition and abscisic acid (ABA) accumulation in leaf tissues at 12 and 24 h after application respectively and induced a high level of resistance against TNV. Treatment with the ABA- inhibitor, nordihydroguaiaretic acid (NDGA), before CHT application, reduced callose deposition and resistance of plants to TNV, indicating the involvement of ABA in induction of resistance by CHT. ABA by itself could induce significant level of resistance. The results indicated the enhancement of ABA synthesis induced by chitosan plays an important role in increasing callose deposition, a factor that is involved in the buildup of resistance to virus disease (Iriti and Faoro 2008).

8.2 Synthetic Organic Compounds as Biological Control Agents

Several organic compounds have been tested for their ability to suppress disease development and most of them have been shown to act on pathogen indirectly by stimulating the defense mechanisms of the host plants. These compounds induce the

same spectrum of systemic acquired resistance (SAR) genes to levels comparable with those induced by the biotic inducers of disease resistance (Métraux et al. 1991; Ward et al. 1991). Several organic compounds such as, salicylic acid (SA), acibenzolar-*S*-methyl (ASM) and ß-amino butyric acid (BABA) have been investigated for their ability to induce systemic resistance in plants against diseases caused by microbial pathogens.

8.2.1 Salicylic Acid

The model plant *Arabidopsis thaliana* has been used to gain an insight into the molecular mechanisms underlying SAR. The ability to accumulate salicylic acid (SA) is indispensable for the development of SAR, since *Arabidopsis* SA biosynthesis mutants *SA induction deficient 1* and *2* (*sid 1* and *sid 2*) and transgenic plants expressing the SA-degrading enzyme NahG are SAR defective (Wildermuth et al. 2001; Newrath et al. 2002). The SAR regulatory protein nonexpressor of PR genes (*NPR1*) is activated by SA through redox changes. These changes, in turn, drive systemic expression of antimicrobial PR proteins and facilitate their secretion by upregulating protein secretory pathway genes (Mou et al. 2003; Wang et al. 2005). Further, the long-distance signaling in *Arabidopsis* appears to depend on a peptide signal system mediated by the Asp protease constitutive disease resistance (CDR1) (Xia et al. 2004). The reactive oxygen species (ROS)-mediated systemic signaling network is known to contribute to SAR. In addition, environmental conditions and plant developmental plasticity are also able to significantly influence SAR development (Zeier et al. 2004; Zeier 2005). SA-induced defense expression via nonexpressor of PR genes-1 (*NPR1*), a key mediator of SAR is observed in both dicotyledons and monocotyledons (Dong 2004). In *Arabidopsis*, AtNPR1 is present predominantly in a monomeric form that is able to translocate into the nucleus, where it activates defense genes expression through interaction with transcription factors (Subramaniam et al. 2001; Zhang et al. 2003).

Exogenous application of SA triggers the translocation of NPR1 into the plant cell nucleus. After entry into the nucleus, NPR1 interacts with TGA expression factors to mediate gene expression. The expression of a large number of genes is altered, following SA treatment (Glazebrook et al. 2003; Yao et al. 2003). The PR genes are the most studied NPR1 targets and they encode small secreted or vacuole-targeted proteins with antimicrobial properties. By employing Affymetrix GeneChips (8,200 genes), putative NPR1 target genes were identified by comparing transcriptional profiles of *npr1* and *npr1/35S::NPR1-GR* that were treated with SA and then the translation inhibitor cycloheximide (Chx). The induced genes were classified into two groups, according to their known deduced functions. One group contains genes involved in defense, including several PR genes. Another group encloses genes encoding, members of the protein secretory pathway, most of which are endoplasmic reticulum (ER)-localized proteins. These secretion-related genes include those encoding the Sec61 translocon complex. NPR1 is also able to regulate many genes encoding ER-resident chaperones, such as BiP2 and glucose-regulated protein 94

(GRP94). Induction of many genes by SA via the endogenous NPR1 was confirmed by RNA blot analysis, real-time RT-PCR assay (Wang et al. 2005).

8.2.1.1 Fungal Diseases

Exogenous application of salicylic acid (SA) to pea leaves induced systemic resistance to the powdery mildew disease caused by *Erysiphe pisi* (Frey and Carver 1998). A positive relationship between induction of SAR by SA and 2,6-dichloroisonicotinic acid (INA) and systemic induction of several genes in *Arabidopsis thaliana* (At) was observed (Dempsey et al. 1993; Mauch-Mani and Slusarenko 1996). The interaction between *Fusarium oxysporum* (*Fo*) and *Arabidopsis thaliana* (*At*) was investigated to have a better understanding of the nature of host defenses that might be effective against Fusarium wilt pathogen. The expression of salicylate- and jasmonate-responsive defence genes in *Fo*-challenged roots of *At* plants as well as in the roots of plants whose leaves were treated with salicylate or jasmonate was analyzed. The genes (*PR1. PDF1.2* and *CHIB*) encoding proteins with defense functions or transcription factors (*AtERF1*, *AtETF2*, *AtERF4* and *AtMYC2*) known to positively or negatively regulate defenses against *Fo* were not activated in *Fo*-inoculated roots. In contrast, the jasmonate-responsive gene PDF1.2 was induced in the leaves of plants whose roots were challenged with *Fo*, but salicylate-responsive PR1 gene was not induced in the leaves of inoculated plants. Exogenous SA treatment prior to inoculation, however, activated *PR1* and *BGL2* defense gene expression in leaves and provided increased *Fo* resistance, as evidenced by foliar necrosis and plant death. Exogenous SA treatment of the foliar tissues did not activate defense gene expression in the roots of treated plants. The finding suggested that salicylate-dependent defenses may function in foliar tissues to reduce the development of pathogen-induced wilting and necrosis. Although jasmonate application induced defense gene expression in leaves, it did not enhance the level of resistance to *Fo*. The results suggested that genetic manipulation of plant defence signaling pathways could be exploited for protection of plants against Fusarium wilt diseases (Edgar et al. 2006).

If plants are conditioned by pretreatment with inducers of SAR, the systemically protected plants may be able to resist the pathogen more effectively. Pretreatment of parsley cell cultures with SA or INA markedly enhanced the ability of the cultures to produce coumarins, following treatment with an elicitor from *Phytophthora megasperma* f.sp. *glycinea* (Kauss et al. 1992). Application of INA on green bean (*Phaseolus vulgaris*) resulted in marked increase in the activities of chitinase and ß-1,3-glucanase and accumulation of SA (Dann et al. 1996). Treatment of *Arabidopsis* with INA, followed by challenge inoculation with a virulent race of *Phytophthora parasitica* resulted in lignification of intercellular hyphae and consequent retarded development of pathogen (Sticher et al. 1997). INA may induce defense response prior to infection or potentiate defense responses, following infection. INA upon application, conditioned sugar beet plants, leading to production of PR-proteins at a faster rate, when infected by the fungal pathogen *Cercospora beticola* (Nielsen et al. 1994). Similar effects were observed in cucumber-*Colletotrichum*

lagenarium pathosystems (Siegrist et al. 1994). The effect of exogenous application of salicylic acid (SA) on host resistance to rice blast pathogen *Magnaporthe grisea* was investigated. Blast lesion formation on rice leaves applied with SA (8 mM) was suppressed. Activities of peroxidase and polyphenol oxidase were stimulated by SA application. Chemical analysis of crude leaf extracts detected four rice phytoalexins, oryzalexin A, C and F and momilactone A, following root application of SA. The hydrolysates of conjugated antifungal compounds from SA-treated rice plants caused greater inhibition of conidial germination of *M. griesea* than those from control plants. The results indicated that exogenous SA application could increase the concentration of products involved in resistance and also antifungal compounds which might arrest pathogen invasion (Daw et al. 2008).

Systemic resistance to the cotton leaf spot pathogen, *Alternaria macrospora* was induced in cotton leaves, following treatment of the cotyledons with conidia of *A. macrospora* or a formulation containing 2,6-dichloroisonicotinic acid (INA) (Brock et al. 1994). In a later study, a wettable powder (WP) formulation providing 5–25 µg/ml of INA and 15–75 µg/ml of WP applied to cotton cotyledons significantly enhanced the resistance of the next two true leaves to challenge inoculation with *A. macrospora*. Each treatment enhanced the activities of ß-1,3-glucanase in unchallenged leaf and stem tissues of cotton. Each of the components of the wettable powder without INA applied to cotyledons increased the enzymatic activities in the next leaves. Individual components, as suspensions of silicic acid and kaolin and solutions of the detergent Attisol II, the wetting agent Ultravon W300 and pure INA-applied to cotyledons increased the resistance of the next leaves to *A. macrospora*. The elevated activities of ß-1,3-glucanase in the first and second leaves of cotton by treatment with INA indicated that systemic resistance could be induced in cotton by treatment with INA or conidia of *A. macrospora* (Colson-Hanks and Deverall 2000). Exogenous application of salicylic acid (SA) and benzothiadiazole (BTH) solutions induced systemic acquired resistance (SAR) in faba bean against rust disease (*Uromyces viciae-fabae*) and Ascochyta blight (*Ascochyta fabae*). Under field conditions, incidence of rust and Ascochyta blight diseases were significantly reduced by treatment with SA and BTH on susceptible accessions. Moderately resistant accessions became immune to Ascochyta blight with BTH treatment and showed lower disease severity for rust, after SA or BTH treatment. No effect could be seen on resistant accessions due to treatment with the resistance inducers (Sillero et al. 2012).

Resistance of asparagus (*Asparagus officinalis*) was induced by nonpathogenic *Fusarium oxysporum* (*npFo*) to *Fusarium oxysporum* f.sp. *asparagi* (*Foa*), through activation of defense-related responses. To elucidate the putative *npFo*-mediated defense pathways, the effect of salicylic acid (SA) was investigated, using a split-root system of asparagus, where one half of the seedling root system was drenched with SA and the activation of defense responses was measured subsequently on the remaining roots. Treatment of asparagus roots with SA by soil drench (20 mg/l) at 2 days before challenge inoculation with *Foa*, was sufficient to protect the plants systemically. Diphenyleneiodonium chloride (DPI), an SA synthesis inhibitor, prevented the induction of *Fusarium* resistance by *npFo*. In vitro assays showed that SA did not exert any direct inhibitory effect on germination of conidia and mycelial

growth of *Foa*. SA-treated plants showed enhanced systemic resistance, with significant reduction in disease severity of the roots inoculated with *Foa*, compared with untreated control plants. SA activated peroxidase and phenylalanine ammonia lyase, as well as lignification upon *Foa* infection, in a manner similar to that observed with *npFo* pretreatment. The pretreatment of asparagus roots with SA or *npFo* primed the plants for a potential defense response to *Foa*. The results suggested the involvement of an SA-dependent systemic acquired resistance (SAR) pathway in the *npFo*-induced potential defense responses and resistance to *Foa* in asparagus (He and Wolyn 2005). The ability of salicylic acid to suppress tomato Fusarium wilt disease caused by *Fusarium oxysporum* f.sp. *lycopersici* (FOL), by inducing resistance in tomato, was determined. Salicylic acid (SA) at a concentration of 200 mM was applied through root feeding and foliar spray on tomato plants. Endogenous accumulation of free SA in tomato roots was detected by high performance liquid chromatography (HPLC) technique and its identity was confirmed by LC-MS/MS analysis. The endogenous level of SA in the roots increased at 168 h after application to about ten times higher than in untreated control plants. Similar increase in SA content in leaves also occurred, when applied as foliar spray. The activities of PAL and PO were stimulated, following application of SA through roots or foliage. The SA-treated tomato plants challenged with FOL showed significant reduction in the intensity of vascular browning, leaf yellowing and wilting. However, the mycelial growth of FOL was significantly affected by SA in in vitro tests. The results indicated that SA-induced systemic acquired resistance (SAR) might be responsible for the suppression of wilt disease development in tomato (Mandal et al. 2009).

The responsiveness of the defense-related PR-genes to the exogenous application of salicylic acid (SA), methyl jasmonate (MeJA), biologically active derivative of jasmonic acid (JA) was studied in wheat seedlings on infection at 1, 2, or 3 weeks, following emergence. The transcription levels of defense-related genes were assessed. Application of SA or MeJA coordinately activated transcripts of different groups of defense-related proteins and reduced infection by common bunt disease of wheat caused by *Tilletia laevis*. The transcripts of chi1, chi3, chi4, PR-1, PR-1.2, Glu1 and a lipase were up-regulated to MeJA treatment and their expression was potentiated by infection by *T. laevis*. The highest transcript levels were associated with the 3-week stage. Transcripts of Glu2 responded almost exclusively to SA, were not potentiated by infection and the highest expression was observed in the 3-week seedling stage. On the other hand, transcripts of ns-LTP-1, ns-LTP-2 and Glu3 were up-regulated in response to both SA and MeJA, not potentiated by infection and the highest transcripts levels were recorded in the 1-week seedling stage (Lu et al. 2006). Perception of both general and specific pathogen-associated molecules triggers defense responses in plants via signal transduction cascades and transcriptional activation of numerous genes. In chickpea (*Cicer arietinum*), putative genes potentially involved in defense responses, including the rapid synthesis of PR-proteins, presence of an oxidative burst and synthesis of putative cell wall-strengthening proteins and antimicrobial proteins have been identified (Coram and Pang 2006). The responses of three chickpea genotypes treated with defense signaling compounds SA, MeJA and aminocyclopropane carboxylic acid (ACC) to *Ascochyta*

rabiei, were investigated, using microarray technology. The observations were validated by quantitative RT-PCR assay. Of the 715 experimental microarray features, 425 (59.4 %) were differentially expressed (DE) at least in one condition. According to treatment applied, 69 , 15.8 and 57.6 % were differentially expressed respectively by ACC, MeJA and SA. The coregulation of transcripts between treatments for each genotype with varying levels of disease resistance showed large proportions of transcripts were independently regulated by ACC, MeJA or SA. Of the coregulated transcripts, the ACC-SA category contained the most for all genotypes lending support to the suggestion of cross-talk and overlap between signaling pathways (Salzman et al. 2005; Jalali et al. 2006).

The effectiveness of protection provided by salicylic acid (100 μM), chitosan (0.02 %) and the nutrient chelate product Alexin (1 %) to greenhouse carrot plants against the necrotrophic fungal pathogens *Alternaria radicina* and *Botrytis cinerea* was assessed. The plants were challenged by pathogen inoculation at 10 h after treatment with the chemicals. Significant reduction in disease development was observed at 10 days after treatment, chitosan providing the best protection against the pathogens and the least effective was salicylic acid treatment. Additional application reduced the disease severity further down. Treated plants showed elevated transient levels of PR protein PR-1, lipid transfer protein (LTP), chalcone synthase, nonexpressor of PR-1 and PR-5 genes, compared with control plants, when assayed at 10–70 h after treatment. The activities of PO, PPO, PAL, chitinase, ß-1,3-glucanase and lipoxygenase was significantly increased in elicitor-treated plants, compared with untreated control plants. Protein extracts from elicitor-treated plants exhibited inhibition of the spore germination and germ tube elongation (30–45 %). Accumulation of total phenolics, 6-methoxymellin and H_2O_2 in treated plants was a distinctive change observed. As all the three chemicals induced similar changes in treated plants, it is likely that the elicitors, chitosan and Alexin might activate the salicylate pathway, resulting in the induction of defense genes, enzymes, phytoalexins and phenolics which could contribute to the suppression of development of the fungal pathogens infecting carrot (Jayaraj et al. 2009).

The possibility of eliciting resistance response in postharvest produce by applying inducers of disease resistance has been demonstrated. The efficacy of the chemical elicitor 5-chloro-salicylic acid (5CSA) and the fungal antagonist, *Ulocladium oudemansii* (*Uo*) in suppressing the development of the gray mold pathogen *Botrytis cinerea*, infecting Chardonnay grapevines was compared, when applied alone or in combination under greenhouse conditions. Treatment with 5CSA (1 mM) induced resistance in potted plants, as reflected by reduction in lesion diameter. Reduction in lesion size was the greatest in plants that were treated with 5CSA at 7 days, before challenge inoculation with *B. cinerea*. 5CSA did not significantly reduce the conidial germination and germ tube vigor of the pathogen. The chemical elicitor and fungal antagonist were tested for their interactive behavior. Application of 5CSA, *Uo* and 5CSA + *Uo*, every 10–14 days from flowering until 1 week preharvest significantly reduced Botrytis bunch rot severity in the greenhouse. Levels of disease suppression by the treatments did not significantly differ, but they were significantly more effective in reducing bunch rot, compared with untreated controls. After 14 days of postharvest

incubation in humidity chambers, Botrytis severity increased to 83 % on untreated bunches, compared with 37–41 % on those treated with 5CSA and *Uo* alone. The combination of 5CSA and *Uo* proved to be significantly more effective than each component alone with bunch rot severity being less than 15.2 %. The results showed that the elicitor and the antagonist had potential for effective control of Botrytis bunch rot disease of grapevines (Reglinski et al. 2005). The level of protection provided by salicylic acid (SA) at 2.5 mM to YaLi pear (*Pyrus bretschneideri*) against infection by *Penicillium expansum* was determined by spraying three times around 30, 60 and 90 days, after full flowering. The fruits were inoculated with *P. expansum* and incubated at 20 °C, 95–100 % RH. SA-treated fruits had 58 and 26.5 % disease incidence and lesion diameter of 58.4 and 29 % lower than that on untreated fruits respectively on 12 and 17 days after incubation. Enhancement of H_2O_2 accumulation and activities of defense-related enzymes PO, PAL, chitinase or ß-1,3-glucanase was observed in the young SA-treated fruits, compared with untreated fruits. Activities of the antioxidant enzymes, including catalase and ascorbate peroxidase in young fruits were significantly reduced by SA spraying. However, the activity of another antioxidant enzyme glutathione reductase was significantly enhanced in SA-treated fruits. Application of SA at preharvest stage could be an effective approach for protecting the fruits against infection by *P. expansum* at pre- and post-harvest stages (Cao et al. 2006).

The effects of application of methyl jasmonate (MeJA) and the yeast *Cryptococcus laurentii* alone or in combination on the development of brown rot disease of peach fruit caused by *Monilinia fructicola* and blue mold disease caused by *Penicillium expansum* were assessed. Treatment with MeJA at 200 µmol/l enhanced the population of the yeast *C. laurentii* both in vitro and the wounds of peach fruit. Enhancement of resistance by *C. laurentii* in peach fruit was positively correlated with the population of *C. laurentii*. Treatment of peach fruit with *C. laurentii* at 5×10^7 CFU/ml in combination with 200 µmol/l MeJA induced stronger activities of chitinase, ß-1,3-glucanase, PAL and PO in peach fruit, than when MeJA or the yeast was applied alone. The combined treatment significantly reduced lesion diameter on fruit caused by *M. fructicola* and *P. expansum* at 25 and 0 °C respectively. The onset of disease resistance against *M. frutcticola* and *P. expansum* paralleled closely the increase in chitinase, ß-1,3-glucanase, PAL and PO activities. MeJA did not affect the mycelial growth of *M. fructicola* significantly. But it had direct inhibitory effect on the growth of *P. expansum* at 25 and 0 °C. The results indicated that reduction of lesion diameter caused by brown rot and blue mold was primarily, due to induction of resistance by MeJA in peach fruit as well as enhancement by MeJA of population of *C. laurentii* in wounds in peach fruit (Yao and Tian 2005a, b).

The efficacy of suppressing the development of *Monilinia fructicola*, infecting sweet cherry fruits was assessed by applying salicylic acid (SA) and methyl jasmonate (MeJA) as pre- and post-harvest treatments. Preharvest treatments with 2 mM SA and 0.2 mM MeJA significantly reduced lesion diameters induced by *M. fructicola* on sweet cherry fruits, compared with effectiveness of postharvest treatments. Preharvest treatment of sweet cherry with SA or MeJA induced ß-1,3-glucanase, PAL and PO activities during the early stages of storage time. SA and MeJA enhanced the activities of the defense-related enzymes to a greater level, when cherry fruits

were stored at 25 °C than at 0 °C. SA at concentration of 2 mM exhibited fungitoxicity on *M. fructicola* by inhibiting mycelial growth and conidial germination in in vitro assays. On the other hand, MeJA was not inhibitory to *M. fructicola* at a concentration of 0.2 mM used for treating the cherry fruits. MeJA as preharvest spray stimulated the activities of ß-1,3-glucanase and PAL to a greater level than SA and untreated control fruits during early storage time (Yao and Tian 2005a, b). The role of exogenous salicylic acid (SA) in regulating an antioxidative defense response of sweet cherry (*Prunus avium* cv. Hongdeng) fruit inoculated with *Penicillium expansum* was investigated by detecting carbonylated proteins employing immunological techniques. In SA-treated fruits challenged with *P. expansum*, accumulation of carbonylated proteins (29–45-kDa) was less than in untreated control fruits. The activities of catalase (CAT), glutathione peroxidase (GPX), chitinase and ß-1,3-glucanase were stimulated in SA-treated fruits. Furthermore, the expression of CAT, GPX and ß-1,3-glucanase genes were at a higher level, following treatment with SA. As SA did not show any inhibitory effect on the mycelial growth of *P. expansum*, SA-activated antioxidant defense responses of sweet cherry fruit might have a role in building up of resistance against the fungal pathogen (Xu and Tian 2008). The efficacy of methyl jasmonate (MeJA), (44.8 µl/l concentration) in suppressing the development of anthracnose rot of tomato fruit caused by *Colletotrichum coccodes* was assessed, along with chloride (48 ml/l) during storage at 12 °C and 95 % RH. Fruits treated with MeJA vapors reduced fungal spore germination/production, but had no effect on fungal mycelial growth. Fruit lesion development was at a faster rate after exposure in pure (100 %, v/v) volatile vapors. On the other hand, sanitary dips of pre-treated fruit in MeJA, resulted in 20 % suppression of fungal development in wound-inoculated fruit and storage in 'ambient air'. The benefits associated with volatile-enrichment, are suppression of spore germination and reduction of spore production. The volatile vapor did not have any inhibitory effect on the pathogen grown on potato dextrose agar (PDA) medium. It was implied that suppression of the pathogen was due to primarily to the impact of volatiles on fruit-pathogen interactions and/ or 'memory' effects in fruit tissues. The results showed that volatiles had potential to be considered as an alternative to the traditional postharvest sanitizing methods (Tzortzakis 2007).

Induction of resistance by methyl jasmonate (MeJA) in tomato fruits against the gray mold pathogen *Botrytis cinerea* was studied. Green mature tomatoes cv. Lichun were treated with 100 µM MeJA and nordihydroguaiaretic acid (NDGA, LOX inhibitor) for 5 min and incubated at 25 ± 1 °C, 85–90 % RH. Treatment with MeJA reduced disease symptoms in tomato fruit, soon after challenge inoculation with *B. cinerea*. Lesion size was reduced by 42.5, 27.9 and 13.9 % respectively in fruits inoculated at 1, 3 and 6 days after treatment. Inhibitory effect of MeJA application was not perceptible, when fruits were inoculated at 9 and 12 days after treatment. Ethylene biosynthesis was activated in green mature tomatoes in response to MeJA treatment. The increase in ethylene production was closely related with the conversion of ACC to ethylene. The development of ethylene biosynthesis was accompanied by a sufficient increase in LOX activity. The results indicated that resistance induced by MeJA against *B. cinerea* was desirable and its utility for wide application might

be a desirable approach (Yu et al. 2009). The extent of protection offered by jasmonic acid (JA) to sugar beet roots against fungal pathogens commonly causing storage rots was assessed. Harvested sugar beet roots were treated with JA at different concentrations (0.01–100 μM) and inoculated with *Botrytis cinerea, Penicillium claviforme* or *Phoma betae*. After incubation at 20 °C and 90 % RH, the severity of rotting symptoms was recorded. JA at all concentrations tested, reduced the rot severity due to all three pathogens. All concentrations of JA provided protection to the same level against *B. cinerea* and *P. betae* and reduced the amount of rotted tissue, due to these pathogens at an average of 51 and 71 % respectively. The extent of protection provided by JA against *P. claviforme* increased with increase in its concentration. JA at 100 μM concentration reduced the rot by 65 %. Treatment with JA did not affect the disease incidence, but reduced the rot severity by reducing the progress of disease symptoms in root storage tissue (Fugate et al. 2012).

8.2.1.2 Viral Diseases

Inducers of disease resistance have been evaluated for their efficacy against plant viruses. Induction of systemic acquired resistance (SAR) in tobacco cv. Samsun NN was demonstrated against *Tobacco mosaic virus* (TMV). The level of SAR was determined, based on the extent of reduction in number and/ or diameter of local lesions formed on leaves of Samsun NN, following challenge inoculation with TMV (Ross 1961a, b). Production of PR-proteins in tobacco inoculated with TMV was related to the development of resistance (Gianinazzi 1983). The PR-protein induced in cucumber by *Tobacco necrosis virus* (TNV) was a chitinase and the plant exhibited resistance also to the bacterial pathogen *Pseudomonas syringae* pv. *lachrymans* (Métraux et al. 1988). PR-proteins (PRs) mostly accumulated in the extracellular space or in the vacuole in the cells of tobacco. Systemic induction of PRs may be due to enormous increase in endogenous levels of SA. The increase in SA levels paralleled PR-1 production in TMV-resistant Xanthi nc (NN) tobacco plants, but not in susceptible (nn) tobacco (Yalpani et al. 1991). Exogenous application of SA induced PR-1 production in Xanthi nc tobacco. In contrast, no detectable change occurred in the constitutive expression of high levels of PR-1 proteins in the hybrid of *Nicotiana glutinosa* × *N. debneyi*, highly resistant to TMV. The results strongly indicated the regulatory role of SA in disease resistance and PR-protein synthesis (Yalpani et al. 1993). SA induces a range of defense genes, especially those encoding the PR-proteins. The mechanism of development of SA-mediated resistance to plant viruses is not clearly understood. Treatment with SA resulted in accumulation of PR-proteins and *Tobacco mosaic virus* (TMV) titer was reduced in directly inoculated leaves of tobacco. In addition, the ratio of viral genomic RNA to coat protein (CP) mRNA and the ratio of plus-to-minus-sense RNAs were also affected by SA application (Chivasa et al. 1997), suggesting that SA-induced interference with the activity of TMV-RNA-directed RNA polymerase (RdRp). Salicylhydraoxamic acid (SHAM), an inhibitor of plant mitochondrial alternative oxidase (AOX), inhibited the delay in symptom expression in

TMV-inoculated tobacco plants treated with SA. Further, the SA-induced SAR in tobacco plants with *N* gene was also inhibited (Chivasa et al. 1997). In the case of *Potato virus X* (PVX), RNA accumulation in inoculated tobacco leaf tissue was reduced by SA treatment and resistance was also found to be dependent in the SHAM-sensitive signaling pathway. In contrast, symptom development in SA-treated and *Cucumber mosaic virus* (CMV)-inoculated plants was also delayed. This was not due to inhibition of replication, but rather to inhibition of systemic movement of the virus. SA-induced resistance to CMV was lost, due to the action of SHAM. In the case of virus diseases, SHAM-sensitive signaling pathways seem to activate both resistance mechanisms: inhibition of long-distance movement and inhibition of virus replication as in TMV and PVX (Naylor et al. 1998).

Salicylic acid (SA)-induced resistance to *Cucumber mosaic virus* (CMV) in tobacco was found to be due to inhibition of systemic movement of the virus and the resistance was induced via a signal transduction pathway that also can be triggered by antimycin A, are inducer of mitochondrial enzyme alternative oxidase (AOX). In *Arabidopsis thaliana*, inhibition of CMV systemic movement was also induced by SA and antimycin A. The results indicated that the mechanisms underlying induced resistance to CMV in tobacco and *A. thaliana* were similar. In contrast, in squash (*Cucurbita pepo*), SA-induced resistance to CMV resulted from inhibition of virus accumulation in directly inoculated tissue, most likely through inhibition of cell-to-cell movement. Furthermore, neither of the AOX-inducers, antimycin A or KCN induced resistance to CMV in squash. In addition, AOX inhibitors, that compromise SA-induced resistance to CMV in tobacco, did not inhibit SA-induced resistance to the virus in squash. The results indicated that different plant host species appear to employ distinctly different approaches to resist infection by the same virus (Mayers et al. 2005). The effects of salicylic acid (SA) and 1-aminocyclopropane-1-carboxylic acid (ACC), a precursor of ethylene (ET) biosynthesis, on *Potato virus Y* (PVY) isolate N:O-induced systemic symptom expression in tobacco plants were assessed. Treatment of tobacco seedlings with SA delayed virus-induced necrosis in stems by 1–2 days. SA, but not ACC, was able to suppress severity of symptoms on stems significantly. However, both compounds did not affect the recovery of plants from severe phase of disease symptoms. SA application enhanced peroxidase activity in stems and PR-genes were activated in both stem and leaves of tobacco. The results suggested that SAR has a critical role in suppressing development of symptoms induced by the strain $PVY^{N:O}$, through SA-mediated and ET-dependent pathways. Reduced replication/accumulation of the virus was reflected by the suppression of disease symptom in SA-treated plants (Nie 2006).

Elimination of viruses from infected plants or planting materials is one of the important strategies for effective management of virus diseases. Thermotherapy has been successfully applied to eliminate the viruses from plants and planting materials. Enhancement of heat tolerance of plants by treating with chemicals may be expected to increase the chances of obtaining higher percentage of virus-free plants. The ability of salicylic acid (SA) to increase the heat tolerance of potato microplants infected with *Potato virus X* (PVX) was assessed. Potato microplants were cultured with or without 10^{-5} M SA for 4 weeks, then subcultured without SA and exposed to 42 °C

for 30 days. Survival of SA-treated microplants was more consistent, compared to untreated control plants. SA treatment also improved the post-thermotherapy subculture. Among the surviving microplants, SA increased the virus-free yield to 100 % from 40 to 65 % in the controls. In another group of 30 potato genotypes infected with PVX, 98 % of the surviving SA-treated microplants became PVX-free after thermotherapy, compared to 75 % of untreated control plants. SA-treated microplants had lower catalase activity and higher H_2O_2 levels (López-Delagado et al. 2004).

8.2.2 Benzothiadiazole

The bioassay of benzo (1,2,3)-thiadiazole-7-carbothioic acid-*S*-methyl ester (BTH), a nontoxic synthetic functional analogue of salicylic acid, has been employed for assessment of its potential for protecting crop plants against diseases caused by microbial pathogens. Induction of resistance in plants against diseases has been demonstrated to be the primary mechanism of suppressing the development of diseases affecting various crops.

8.2.2.1 Fungal Diseases

Induction of systemic resistance by BTH in wheat to powdery mildew pathogen *Blumeria graminis* f.sp. *tritici* (*Bgt*) was reported by Stadnik and Buchenauer (2000). The white mold and gummy stem blight diseases could be reduced effectively by inducing resistance in melons by applying BTH (Buzi et al. 2004). In a later investigation, BTH was found to arrest the progress of powdery mildew disease at 27 days after application. The induced resistance was found to be long-lasting and the disease severity was reduced by 2–53 %, compared with untreated control, depending on the year of a 3-year small-plot experiment (Vechet et al. 2009). Benzothiadiazole (BTH) and chitin (CHT) were evaluated for their ability to induce resistance, when applied as foliar spray to barley plants against powdery mildew pathogen *Blumeria graminis* f.sp. *hordei* (*Bgh*). After an induction phase (IP) of 3 days (the time elapsed between treatment and pathogen inoculation), both compounds significantly reduced *Bgh* infection on the primary leaf. BTH was more effective than CHT with 68.9 % inhibition of infection, as against 55 % inhibition by CHT. A 5-day IP further reduced the infected areas in BTH-treated plants (−77.2 %), but not in CHT-treated plants (−47.1 %). Both BTH and CHT induced oxidative burst and phenolic compound deposition in treated leaves, creating an environment hostile to the pathogen, resulting in retardation of invasion due to impairment of haustorium development. The greater efficacy of BTH could be attributed to a greater reinforcement of papilla, higher level and homogenous diffusion of H_2O_2 in the treated leaf tissues and an induced hypersensitive-like response in many penetrated cells (Faoro et al. 2008).

The effect of root treatment with BTH was assessed in papaya plants challenged with *Phytophthora palmivora*. The activities of chitinase and ß-glucanase were enhanced in treated papaya plants. The *NPR1 gene* (*CpNPR1*) was isolated by homology to a conserved region of *Arabidopsis* and tobacco *NPR1* genes and four partial PR-1(*PR1*) cDNAs from papaya identified by homology to region conserved in all known *PR1* gene family members. The CpPR1b and CpPR1d were induced by BTH. The CpPR1d with highest level of homology to tobacco PR1a was considered as a convenient marker of SAR induction in papaya (Zhu et al. 2003). In a later investigation, 25 additional papaya genes showing elevated systemic expression at 3 days after BTH treatment were identified by suppression subtraction hybridization and confirmed and quantified by northern blots and quantitative PCR assay. The induced genes of papaya included several PR genes and two genes with likely involvement in altering cell wall porosity and lignification. In addition, six genes with potential roles in establishing reducing conditions, following the oxidative burst were also induced. The results suggested that the regulation of cellular redox status may play a role in SAR induction in papaya (Qiu et al. 2004). The responses of two rice cultivars Tetep [resistant to *Magnaporthe grisea* (*Mg*) and *Cochliobolus miyabeanus* (*Cm*), causing blast and brown spot diseases respectively] and Nakdong (susceptible to both pathogens) to treatment with BTH and the conidial germination fluid (CGF) of *Cm* were assessed. Treatment with CGF induced rapid cell death in both cultivars, possibly due to the phytotoxins produced by *Cm*. Pretreatment with CGF induced significant resistance to *Mg* in cv. Nakdong, but not to *Cm*. Likewise, BTH significantly enhanced resistance to only *Mg*, but it failed to protect rice cv. Nakdong against *Cm*. Enhancement of resistance of cv. Nakdong to *Mg* by BTH or CGF treatment was correlated with more rapid induction of three monitored PR genes. Expression patterns of three PR genes suggested that rice defense mechanisms against rice brown leaf spot disease are distinct from those against blast disease and their expression patterns in response to *Cm* infection were nearly identical in both susceptible and resistant cultivars. Rice appears to employ distinct mechanisms for its defense against these two important fungal pathogens (Ahn et al. 2005).

The severity of dry rot disease caused by *Fusarium semitectum* in inoculated potato tuber was reduced by BTH treatment of tubers. The activity of ß-1,3-glucanase in leaves, stem, tubers and stolons of BTH-treated plants was increased (Bokshi et al. 2003). The efficiency of benzothiadiazole (BTH) in inducing resistance in cocoyam (*Xanthosoma sagittifolium*) against root rot pathogen *Pythium myriotylum* was assessed. Under controlled conditions, BTH (0.2 mg/ml) applied on leaves induced resistance to the pathogen effectively, resulting in significant reduction in disease incidence and severity. The activities of peroxidase and polyphenoloxidase and total phenolic contents were enhanced. The increase in peroxidase activity was correlated with two new isoforms in a white (sensitive) cultivar inoculated, after stimulation. In a yellow (resistant) cultivar, stimulation was characterized by the appearance of one isoform. Qualitative analysis of phenolic compounds by HPLC showed an increase of hydroxycinnamic and flavonoid derivatives after inoculation. Presence of a new caffeoylshikimic acid derivative was also detected after stimulation, following inoculation of both cultivars. The

pattern of induction of resistance to *P. myriotylum* appeared to be cultivar-dependent (Mboubda et al. 2010). Effectiveness of benzothiadiazole (BTH), as foliar spray, was investigated for reducing infection of *Nicotiana benthamiana* by *Colletotrichum orbiculare*. BTH reduced the number of lesions per leaf area caused by *C. orbiculare* by 98 % and it was more effective than (2R, 3R) butanediol or PC1, an isoparaffin-based mixture. Foliar application of BTH induced expression of genes for the acidic PR proteins, NbPR-1a, NbPR-3Q and acidic NbPR-5. In contrast, soil application of (2R, 3R) butanediol or PC1 primed expression of genes for the basic PR proteins NbPRb-1b, basic NbPR-2 and NbPR-5 dB. These results were consistent with the activation of SA-dependent SAR by BTH and that of jasmonate/ethylene-dependent induced systemic resistance (ISR) by (2R, 3R) butanediol or PC1 and showed that (2R, 3R) butanediol and PC1 can affect gene expression similarly to PGPR (Cortes-Barco et al. 2010).

Application of BTH on strawberry plants delayed the development of gray mold disease on harvested fruits caused by *Botrytis cinerea* and increased the storage life of fruits as well (Terry and Joyce 2000). The resistance-inducing potential of BTH was assessed against *Penicillium expansum*, infecting peach fruits. Harvested peach fruits were treated with BTH solution (200 mg/l) for 5 min immediately after harvest, incubated at 22 °C and 85–95 % for 60 h and then inoculated with *P. expansum* (1.2×10^4 conidia/ml). The disease incidence and lesion area (disease severity) in BTH-treated fruits were 49.5 and 64.1 % respectively lower than that of untreated controls. BTH treatment on fruits challenged by pathogen inoculation, enhanced the activities of PAL, PPO and PO, as well as the levels of total phenolic compounds and H_2O_2. In addition, the activities of superoxide dismutase (SOD) and ascorbic acid level showed increases in BTH-treated fruits during the middle and later periods of inoculation. Application of BTH was considered to have the potential for substituting the fungicides applied for the control of postharvest diseases (Liu et al. 2005). The efficiency of protection of mango fruits by application of BTH against the anthracnose pathogen *Colletotrichum gloeosporioides* was assessed. The mangoes were treated by vacuum infiltration of BTH (1.0 mM) after harvest. The fruits were inoculated after 72 h with 15 µl of conidial suspension (10^5/ml) and incubated at 13 °C, 85–90 % RH. Disease incidence and lesion diameter were significantly reduced by BTH treatment, compared with untreated controls. Enhancement of defense-related enzymes in mango fruits treated with BTH, inhibition of oxidative enzyme catalase and increase in level of H_2O_2 were observed in BTH-treated fruits, indicating the induction of processes involved in disease resistance. The results indicated that BTH application might be considered as an alternative to traditional fungicide treatment of mango fruits during storage (Zhu et al. 2008).

Benzothiadiazole (BTH) was shown to protect apple from fire blight disease caused by *Erwinia amylovora* (*Ea*). BTH did not have any direct toxic effect on *Ea* in agar plates, consistent with BTH being an inducer of SAR. BTH was able to protect Jonathan apple under field conditions and also to Golden Delicious seedling, scions and trees following artificial inoculation of *Ea* (Brisset et al. 2000; Maxson-Stein et al. 2002). The effect of BTH was shown to be associated with increased gene expression and accumulation of defense-related enzymes. Transcripts of three PR-related genes

PR-1, PR-2 and PR-8 were strongly induced by high concentrations of BTH in Jonathan seedling (Maxson-Stein et al. 2002). Peroxidase and ß-1,3-glucanase activities (PR-2) in BTH-treated Golden Delicious seedlings were stimulated. Systemic stimulation of the enzymatic activities was also seen later (Brisset et al. 2000). Application of BTH on 2-year old pear plants protected them from fire blight both in terms of disease incidence and severity. Although the treated plants challenged with *E*a harbored viable pathogen cells, as revealed by PCR-based detection, little or no symptoms of the disease could be seen at 6 months after inoculation. Expression of a member of the PR-1 family in pear plants treated with *Ea* was found to be constitutive and unaffected by treatments, suggesting that molecules other than PR-1 may be required for induction of systemic resistance by BTH (Sparla et al. 2004).

The ability of benzothiadiazole (BTH) to decrease the susceptibility of pea seedlings to leaf pathogens was assessed. The avirulent strain of *Pseudomonas syringae* pv. *pisi* (*Psp*) was included for comparison. The abiotic and biotic agents decreased the susceptibility of leaves of pea at 7 or 14 days after application of BTH to challenge inoculation with the severe strain of *Psp* or the fungal pathogens *Mycosphaerella pinodes* and *Uromyces vicia-fabae*. Treatment with BTH or avirulent strain of *Psp* enhanced activities of ß-1,3-glucanase and chitinase in untreated upper leaves at 6 days after application. Higher concentration of BTH (100 µg a.i./ml) caused significant greater disease minimization and stimulation of the two defense-related enzymes than the lower concentration (20 µg a.i./ml) (Dann and Deverall 2000). The effectiveness of BTH and an avirulent strain of *Pseudomonas syringae* pv. *maulicola* (*Psm*) in inducing SAR in *Brassica napus* (canola) against virulent strains of *Psm* and the fungal pathogen *Leptosphaeria maculans* was assessed. Application of BTH enhanced resistance against virulent strains of *Psm* and *L. maculans* to a greater extent, than localized preinoculation of plants with avirulent strain. Pretreatments with BTH and avirulent strain resulted in accumulation of PR genes including *BnPR-1* and *BnPR-2*, the levels of transcript accumulation being greater in BTH-treated plants. Development of SAR in *B. napus* plants expressing a bacterial salicylate hydroxylase transgene (*NahG*) that is known to metabolize salicylic acid (SA) to catechol was appreciably compromised. These plants accumulated only reduced levels of PR gene transcripts, compared with non-transformed controls. The results indicated that SAR development in *B. napus* had many hallmarks of classical SAR including long-lasting and broad host range resistance, associated with PR gene activation and requirement of SA (Potlakayala et al. 2007).

The efficacy of benzothiadiazole (BTH) to induce systemic acquired resistance (SAR) in tomato cv. Volledung plants against a yellow strain of *Cucumber mosaic virus* (CMV-Y) was assessed. Application of BTH, as a drench, at 7 days before inoculation with CMV-Y, protected plants against the necrosis caused by CMV-Y. The resistance to CMV-Y was revealed by the decrease in disease incidence and severity in BTH-treated plants. At 21 days after challenge inoculation with CMV-Y, the disease incidence in plants with SAR did not exceed 12.5 %, whereas the control plants showed high levels of severe infection (91.7 %). In BTH-treated plants, the primary symptoms were delayed for 7 days. The disease spread rapidly in control plants and by the end of the experiment, almost all control plants showed severe

mosaic and leaf necrosis. Interference in replication of CMV-Y was indicated by the results of enzyme-linked immunosorbent assay (ELISA), following treatment of leaves with BTH. Analysis of the newly developed leaves of BTH-treated plants for virus antigen revealed that symptomless plants failed to support the replication of CMV-Y and the concentration of virus in these plants was similar to that in uninoculated control plants (Anfoka 2000).

Acibenzolar-*S*-methyl (ASM, a derivative of BTH) has been shown to be effective in inducing resistance in several crop plants against various diseases caused by microbial pathogens. Application of the defense activator ASM could enhance resistance to crown rot and red stele of strawberry caused by *Phytophthora cactorum*. Both ASM and chitosan reduced crown rot symptoms and the effect was enhanced, when the time between treatment and challenge inoculation with *P. cactorum* was increased from 2 to 20 days. There were no significant differences between treatments where the concentration of ASM was increased from 10 to 1,000 μg a.i./plant. Inoculation of alpine strawberry plants (*Fragaria vesca* var. *alpina*) cv. Alexandria with *P. fragariae* var. *fragariae*, after treatments with acibenzolar-S-methyl (ASM), chitosan or fosetyl-Al (fungicide) showed that ASM provided good protection to alpine strawberry, while chitosan had no effect at all. There was no significant difference between ASM and fosetyl-Al, when applied at the same time. ASM (500 μg a.i./ml) reduced the growth rate of *P. fragariae* var. *fragariae*, but not that of *P. cactorum*. On the other hand, chitosan showed inhibitory effect on both fungal pathogens. The results indicated the potential of ASM for use against the root-infecting fungal pathogens of strawberry (Eikeno et al. 2003). The potential of acibenzolar-*S*-methyl (ASM), to induce resistance in cotton against *Thielaviopsis basicola*, causative agent of black root rot disease was determined, using naturally infested soil. In the greenhouse, soaking seeds in solutions of ASM (25 or 50 μg/ml) for 3–5 h, before planting, consistently reduced disease severity on tap roots by 20–30 %. In field experiments, ASM was applied either as sprays over the seed during sowing (in-furrow spray), as seed (soaking) dressing or as foliar sprays over seedlings. Seed-soaking reduced the disease severity on tap roots by 33 %, while in-furrow spray reduced the severity of symptoms by 24 %, increased the number of relatively healthy lateral roots by 350 % and increased the boll number by 29 %. Foliar sprays were not effective in reducing the disease severity. ASM treatment effect varied, depending on the mode of application. Hence, it is necessary to select the suitable method of application (delivery) and required concentration of ASM for inducing resistance against the target pathogen(s) (Mondal et al. 2005).

The effectiveness of acibenzolar-*S*-methyl (ASM) in suppressing the development of Fusarium wilt disease of cyclamen caused by *F. oxysporum* f.sp. *cyclaminis* was assessed under greenhouse conditions. The estimates of area under disease progress curve (AUDPC) showed a negative relationship with the rate of ASM applied to the potting mix infested with the pathogen. The major benefit of ASM was that it delayed the onset of wilt symptoms in most of the treated plants for up to 3 weeks and a few plants were entirely asymptomatic. Although no visible phytotoxicity on leaves could recognized, the decline in dry mass of treated plants suggested some level of plant growth inhibition by ASM. However, ASM might be

considered as a component of integrated cyclamen wilt disease management program (Elmer 2006). ASM exhibited antifungal activity against *Rhizoctonia solani* AG-4 infecting soybean causing root rot disease. The antifungal activity of ASM reached 40 %, in comparison with the control. At concentrations of 0.08 and 0.5 g/l, ASM significantly induced resistance responses in the soybean hypocotyls, resulting in reduction of disease intensity of rotting symptoms. The reduction in disease intensity due to ASM treatment was correlated with a stimulation of chitinase activity. The protective effect of ASM against *R. solani* AG-4 was, probably due to a combination of induced resistance and its direct antifungal activity against the pathogen. Adverse effect on plant growth, following treatment with ASM was also observed. A dose-dependent inhibition of the seminal root growth was recorded, following seed treatment with ASM. However, the growth reduction of soybean was only transitional and the treated plants rapidly recovered in optimal growth conditions, except the plants treated with higher dose of ASM (0.5 g/l). The results indicated the need to determine the optimum concentration of inducers of resistance to avoid the adverse effects on plant growth (Faessel et al. 2008).

Acibenzolar-*S*-methyl (ASM, Bion WG, Novartis Ltd., Switzerland)-mediated systemic priming of PAL, chitinase activity and phytoalexins accumulation in cowpea occurred, resulting in enhanced resistance to anthracnose caused by *Colletotrichum destructivum* (Latunde-Dada and Lucas 2001). In cucumber plants treated with ASM, *pal* gene was systemically primed, following inoculation with *C. orbiculare* (Cools and Ishii 2002). ASM was found to be effective in reducing scab disease of Japanese pear caused by *Venturia nashicola* in the field, though several rounds of application with ASM was required (Brisset et al. 2000; Ishii et al. 2002). The tripartite interaction among Japanese pear-ASM-*V. nashicola* was studied to understand the molecular mechanism involved in the induction of resistance, since ASM did not have any antifungal activity. The defense responses induced by ASM included transcripts encoding polygalacturonase-inhibiting protein (PGIP) that were highly and transiently promoted, after scab inoculation of plants pretreated with ASM. Enhancement of PAL activity, SA accumulation and induction of several PR-proteins in ASM-treated plants suggested that resistance triggered by ASM may be SA-independent (Faize et al. 2004). In a later study the gene expression during ASM-induced priming for potential responses to *Venturia nashicola*, causing scab disease of Japanese pear, was investigated. Pretreatment of leaves of Japanese pear plants with SAR-activator ASM significantly reduced the severity of scab disease. Three defense-related genes that were involved in the signaling network from Japanese pear, including a non-expressor of PR-protein1 (jpNPR1), a respiratory burst oxidase homologues (jpRBOH) and a nitrogen-activated protein kinase (jpMAPK) were cloned. The RT-PCR assay revealed that transcript level of jpNPR1 was not significantly elevated in Japanese pear leaves, following treatment with ASM and during infection with *V. nashicola* and was highly and transiently primed in ASM-treated leaves, after scab inoculation. Transcripts encoding jpRBOH and jpMAPK were directly responsive to ASM and they were also potentiated early after challenge inoculation with *V. nashicola* (Faize et al. 2009).

The effects of application of ASM on infection behavior of Japanese pear scab pathogen *V. nashicola* race 1 were studied, based on the utrastructural changes in the pathogen in plant tissues. On ASM-pretreated leaf surfaces, conidial germination and appressorial formation did not show any obvious variation, compared with leaf surfaces treated with distilled water (DW). After penetration, subcuticular hyphae in the epidermal pectin layers and middle lamellae of ASM-treated leaves were observed at a lower frequency than in DW-treated leaves, indicating suppression of pathogen growth in ASM-treated pear leaves. Morphological modification of pathogen hyphae in the pectin layers were observed in ASM-pretreated leaves. The hyphae exhibited cell collapse including plasmolysis, necrotic cytoplasm and cell wall destruction. The cell walls of collapsed hyphae broke into numerous fibrous and amorphous pieces, suggesting that ASM-induced scab resistance might be associated with cell wall-degrading enzymes from pear plants. The subcuticular hyphae proliferated much more rapidly in the pectin layers of DW-treated leaves than in ASM-treated leaves. The inhibition of hyphal invasion into the middle lamella might be due to ASM-induced suppression of hyphal growth in the pectin layers. The activity or production of pectin-degrading enzymes might be inhibited in ASM-treated leaves (Jiang et al. 2008). In cucumber plants treated with acibenzolar-S-methyl (ASM), systemic resistance was induced against anthracnose pathogen *Colletotrichum orbiculare* and powdery mildew pathogen *Spaerotheca fuliginea*. The phenolic compounds were extracted from ASM-treated and inoculated plants. The glucoside-linked phenolic compounds from cucumber leaves were evaluated for their antifungal activity. The mycelial growth of *C. orbiculare* was inhibited by the phenols to the maximum extent, followed by that of the scab pathogen *Cladosporium cucumerinum*. But the phenolic compounds were ineffective against *Corynespora casiicola*, causing Corynespora leaf spot disease. The accumulation of active compounds seemed to increase with growth stages of cucumber plants, irrespective of ASM treatment. No increase in phytoalexin-like phenol compounds occurred in ASM-treated cucumber plants inoculated with either anthracnose or powdery mildew pathogen (Lin et al. 2009).

The potential of acibenzolar-*S*-methyl (ASM), when applied alone or in combination with fungicides or antibiotics was assessed for the control of coffee rust (*Hemileia vastatrix*) and brown leaf spot (*Phoma costarricencis*) under field conditions and also for the control of brown eye spot (*Cercospora cofffeicola*) and bacterial blight (*Pseudomonas syringae* pv. *garcae*) on coffee seedlings. ASM protected coffee seedlings against brown eye spot disease, when applied at 2.5 and 5.0 g of a.i. per liter of water, providing 34–55 % of disease control. Application of fungicides effectively reduced brown spot disease and they were superior to ASM. There was no synergistic effect of the combination of ASM with azoxystrobin, cyproconazole or cupric fungicides for the control of the disease under field conditions (Patrício et al. 2008). The effectiveness of activation of host defense systems in reducing Eucalyptus rust disease caused by *Puccinia psidii* by applying acibenzolar-*S*-methyl (ASM) was investigated. Greenhouse assessments revealed that the *E. grandis*×*E. urophylla* hybrid CO (moderately resistant) had lower disease severity than VR hybrid (susceptible), following application of ASM or the extract of *Saccharomyces cerevisiae*.

The enhanced resistance to the rust disease was associated with the induction of a hypersensitive reaction which appeared to be effective in reducing the disease severity in both hybrids (Boava et al. 2009). The comparative efficacy of ASM and salicylic acid (CSA) in inducing resistance in tea plants against the blister blight pathogen *Exobasidium vexans* was assessed. Significant enhancement in the activities of defense-related enzymes like PAL, PO and ß-1,3-glucanase was observed in the elicitors-treated tea leaves. ASM (0.14 %) registered lowest disease severity (25.2 %) and SA treatment was found to be less effective. Under field conditions, application of ASM (0.14 %) resulted in disease protection of 25 %. The combined application of ASM and standard fungicide alternatively was more effective in increasing the protection level to 46.8 %. The results indicated the feasibility of including ASM as a component of disease management program for tea (Ajay and Baby 2010).

Basil downy mildew caused by *Peronospora belbahrii* is considered as a major threat to sweet basil production in Florida, USA. Resistance inducers, acibenzolar-S-methyl (ASM, Actigard 50 WG), DL-3-aminobutyric acid (BABA), isonicotinic acid (INA), salicylic acid (SA) and sodium salicylate (SS) were evaluated for their efficacy in suppressing development of downy mildew pathogen under greenhouse conditions. These inducers of systemic acquired resistance (SAR) differed in their efficacy depending on the rate, method and time of application. Foliar sprays of ASM applied pre-, post- or pre- + post-inoculation at rates ranging from 25 to 400 mg/l significantly reduced disease severity ($P = 0.05$), compared with non-treated control in all experiments. ASM sprayed at 50 mg/l three times on a weekly basis commencing from 3 to 7 days post-inoculation resulted in 93.8 and 47.1 % reduction in disease severity respectively. On the other hand, foliar sprays of BABA as pre- + post-inoculation at rates equal or higher than 125 mg/l significantly suppressed downy mildew, compared with nontreated controls. Foliar treatments of ASM or BABA, followed by one or two post-inoculation sprays of a mixture of potassium phosphate (Prophyt) and azoxystrobin (Quadris) significantly improved effectiveness of disease control. Sporangial production on ASM treated leaves was significantly reduced relative to the unrelated controls (Mersha et al. 2012).

The plant activator acibenzolar-*S*-methyl (ASM) was evaluated for its potential to suppress development of major diseases of stored melons caused by *Fusarium* spp., *Alternaria* spp., *Rhizopus* spp. and *Trichothecium* sp. Preflowering foliar spray of ASM (50 mg a.i./l) combined with a fruit dip in guazatine (500 mg a.i./l) at harvest substantially decreased the diseases. The treatment with ASM alone was significantly effective in reduction of the severity in many, but not all situations. The fungicide guazatine was less effective in reducing the diseases caused by *Alternaria* spp. and *Rhizopus* spp. (Huang et al. 2000). Infiltration of ASM (0.5 mM) into Yali pear fruit (*Pyrus bretschneideri*) after harvest significantly reduced incidence of disease and lesion area in/on the fruit inoculated with *Penicillium expansum*. The duration of protection provided by ASM lasted over 15 days. ASM did not inhibit the mycelial growth of *P. expansum* in vitro. But significant enhancement of the activities of the principal defense-related enzymes, including PO, PAL, chitinase and antioxidant enzymes including superoxide dismutase and catalase was observed in the fruit during infection. Two types of secondary metabolites total phenolic compounds and

flavonoids and two products of lipid peroxidation, H_2O_2 and malondialdehyde were also involved in the resistance and significantly accumulated in ASM-treated fruit during infection. The inhibitory effect of ASM on the disease may be related to its ability to enhance defense responses in the treated fruits (Cao et al. 2005). The ability of ASM and methyl jasmonate (MeJA) to protect the cut flowers of Freesia hybrid against *Botrytis cinerea* was assessed. The chemicals were applied as spray or pulse treatments. Lesion numbers on non-inoculated freesia flowers and diameters of lesions on attached petals of inoculated flowers were reduced significantly, compared with untreated controls. However, ASM treatment was not effective, when inoculated flowers were incubated at 20 °C. ASM had direct antimicrobial activity by reducing mycelial growth and conidial germ tube elongation of *B. cinerea*. ASM did not adversely affect the life of cut flowers (Darras et al. 2007).

Application of acibenzolar-*S*-methyl (ASM) has been demonstrated to be effective in reducing the incidence and severity of certain bacterial diseases of plants. ASM applied at 100 and 200 mg a.i/l was able to protect Golden Delicious apple seedlings, scions and trees effectively against fire blight disease, when applied before inoculation of *Erwinia amylovora*. ASM provided protection to apple seedlings to the level comparable to the standard antibiotic plantomycin (100 mg/l, streptomycin sulfate) applied just before inoculation with the pathogen. Protection of apple seedlings by ASM was consistently associated with the activation of two families of defense-related enzymes, peroxidases and ß-1,3-glucanases. Accumulation of both enzymes was induced locally in treated leaves and systemically, especially for ß-1,3-glucanases in upper untreated leaves and was sustained for at least 17 days. The results suggested that ASM induced systemic resistance in apple by increasing defense-related compounds (Brisset et al. 2000). The potential of acibenzolar-*S*-methyl (ASM) and DL-3-aminobutyric acid (BABA) for suppressing the development of apple fire blight disease caused by *Erwinia amylovora* was assessed. ASM or BABA alone or in sequence was applied as foliar sprays on apple seedlings at different days before inoculation (dbi). ASM was slightly more effective than BABA in suppressing the development of fire blight disease, expressed as browning discoloration index (BDI) and stem bending index (SBI). Spray treatments of apple seedlings with BABA (4 dbi) and ASM (2 dbi) in sequence drastically reduced the fire blight symptoms, while single treatment separately with ASM or BABA was less effective. Further, BABA/ASM treatment induced long-lasting resistance in apple seedlings, as indicated by the absence of disease symptoms on treated apple seedlings for more than 2 months. The change in sequence of application of ASM first (4 dbi), followed by BABA (2 dbi) also was equally effective in reducing disease symptoms. The marked effects of induced resistance following treatments were consistent. ASM induced the same set of genes that are activated by SA and it induced the same spectrum of disease resistance that is mediated by SA-dependent pathway. Application of BABA markedly enhanced free and total levels of SA, suggesting that BABA might induce resistance in apple seedlings also through activation of a signaling pathway that depended on SA accumulation. The results suggested an additive or synergistic relationship in the activity of ASM and BABA, resulting in enhancement of effectiveness of disease suppression (Hassan and Buchenauer 2007; Appendix 8.1).

Pepper (chilli) plants sprayed with acibenzolar-*S*-methyl (ASM) and copper hydroxide were able to protect pepper fruit against infection by *Xanthomonas axonopodis* pv. *vesicatoria*. As the protection offered by ASM was observed, when the inducer was entirely degraded, it appeared that the protection was due to activation of systemic acquired resistance (SAR) (Buonaurio et al. 2002). Pepper plants carrying one or more major genes for resistance (R) to *Xcv* were sprayed with ASM, followed by challenge with incompatible strains of *Xcv*. At 3 weeks after inoculation, the ASM-treated plants showed significantly fewer lesions induced by both incompatible (hypersensitive) and compatible (susceptible) responses than the number of lesions formed on untreated plants. Disease lesions contained race-change mutants. The results indicated the usefulness of SAR inducers to increase the durability of genotype-specific resistance conferred by R genes (Romero and Ritchie 2004). The efficacy of ASM and *Pseudomonas fluorescens* (*Pf*) applied alone or in combination for seed and/ or seedling treatment for suppressing the development of bacterial spot disease of tomato caused by *Xanthomonas axonopodis* pv. *vescatoria* was assessed. The seed treatment with chemical or bacteria improved seed germination and seedling vigor relative to the untreated controls. Under field conditions, combination of ASM and *Pf* significantly reduced the severity of bacterial spot disease and increased seedling biomass and tomato yield, compared with untreated controls (Abo-Elyousr and El-Hendawy 2008). The effect of ASM and *Bacillus cereus* isolate L2-1 applied either alone or in combination on the suppression of cotton bacterial blight caused by *Xanthomonas axonopodis* pv. *malvacearum* (*Xam*), was determined. Treatment with ASM reduced the disease severity to the maximum extent (57.8 %), while L2-1 reduced the severity to 25.48 % of the control. The combination of ASM + L2-1 was less effective, compared to ASM treatment alone (Table 8.6). ASM alone induced the highest increases in the activities of PAL, peroxidase and ß-1,3-glucanase, as well as lignin deposition, especially at 14 days after treatment (Ishida et al. 2008).

The ability of acibenzolar-*S*-methyl (ASM) to induce resistance in tomato against *Ralstonia solanacearum (Rs*), causing bacterial wilt (BW) disease was determined. Tomato plants were treated with ASM by foliar spray (25 µg/ml) and soil drench (12.5 µg/ml) and inoculated with different concentrations of *Rs*. Uninoculated and untreated tomato plants were maintained as controls. Bacterial wilt disease incidence in different treatments was recorded at weekly intervals, after inoculation and quantified as percentage of plants wilted. Growth parameters were also recorded at weekly interval, after ASM treatment and inoculation with *Rs*. Height and shoot biomass decreased rapidly in plants not treated with ASM, but inoculated with low or high concentration of *Rs*. Nutrient concentrations such as Ca, B and P declined in leaves of inoculated plants, when compared with untreated control. Application of ASM significantly reduced bacterial wilt disease incidence, when plants were inoculated with low concentration of *Rs*. The results suggested that application of ASM might be effective under low disease pressure conditions (Haciasalihoglu et al. 2007). The induced defense responses against tomato bacterial wilt disease by application of ASM and *Pseudomonas fluorescens* (Pf2) alone or in combination were investigated. Seedling treatments with either Pf2 or ASM significantly reduced disease severity

Table 8.6 Effect of treatment of tomato with acibenzolar-*S*-methyl (ASM) and *Bacillus cereus* L2-1 isolate on severity of cotton bacterial blight disease, as reflected by the area under disease progress curve (AUDPC) (Ishida et al. 2008)

Treatments	AUDPC[x]	Percentage of control relative to the inoculated control
Absolute control	0.0a	–
ASM	54.65b	57.81
ASM + L2-1 isolate	66.37bc	48.76
L2-1 isolate	96. 53c	25.48
Inoculated control	129.53d	–
CV (%)	19.58	–

[x]Means followed by the same letter in one column are not statistically significant as per Turkey's multiple range test at 5 % probability

by 58 and 56 % respectively. Combined application of ASM and Pf2 resulted in maximum disease reduction (72 %). Application of ASM alone increased seedling biomass relative to infected control by 64.3 %. Alterations in the defense-related enzymes were assessed, following treatment with ASM and Pf2. Significant changes ($P \leq 0.05$) in the activities of polyphenoloxidase (PPO), ß-glucosidase (ß-GL) and peroxidase (PO) were observed in tomato plants treated with ASM or Pf2. The results indicated the usefulness of application of ASM and *P. fluorescens* as foliar sprays and soil drench for the control of bacterial wilt disease of tomato caused by *R. solanacearum* under field conditions (Abo-Elyousr et al. 2012).

The effect of acibenzolar-*S*-methyl (ASM) on the development of tomato bacterial canker disease caused by *Clavibacter michiganenesis* subsp. *michiganensis* (*Cmm*) was assessed. Pretreatment of tomato plants with ASM reduced the severity of the canker disease and the growth of the bacterial pathogen was reduced in planta. Development of resistance in ASM-treated plants required an interval of 1–7 days between inducer application and challenge inoculation with *Cmm*. Best protection against *Cmm* was recorded, when challenge inoculation was done at 3 days after ASM application. Reduction in disease severity (up to 75 %) was correlated with the suppression of bacterial growth during the time course of infection. The ASM-treated tomato plants showed higher activities of peroxidase, chitinase, superoxide dismutase and glutathione-S-transferase, compared with untreated control plants. ASM appeared to suppress disease development by directly limiting the pathogen population and also by enhancing the activities of defense-related enzymes, resulting in higher level of resistance to the canker disease in tomato (Soylu et al. 2003; Baysal et al. 2003). The efficiency of ASM to protect lettuce plants against the bacterial speck disease caused by *Xanthomonas campestris* pv. *vitians* (*Xcv*) was assessed. Application of ASM reduced the disease index under greenhouse conditions by inducing resistance to the disease. The resistance induced could be recognized at 7 days after treatment and it lasted for another 4 days. The untreated plants showed rapid development of the disease. The disease severity was reduced by 75 % in ASM-treated lettuce plants. Remarkable decrease in the growth of pathogen in plant tissue was indicated by PCR assays. Treatment of lettuce seedlings with ASM, followed by inoculation with *Xcv*, resulted in significant enhancement of activities of chitinases. The results indicated

that ASM might protect lettuce plants by reducing pathogen development directly and also by increasing host resistance indirectly (Yigit 2011).

The potential of acibenzolar-*S*-methyl (ASM) in reducing the incidence and severity of spotted wilt disease caused by *Tomato spotted wilt virus* (TSWV) in flue-cured tobacco cv. K-326 was assessed. ASM application reduced the final incidence of spotted wilt disease significantly under field conditions (Csinos et al. 2001). In a later study, the mechanism of induction of resistance to TSWV in flue-cured tobacco was investigated. Activation of systemic acquired resistance (SAR) by ASM in flue-cured tobacco was assessed under greenhouse conditions by challenge inoculation with a severe isolate of TSWV. ASM restricted the virus replication and movement and as a result, it reduced systemic infection. As the dosage of ASM increased from 0.25 to 2.0 g a.i./7,000 plants, the number of local and systemic infection and relative levels of TSWV in the treated plants decreased. At 4.0 g of ASM, the highest level of disease resistance was observed, compared with lower dosages of the inducer. But at this dose, the treated plants showed highest level of phytotoxic symptoms and overall stunting of the plants. Activation of resistance was observed within 2 days after treatment with ASM and a high level of resistance was observed at 5 days onward. Expression of PR-protein gene PR3 and different classes of PR-proteins such as PR-1, PR-3 and PR-5 were detected at 2 days post-ASM treatment which inversely correlated with reduction in the number of local lesions induced by TSWV. Accumulation of PR-proteins was weak at 1 day-post-treatment (DPT) with ASM, but a strong accumulation was observed at two DPT. Challenge inoculation of plants at different times after ASM treatment showed that the number of local lesions caused by TSWV was reduced to the maximum extent at 2- DPT. A correlation between resistance induction and PR-protein induction by ASM was noted. Enzyme-linked immunosorbent assay (ELISA) was employed to quantify the TSWV titer in ASM-treated plants. Analysis of ELISA absorbance values showed reduction of relative virus levels in ASM-treated plants suggesting a possible suppressive effect of ASM on virus replication (Table 8.7). Application of gibberellic acid in combination with ASM reversed the stunting caused by higher concentrations of ASM. The results indicated the possibility of using ASM for inducing SAR as a management strategy to reduce the loss due to TSWV infection in flue-cured tobacco (Mandal et al. 2008; Appendix 8.2).

8.2.3 ß-Aminobutyric Acid

The nonprotein amino acid ß-aminobutyric acid (BABA) has been shown to induce resistance in plants against many diseases caused by microbial pathogens. Local treatments of tomato and potato with BABA induced systemic protection in tomato and potato against *Phytophthora infestans*, in tobacco against *Pernospora tabacina* (Cohen 1994; Cohen et al. 1994), in lettuce against *Bremia lactucae* (Pajot et al. 2001) and in cauliflower against *Peronospora parasitica* (Silue et al. 2002). BABA was used to induce resistance in grapevine against downy mildew pathogen *Plasmopara viticola*. The components playing a role in the establishment of BABA-induced

Table 8.7 Differential responses of young and older flue-cured tobacco plants treated with ASM to *Tomato spotted wilt virus* (TSWV) infection (Mandal et al. 2008)

	75-days old plants		45-days old plants	
Type of infection	Treated	Nontreated	Treated	Nontreated
Local infection				
Number of lesions/plant	3.0	20.3	0.2	87.6
Percentage of plants				
Symptomatic	63.3	100.0	10.0	100.0
ELISA-positive	90.0	100.0	60.0	100.0
Systemic infection				
Symptomatic	0.0	0.0	3.3	50.0
ELISA-positive[a]	67.0a	13.3a	6.7a	73.3b

[a]Mean values for ELISA with same letter are not significantly different (P = 0.05)

resistance (IR) and the involvement of different signal transduction pathways in BABA-IR were investigated. Application of BABA resulted in strong reduction in mycelial growth and sporulation in the susceptible cv. Chasselas. Best protection was provided by BABA, followed by jasmonic acid (JA). On the other hand, acibenzolar-*S*-methyl (ASM), salicylic acid (SA) and abscisic acid (ABA) did not increase the level of resistance to downy mildew disease significantly. Marker genes for the SA and JA pathways showed potentiated expression patterns in BABA-treated plants, following infection. The callose synthesis inhibitor 2-deoxy-D-GLUCOSE partially suppressed BABA- and JA- induced resistance against *P. viticola* in Chasselas. Application of PAL-inhibitor and lipoxygenase inhibitor also led to reduced level of resistance due to BABA treatment, suggesting that callose deposition, as well as defense mechanisms, depending on phenylpropanoid and JA pathways, contributing to BABA-IR. The results suggested that primed callose deposition plays a major role in BABA-IR signal transduction pathway (Md. Hamiduzzaman et al. 2005). The mechanism of induction of resistance by ß-aminobutyric acid (BABA) was studied, using the grapevine cv. Chasselas and Solaris respectively susceptible and resistant to the downy mildew disease caused by *Plasmopara viticola*. After treatment with BABA, sporulation of *P. viticola* was strongly inhibited and accumulation of stilbenes increased with time following infection. Induction of trans-piceide, trans-resveratrol and more importantly of trans-ε and trans-δ- viniferin and trans-pterostilbene was observed in BABA-primed Chasselas. On the other hand, in BABA-primed Solaris, trans-resveratrol, trans-δ-viniferin and trans-pterostilbene were found in higher concentrations. Accumulation of stilbenes occurred more strongly in Solaris than in Chasselas, due to treatment with BABA. In addition, BABA-treatment of Solaris resulted in a rapid increase in transcript levels of three genes involved in phenylpropanoid pathway. Treatment of susceptible Chasselas prior to challenge inoculation with *P. viticola* led to the accumulation of specific phytoalexins that could not be detected in untreated susceptible grapevine plants. Such an accumulation of phytoalexins in treated plants provided protection against the downy mildew disease (Slaughter et al. 2008).

The elicitors of disease resistance, ß-aminobutyric acid (BABA) and benzothiadiazole (BTH), extracts of *Solidago canadensis* (CanG) and *Penicillium chrysogenum* (PEN), linoleic acid (LIN) and the BCA *Aureobasidium pullulans* (Aureo) were compared for their effectiveness in suppressing the development of grapevine downy mildew disease caused by *Plasmopara viticola* on plants grown in pots. BABA, BTH and CanG provided protection of more than 80 %, whereas PEN, LIN and Aureo could provide only minimal protection. BABA and Aureo did not inhibit zoospore germination, whereas a concentration-dependent inhibition of zoospore mobility was recorded for all other treatments. BTH, CanG, PEN and LIN induced the synthesis of a broad-spectrum of resistance-related metabolites, while Aureo did not induce any defense response in treated plants. BABA treatment caused formation of necrotic spots and induced synthesis of PR-proteins soon after challenge inoculation. The results indicated the possibility of exploiting synergistic effects of the resistance elicitors and fungicides for reducing the ecological burden, due to application of fungicides exclusively and repeatedly (Harm et al. 2011). In pearl millet treated with BABA (50 mM), reduction in severity of downy mildew disease caused by *Sclerospora graminicola* and induction of defense-related enzymes PAL, PO, ß-1,3-glucanase activities and contents of cell wall hydroxy-proline-rich glycoproteins (HRGP) were observed. Application of BABA resulted in increased seedling vigor (Shailasree et al. 2007). The protective effect of ß-aminobutyric acid (BABA) against another downy mildew pathogen *Bremia lactucae*, infecting lettuce was investigated. Application of BABA induced local and systemic resistance in lettuce against *B. lactucae*. Systemic translocation of ^{14}C-BABA and systemic protection against lettuce downy mildew were found to be tightly correlated. BABA did not have any inhibitory effect on spore germination, appressorium formation or penetration of *B. lactucae* into the host. Epifluorescence and confocal microscopy revealed that BABA induced rapid encasement with callose of the primary infection structures of the pathogen, thus preventing it from further developing intracellular hyphae and haustoria. Invaded host cells treated with BABA did not accumulate phenolics, callose and lignin or express HR. In contrast, cells of genetically resistant cultivars accumulated phenolics, callose and lignin and exhibited HR within 1 day after inoculation. The callose synthesis inhibitor DDG did not inhibit callose encasement nor compromised the resistance induced by BABA. PR-proteins accumulated too late to be responsible for the induced resistance. Induction of rapid accumulation of H_2O_2 in the penetrated epidermal cells of lettuce inoculated with *B. lactucae* was also observed in BABA-treated plants (Cohen et al. 2010).

The protective effect of ß-aminobutyric acid (BABA) against potato late blight disease caused by *Phytophthora infestan*s was demonstrated using two potato cultivars Bintje and Pampeana with different horizontal resistance. Foliar pretreatment at 30 days after emergence provided a 60 % protection in cv. Pampeana against *P. infestans*. The expression of defense molecules such as glucanases, chitinases and phenolic compounds was stimulated, following BABA-treatment (Altamiranda et al. 2008). The responses of potato cultivars with different levels of resistance to BABA application-induced resistance against *P. infestans* were investigated. In addition, the effect of BABA on the activity of a potential pathogenic factor of *Fusarium solani* infecting potato tubers was also assessed. Plants receiving four

applications of BABA throughout the crop cycle, produced tubers with greater resistance to both *P. infestans* and *F. solani* than those from non-treated plants. Tuber slices from treated plants, inoculated with *P. infestans*, showed an increase in phenolic and phytoalexins contents. The aspartyl protease StAP1 accumulation was also higher in tubers obtained from treated plants and inoculated with *P. infestans*. In the potato-*F. solani* interaction, infected tubers from BABA-treated plants exhibited minor fungal proteolytic activity than infected and non-treated tubers. Application of BABA improved the growth of potato plants, in addition to protection provided against the fungal pathogens. Further, treatments with BABA increased the resistance of potatoes, but the degree of enhancement depended on the natural level of resistance of the potato cultivar concerned (Olivieri et al. 2009).

Different factors influencing the potential of the ß-aminobutyric acid (BABA) to induce resistance to the late blight pathogen *Phytophthora infestans*, infecting potato were studied. In the greenhouse, the durability of resistance induced by BABA, the dose–response relationships in susceptible (Bintje) and partially resistant (Ovatio and Superb) cultivars and effects of combined application of BABA and fungicides were investigated. BABA treatment significantly reduced the lesion size by an average of 40–50 %, compared with nontreated controls. The induced resistance lasted for only 4–5 days and the efficacy was reduced progressively with lapse of time. Combination of BABA and fungicides appeared to have an additive effect both under greenhouse and field conditions. With increase in the concentration of BABA, the protective effect became stronger proportionally. The partially resistant potato cultivars Ovatio and Superb reacted to lower concentrations of BABA, whereas no significant effect of BABA application was discernible under field conditions. BABA was able to compensate a 20–25 % reduction in the dose of the fungicide Shirlan, as the combination was as effective as full dose of Shirlan alone. The results indicated that BABA could be applied as a component of disease management system directed towards the major disease of potato (Liljeroth et al. 2010).

The protective ability of ß-aminobutyric acid (BABA) against a biotrophic pathogen *Pseudoperonospora cubensis*, causing downy mildew and necrotic pathogen *Colletotrichum lagenarium* causing anthracnose disease of cucumber plants was assessed. A fast and reproducible leaf disc assay was developed to evaluate the effect of BABA on cucumbers infected by these two pathogens. Accumulation of callose was observed in the interactions with both pathogens, following treatment of cucumber plants with BABA. In addition, a localized rapid cell death and production of reactive oxygen intermediates were detected, after infection by downy mildew pathogen. In contrast, presence of degenerated primary hyphae was observed in BABA-treated leaf tissues inoculated with *C. lagenarium*. The results revealed the differential effects of BABA in plants infected by two pathogens with different modes of parasitism (Walz and Simon 2009). *Fusarium verticillioides*, causing Fusarium ear rot disease of maize, drastically reduces the quantity and quality of grains and contaminates the grains with fumonisin (mycotoxin) harmful to humans and animals. The effectiveness of chemical elicitors in reducing infection by *F. verticillioides* was investigated. ß-aminobutyric acid (BABA), benzothiadiazole (BTH), harpin protein, 2,6-dichloroisonicotinic acid (INA) and methyl jasmonate (MeJA) were evaluated in

field trials, in addition to the fungicides difenconazole (triazole) and azoxystrobin (strobilurin). Maize plants were inoculated with *F. veriticillioides* isolate MRC826 (a high fumonisin producer), after treatment with elicitors and fungicides. Visual rating of Fusarium ear rot was carried out and the fumonisin B_1, B_2 and B_3 contents in grains were quantified with high performance liquid chromatography (HPLC) technique. Neither the elicitors nor fungicides consistently reduced the Fusarium ear rot and/ or fumonisin contamination significantly. Treatment effects on fumonisin content were influenced by maize genotype and trial location. Although the elicitors were effective in reducing maize foliar disease, they were found to be ineffective in reducing infection of maize plants by *F. verticillioides* and fumonisin contamination of grains (Small et al. 2012).

The efficacy of BABA in protecting two artichoke cultivars C3 and Explorer was assessed against the white mold pathogen *Sclerotinia sclerotiorum*. Treatments with BABA by soil drenching induced a high level of resistance against *S. sclerotiorum* in artichoke plantlets of both cultivars C3 and Explorer with similar level of protection. Further, a consistent increase in peroxidase activity paralleled with the differential induction of alkaline isoenzyme with a pI of 8.6 also occurred. The results indicated that a correlation between BABA-induced resistance (BABA-IR) and an augmented capacity to express basal defense responses were more pronounced in the cultivar C3 (Marcucci et al. 2010). The comparative efficacy of abiotic inducers of disease resistance, ß-aminobutyric acid (BABA), benzothiadiazole (BTH) and salicylic acid (SA) was assessed against pea rust pathogen *Uromyces pisi*. Exogenous application of SA (5–10 mM) prior to rust inoculation did not protect pea plants against rust locally, but reduced infection systemically in the first upper leaf node only. Phytotoxicity symptoms were observed on SA-treated plants. Application of BTH (1–10 mM) provided locally a 30–40 % reduction in infection frequency (IF). At least 5 mM was required to reduce rust infection systemically in the first upper leaf. Application of BABA reduced rust infection more effectively at each concentration and pair of leaves tested. On the first leaf BABA reduced IF values between 46 % (at 5 mM) and 58 % (at 50 mM), compared with untreated control. BABA effectively induced systemic resistance in the second and third nodes and the IF values were reduced by 50 % of the values in control treatment. No phytotoxic effects were evident in plants treated with BABA at any of the concentrations tested (Table 8.8). The results indicated the greater efficacy of BABA in protecting the pea plants against rust disease and absence of phytotoxicity symptoms, following treatment with BABA (Barilli et al. 2010; Appendix 8.3). The ability of ß-aminobutyric acid (BABA) to protect *Brassica napus* plants against infection by *Leptosphaeria maculans* was assessed. BABA is known to induce resistance in plants against several diseases. In this investigation, BABA displayed direct inhibitory effect against *L. maculans* in in vitro assays. The EC_{50} of BABA was similar to that of the fungicide tebuconazole. Both spore germination and hyphal growth were inhibited. Addition of trypton reversed the toxic effect of BABA in the culture medium, suggesting that BABA might inhibit inorganic nitrogen utilization by the pathogen. Suppression of disease progression in plants and antifungal activity in vitro was weaker for α-aminobutyric acid and negligible for γ-aminobutyric acid. In contrast to benzothiadiazole (BTH), another resistance

Table 8.8 Effect of inducers of resistance on pea rust disease development (Barilli et al. 2010)

	Relative infection frequency (IF)[a]		
Inducer/concentration (mM)	I leaf node	II leaf node	III leaf node
Salicylic acid (SA)			
Control	100.0[y] a (167.5)	100.0a (171.5)	100.0a (169.0)
5.0	80.7a	57.7b	82.2a
7.0	83.0a	48.7b	80.8a
8.5	79.0a	60.7b	82.1a
10.0	80.3a	51.7b	78.4a
BTH			
Control	100.0a (144.5)	100.0a (127.4)	100.0 (94.1)
1.0	66.5b	90.5a	99.0a
5.0	65.5b	50.0b	80.8a
10.0	64.5b	64.7b	63.8b
BABA			
Control	100.0a (104.8)	100.0a (113.4)	100.0a (113.2)
5.0	54.3b	42.3b	42.4b
10.0	54.9b	66.4b	43.9b
20.0	48.0b	59.0b	39.0b
50.0	42.3b	67.8b	51.3b

Real values of control are indicated in parenthesis (pustules/cm^2)
y-Means followed by the same letter in the same column are not significantly different as per LSD test ($P<0.01$)
[a]Infection frequency (IF) is expressed as relative values of the control taken as 100

inducer, the effect of BABA on disease development was nearly independent of the timing of treatment, indicating possible antifungal activity in planta. On the other hand, quantification of multiple hormones and an expression analysis indicated that treatment with BABA induced synthesis of salicylic acid (SA) and expression of SA marker gene PR-1. However, no evidence for priming of SA responses to *L. maculans* was obtained. The antifungal activity of BABA against *L. maculans* could be another possible mechanism of action by which BABA might protect plants against plant pathogens (Šašek et al. 2012).

The ability of ß-aminobutyric acid (BABA) to induce resistance in postharvest produce was assessed. BABA was able to induce disease resistance in grapefruit peel tissue. Application of BABA to specific wound sites on fruit peel surface induced resistance to *Penicillium digitatum*, infecting citrus fruits, in a concentration-dependent manner. BABA was found to be most effective at 20 mM and rather less effective at either higher or lower concentrations. The effect of BABA in inducing resistance to *P. digitatum* in the fruit peel surface was local and limited to the vicinity (within 1–2 cm) of the BABA-treated site. Direct antifungal activity of BABA at higher concentrations was revealed by in vitro assays. Increasing concentrations of BABA (1–100 mM) exhibited inhibition of spore germination and germ tube elongation of *P. digitatum*. Induction of resistance to infection by *P. digitatum* by BABA was accompanied by the activation of defense-related enzyme PAL, after 72 h in grapefruit peel tissue, in addition to stimulation of chitinase gene expression and accumulation of proteins after 48 h (Porat et al. 2003). The mechanism of biocontrol activity of ß-aminobutyric acid

(BABA) against the apple blue mold pathogen *Penicillium expansum* was investigated. Application of BABA provided effective control of the disease, due to direct fungitoxic effect on the pathogen and induction of resistance in apple fruits against the pathogen. BABA exhibited strong inhibitory effect on spore germination and germ tube elongation of *P. expansum*. Observations under fluorescent microscope on propidium iodide (PI)-stained pathogen mycelium treated with BABA revealed the damage to plasma membrane of spores and leakage of protein and sugar at high levels. Furthermore, BABA treatment induced a significant increase in the activities of the defense-related enzymes chitinase, ß-1,3-glucanase and peroxidase in apple fruit. The results suggested that disease suppression by BABA might be associated with its direct antifungal property and ability to elicit disease resistance, as reflected by stimulation of defense-related enzymes in apple fruits (Zhang et al. 2011).

The effectiveness of biotic and abiotic biocontrol agents alone or in combination for the suppression of development of apple blue mold disease caused by *Penicillium expansum* was assessed. The biotic BCAs *Trichoderma atroviride, T. harzianum, T. reesei* and *Pseudomonas syringae* pv. *syringae* (*Pss*) and abiotic resistance inducing ß-aminobutyric acid (BABA), acibenzolar-*S*-methyl (ASM) and methyl jasomonate (MeJA) were tested for their efficacy in suppressing the development of apple blue mold disease. The biotic and abiotic agents had direct inhibitory effects on the development of *P. expansum*, resulting in significant reduction in the percentages of infection and/ or size of lesions formed on fruits. The antifungal activities of ASM, BABA and MeJA were observed in in vitro tests. Treatments with resistance inducers and biotic agents did not control infection in the untreated wounds that were made one cm away from the original treated wound sites. However, RT-PCR assays indicated that treatments with *Pss* strain1, 3S and ASM induced significant increases in the levels of gene transcripts of PR-proteins PR-1a, PR-2, PR-5 and PR-8 in apple peel, compared with untreated controls. In addition, immunoblotting revealed an accumulation of PR-2 and PR-5 proteins in treated samples. Although the resistant responses in apple fruit were triggered by the biotic and abiotic agents, infection by *P. expansum* was not arrested by them. The results suggested that induction of resistance by biotic and abiotic agents tested, might not be a useful approach for the management of blue mold disease of apple during storage (Quaglia et al. 2011).

Involvement of resistance induced by DL-ß-aminobutyric acid (BABA) in tomato in suppressing the development of bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) was studied. Different doses of BABA (250–100 µg/ml) were applied on 3-week old tomato plants and then inoculated with the bacterial suspension (10^8 CFU/ml). BABA did not show any antibacterial effect in in vitro test. Foliage sprays of 500 µg/ml of BABA did suppress disease development up to 54 % by 14 days after inoculation. Bacterial populations were reduced by 84 % in planta by BABA treatment of tomato plants in comparison with water-treated control plants by 4 days after inoculation. Biochemical analysis showed that inoculated, BABA-treated plants showed higher activities of PAL, PO and higher levels of H_2O_2 than that of control plants at 1 day after treatment. The results suggested that higher levels of defense-related enzymes and H_2O_2 might be associated with induction of resistance by BABA to tomato bacterial canker disease (Baysal et al. 2005). The ability of BABA alone or in combination with

Pseudomonas fluorescens isolate CW2 to suppress the development of tomato bacterial wilt disease caused by *Cmm* was investigated. Soil treatment with BABA or isolate CW2 significantly reduced the incidence of tomato bacterial wilt disease. Application of BABA (0.5 mg/ml, 50 ml/pot) drenched at 4 days before inoculation (dbi) with *Cmm* or CW2 (2×10^8 CFU/ml, 50 ml/pot) applied at 2 dbi reduced leaf wilting index (LWI) by 51.2 and 41.6 % respectively and vascular browning indeed (VBI) by 63.7 and 48.8 % respectively. Combined sequential treatments with BABA applied at 4 dbi and CW2 at 2 dbi or CW2 at 4 dbi and BABA at 2 dbi were found to be still more effective in reducing disease symptoms than individual application. The sequential application of BABA/CW2 or CW2/BABA significantly increased fresh and dry weights of roots and shoots of tomato plants, compared to infected control plants. The roots of tomato plants treated with BABA alone or in combination revealed the presence of highest total and free salicylic acid (SA) contents. The results revealed greater efficacy of combined application of an inducer of disease resistance and biotic agent in, not only providing protection to tomato against bacterial wilt disease, but also in promoting the growth of tomato plants (Hassan and Buchenauer 2008).

8.2.4 Harpin

Harpin is an acidic, heat-stable, glycine-rich, 44-kDa protein encoded by the *hrpN* gene of the bacterial pathogen *Erwinia amylovora*, causing fire blight disease of pear and apple. This is the first known product isolated for the bacteria, capable of eliciting hypersensitive response (HR) in plants (Baker et al. 1993; Mulin et al. 1998). Harpin induced systemic acquired resistance (SAR) in tobacco and *Arabidopsis* (Dong et al. 1999). Harpin has been commercially produced and marketed as "Messenger" which has been suggested for the control of fungal and viral diseases, as well as a plant growth promoter (Wei et al. 1998). Harpin proteins from Gram-negative plant pathogenic bacteria, affecting virulence in host plants, induce hypersensitive cell death (HCD) in nonhosts as well as stimulate defense systems, resulting in enhanced resistance to diseases in plants treated with harpin protein. Nine functional fragments of $HpaG_{xooc}$, a 137-amino acid harpin protein from rice bacterial leaf streak pathogen *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) were obtained by polymerase chain reaction (PCR)-based mutagenesis. These fragments induced different responses in tobacco and rice plants treated with them. The fragments $HpaG_{10\text{-}42}$ and $HpaG_{xooc}$ stimulated stronger defense responses and enhanced more growth in rice than the full-length parent protein $HpaG_{xooc}$. The $HpaG_{10\text{-}42}$ fragment was the most effective in stimulating rice plant growth as well as resistance to *Xoo* and *Magnaporthe grisea* (causing rice blast disease). In addition, $HpaG_{10\text{-}42}$ fragment was more active than $HpaG_{xooc}$ in inducing expression of several genes that regulate rice defense and growth processes and activating certain signaling pathways which may explain the greater beneficial effects observed from treatment with this fragment. The results suggested that the harpin protein fragment $HpaG_{10\text{-}42}$ might be considered as a tool for inducing resistance to major diseases of rice (Chen et al. 2008).

Application of harpin on harvested Red Delicious apples induced resistance to the blue mold disease caused by *Penicillium expansum* (de Capdeville et al. 2002). In a further study, harpin was sprayed on harvested Red Delicious apple at 0, 40, 80 and 160 mg/l as commercial formulation. At 48, 96 and 144 h after treatment, the apples were inoculated with spore suspensions of *P. expansum* at a concentration of 10^3, 5×10^3 and 10^4 conidia /ml. The level of resistance induced by harpin depended on harpin concentration, inoculum concentration and interval between treatment and challenge inoculation with *P. expansum*. The lesion diameters in treatments were directly proportional to the inoculum concentration. Fewer fruits treated with harpin were infected, compared with untreated controls and disease development was appreciably arrested in treated fruits. In another experiment apple trees of the cultivars McIntosh, Empire and Red Delicious were sprayed with different concentrations of harpin (20, 40 and 80 mg/l) at 8 or 4 days before harvest. Fruits were harvested, wounded and inoculated with the pathogen and stored in a commercial cold room. The fruits treated with harpin showed significant reduction in disease incidence. Overall, Red Delicious fruit had the lowest percentage of diseased fruits, both in treated and untreated controls. The effectiveness of harpin treatment persisted even after cold storage, when the non-infected fruits were placed at 20 °C for 7 days. The level of protection provided by harpin was proportional to the concentration applied on trees applied before harvest. However, no difference in the extent of control was seen as a function of interval between the spray time and harvest. Spraying apple trees with harpin a few days (4–8), before harvest appeared to be a feasible disease management strategy for apple blue mold disease during storage (de Capdeville et al. 2003).

8.2.5 Saccharin

The synthetic compound probenazole was reported to be effective against rice blast disease caused by *Magnaporthe grisea* and bacterial blight disease caused by *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Watanabe et al. 1979). Saccharin, a metabolite of probenazole, was shown to be an inducer of systemic resistance in tobacco against *Tobacco mosaic virus* (TMV), in cucumber against *Colletotrichum lagenarium* and in bean against *Uromyces appendiculatus* by Siegrist et al. (1997). The effectiveness of application of saccharin as an inducer of resistance against rust disease caused by *Uromyces viciae-fabae* in broad bean (*Vicia faba*) cv. Aquadulce was assessed. Saccharin was applied at the rate of 200 ml at a concentration of 3 mM at three-leaf stage, either as soil drench or by painting the solution on the first leaf. Plants were challenged with rust pathogen at 1, 6, 10 and 14 days after treatment with saccharin. The saccharin drench was more effective in protecting the plants and the enhanced level of resistance to the rust disease was not apparent until 6 days after treatment. However, systemic protection against rust infection could be observed up to 14 days after treatment as soil drench. No significant growth promotion effect of

saccharin treatment was evident. However, the number of leaves was reduced in saccharin-treated bean plants, indicating limited negative impact of saccharin on plant growth (Boyle and Walters 2005).

Saccharin was demonstrated to provide effective local and systemic protection in barley against powdery mildew disease caused by *Blumeria graminis* f.sp. *hordei*. The percentage of leaf area affected by the disease was significantly reduced on first leaves of plants challenged with the pathogen at 1 day after application of 3 mM to the first leaves of barley seedlings, while powdery mildew-affected area was reduced on the second leaves, after treatment of first leaves with saccharin. Further, disease severity was still reduced in barley plants challenge-inoculated at 14 days after saccharin treatment. Application of saccharin to leaves at 2 h prior to inoculation, did not have any adverse effect on conidial germination or formation of appressoria, indicating the absence of direct antifungal activity of saccharin. Application of saccharin as a drench was effective as foliar application in reducing mildew development. Saccharin at 3 mM concentration reduced the disease by 30–88 %. Saccharin did not induce any significant negative effect on barley growth (Boyle and Walters 2006). Application of saccharin induced systemic resistance in barley against the development of the powdery mildew disease. The physiological and biochemical analyses revealed that phenylalanine ammonia lyase (PAL) activity was reduced significantly in second leaves at 18 and 48 h after inoculation with *Blumeria graminis* f.sp. *hordei* (*Bgh*) in plants treated with saccharin 14 days earlier. Peroxidase activity increased significantly in plants challenged with *Bgh* within 6 days after saccharin application. The changes were, however, not apparent until 48 h after challenge inoculation. Treatment with saccharin resulted in an increase of 33 % in peroxidase (PO) activity, compared with controls. In plants inoculated at 10 or 14 days after saccharin application, cinnamyl alcohol dehydrogenase (CAD) activity increased prior to, and 18, 24 and 48 h after inoculation of barley plants with *Bgh.* CAD activity increased approximately twofold, compared with control. However, in contrast to PO, CAD activity was significantly higher in saccharin-treated plants prior to and after inoculation with the pathogen, suggesting that saccharin might prime CAD prior to challenge inoculation with the pathogen. The results indicated that saccharin reduced the powdery mildew disease severity which might be associated with enhancement of defense-related enzyme activity (Boyle and Walters 2006).

8.2.6 Antimicrobial Peptides

Antimicrobial peptides (AMPs) (12–50 amino acids long) known also as innate immunity host defense peptides or innate defense regulators, are key components of the innate immune system in all organisms, providing a fast-acting defense against invading pathogens (Brogden 2005; Marcos et al. 2008). The amino acid residues most abundant in AMPs are hydrophobic and cationic which results in electrostatic

attraction to negatively charged microbial envelopes, leading to lysis of the cell membrane. The differential electrostatic attraction results in the specificity of attraction to microbes and low toxicity to plant and animal cells lacking charged membrane phospholipids (Makovitzki et al. 2007; Rosenfeld et al. 2008). A subfamily of AMPs with strong antimicrobial activity includes lipopeptides which produced nonribosomally in bacteria and fungi. Cyclic lipopeptides, such as surfactins and fengycins from bacterial BCA *Bacillus subtilis* or massetolide A from *Pseudomonas fluorescens* SS101 were shown to stimulate induction of systemic resistance (ISR) in bean and tomato plants (Ongena et al. 2007; Tran et al. 2007). A new family of synthetic ultrashort peptides with a broad spectrum of antimicrobial activity, affecting phytopathogenic fungi and bacteria without phytotoxicity was developed (Makovitzki et al. 2006). Small antibacterial peptides with lytic activity have been detected in a variety of microorganisms. Cecropin A and B peptides have been shown to have antifungal properties also. The cecropin A-based peptide inhibited germination of *Colletotrichum coccodes* at 50 μm concentrations. The DNA sequence encoding the peptide was cloned in the plasmid pRS413, using the *Saccharomyces cerevisiae* invertase leader sequence for secretion of the peptide and expressed in *S. cerevisiae*. The yeast transformants Y-20 and Y-47 containing the sequences encoding the invertase signal: antimicrobial peptide and YWT-41 containing only the plasmid were compared for their ability to inhibit the germination of *C. coccodes* spore. The extract of transformants did not inhibit germination, but completely prevented the growth of hyphae, after spore germination. Inoculation of tomato fruit with spores of *C. coccodes* treated for 24 h with extracts of Y-20 and Y-27 resulted, in inhibition of symptom development completely at 6 days of incubation at 20 °C, compared with the control transformed yeast YWT-41 and untransformed WT strains. Expression of the antifungal peptide in yeast might result in suppression of the development of decay in tomato fruits (Jones and Prusky 2002).

Plant nonspecific lipid transfer proteins (ns-LTPs) are abundant small, soluble cysteine-rich proteins have been recorded in several plants, including crops like rice. Some of the ns-LTPs have been implicated in plant defense responses to diseases. An association between ns-LTP and bunt disease in wheat was reported (Lu et al. 2005). Phylogenetic analyses revealed two major ns-LTP families in wheat. Eight wheat ns-LTP genes from different clades were cloned into the expression vector pPICZα and transformed into the yeast *Pichia pastoris*. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), Western blotting and in vitro lipid-binding activity assay confirmed that all the eight ns-LTPs were successfully expressed and capable of in vitro binding fatty acid molecules. Three selected ns-LTPs exhibited 50 % inhibition of mycelial growth and spore germination of wheat and nonwheat fungal pathogens (Fig. 8.6). This investigation demonstrated a differential in vitro toxicity of the wheat ns-LTPs to a broad range of fungal pathogens and this toxicity was related to induced permeability changes in the fungal membranes. The different inducible and constitutively expressed ns-LTPs might play a role in an enhanced generalized nonspecific defense response induced, during abiotic and biotic stresses that provide transitory protection against a wide variety of potential pathogens (Sun et al. 2008).

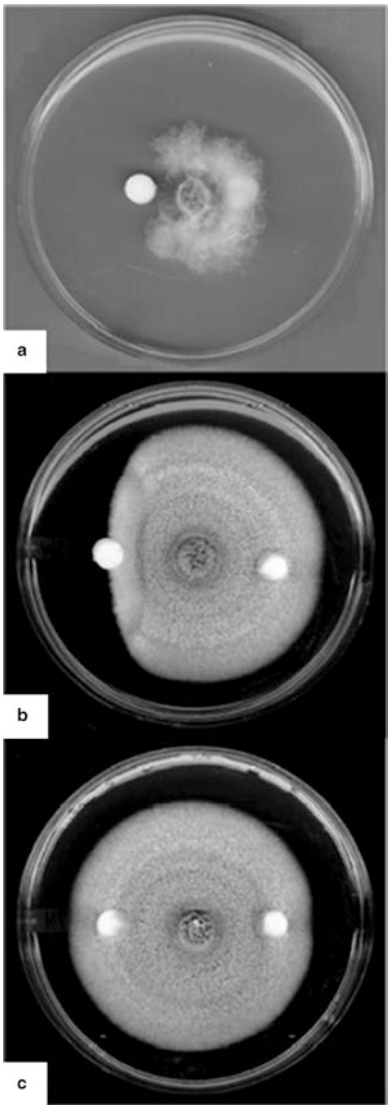

Fig. 8.6 Inhibition of mycelial growth of *Fusarium graminearum* and *Stagonospora nodorum* with nonspecific lipid transfer protein (ns-LTP) in in vitro assays using 96-well plates in potato dextrose broth medium (**a**) TsBs108F7 against *F. graminearum*; (**b**) TaLt10F9 and (**c**) TaLt709L6 against *S. nodorum* (Courtesy of Sun et al. 2008 and with kind permission of The American Phytopathological Society, MN, USA)

Three short peptides P1, P2 and P3 attached to fatty acids of various lengths were evaluated for their potential to suppress the development of fungal and bacterial plant pathogens infecting cucumber. The peptides P1 and P2 could significantly induce systemic resistance against *Botrytis cinerea, Pseudomonas syringae* pv. *lachrymans* and *P. syringae* pv. *tomato* DC3000 to a level comparable to that provided by *Trichoderma asperellum* T203, a known fungal inducer of systemic responses and of microbial leaf compounds in cucumber. The peptide P3 was unable to induce systemic resistance significantly. A direct correlation was observed between lipopeptide activities in medium alkalinization and induction of plant

defense responses with regard to both gene expression and antimicrobial compound synthesis. These peptides showed direct antifungal/antibacterial activities at micromolar concentrations. Gene expression studies with cucumber suggested that expression of specific genes was higher in pathogen-challenged plants pretreated with the lipopeptides. This investigation provided evidence that synthetic ultrashort lipopeptides, similar to *Bacillus subtilis* cyclic lipopeptides could induce defense signaling pathways in plants and systemic protection to foliar bacterial and fungal diseases in cucumber and *Arabdiopsis* plants. Application of synthetic fully biodegradable and low-cost AMPs for protecting plants against microbial plant pathogens appears to be a potential disease management strategy (Brotman et al. 2009).

8.2.7 Miscellaneous Organic Compounds

Imidacloprid is a neo-nicotinoid that breaks down in planta into 6-chloronicotinic acid, a compound closely related to the systemic acquired resistance (SAR) inducer, isonicotinic acid (INA). Imidacloprid (Admire, Bayer) was applied as a systemic insecticide for the control of many pests. Soil application of imidacloprid was effective against citrus canker disease caused by *Xanthomonas citri* subsp. *citri* (*Xcc*). Potted Swingle citrumelo seedlings (*Citrus paradisi* × *Poncirus trifoliata*) were treated with imidacloprid and isonicotinic acid and acibenzolar-*S*-methyl (ASM) (known SAR inducers) as soil drenches or with ASM as a foliar spray 1 week prior to inoculation of immature leaves with *Xcc*. Seedlings were reinoculated four times over a 24-week period. SAR induction was confirmed by expression of the PR-2 gene (ß-1,3-glucanase). Soil drenches of imidacloprid, INA and ASM induced a high and persistent up-regulation of PR-2 gene expression and reduced the number of canker lesions for up to 24 weeks, compared to 4 weeks for foliar ASM. Soil application of SAR inducers reduced canker lesions up to 70 %, compared with the untreated inoculated plants. Lesion size was significantly reduced in treated plants, compared with pustular lesions on untreated plants. Populations of *Xcc* per leaf were reduced, due to soil treatment with imidacloprid and known SAR inducers (Francis et al. 2009).

The immobile phytohormone 24-epibrassinolide (EBL), with antistress activity was tested for its ability to reduce the incidence of *Fusarium oxysporum* f.sp. *cucumerinum* (*Foc*) by treating the roots and foliage of test plants. EBL pretreatment to either roots or shoots significantly reduced disease severity. The treatment improved plant growth, irrespective of method of application of EBL. Application of EBL resulted in reduction in pathogen population on root surfaces and in nutrient solutions, but increased the population of fungi and actinobacteria on root surfaces. PCR-DGGE analysis showed that *Foc* inoculation had significant effects on the bacterial community on root surfaces, as expressed by a decreased diversity index and evenness index. In contrast, EBL applications nullified the effects of the pathogen on bacterial community in the rhizosphere of treated plants. In addition, the *Foc*-inoculated plants had several kinds of decomposing bacteria on their root surfaces, whereas growth-promoting bacteria were associated with the root

surfaces of plants treated with EBL. The results suggested that the differential effects of EBL treatment might favor better plant growth and also result in reduction in the incidence of Fusarium wilt disease in cucumber (Ding et al. 2009). Inducers of SAT, imidacloprid, thiamethoxam and ASM were evaluated for their efficacy in suppressing the development of citrus canker disease caused by *Xanthomonas citri* subsp. *citri* (*Xcc*), using Ray Ruby grapefruit trees as experimental plants. Canker disease incidence on each set of vegetative flushes was recorded as the percentage of the total leaves with lesions. All treatments significantly reduced foliar infection by *Xcc* on the combined spring-summer fall flushes. The standard chemicals copper hydroxide and streptomycin (11 sprays at 3-week interval) effectively reduced canker incidence on shoot flushes produced throughout the season, compared with untreated controls. On the other hand, soil-applied SAR inducers reduced foliar infection, depending on the rate, frequency and timing of application. During tropical storm, SAR inducers were generally ineffective and the protective efficiency varied depending on the concentration and timing of application (Graham and Myers 2011).

The potential of menadione sodium bisulphate (MSB), a water soluble addition compound of vitamin K3 to induce resistance in oilseed rape (*Brassica napus*) cv. Bristol plants against infection by A-type *Leptosphaeria maculans*, causing Phoma stem canker disease, was assessed. Real-time PCR assay was employed to analyze the expression of genes encoding PR-1 and an ascorbate peroxidase (APX) to determine the possible mechanism of induction of resistance by MSB in oilseed rape. Pretreatment of oilseed rape plants with MSB, locally and systemically reduced the number and size of lesions, when inoculated with a suspension of pycnidiospores of *L. maculans*. Real-time PCR gene expression analysis using BTH for comparison demonstrated that MSB did not systemically enhance PR-1 expression of an ascorbate peroxidase, encoding gene APX in response to infection by *L. maculans* and wounding. Increases detected in APX transcript levels were highest at 24/48 h, after inoculation in MSB-treated plants. The results suggested that MSB might induce resistance in oilseed rape plants independent of PR-1, in a way that might involve enhanced production of reactive oxygen species (ROS). This study showed that the MSB could be an effective resistance activator, providing systemic protection to oilseed rape against stem canker disease (Borges et al. 2003).

8.3 Inorganic Compounds as Biological Control Agents

Inorganic compounds have been shown to induce resistance in plants to diseases caused by different microbial pathogens. Induction of resistance to a plant disease, using phosphate salts was demonstrated by Gottstein and Kuć (1989). Cucumber plants sprayed with phosphate solution developed resistance to anthracnose disease caused by *Colletotrichum lagenarium*. Application of 0.1 M phosphate on the upper surface of maize leaves induced systemic resistance to *Puccinia sorghi*, causing rust disease provided an additional advantage of stimulating the growth of

phosphate-treated plants. Phosphates are considered to generate an endogenous SAR signal, because of calcium sequestration at the points of phosphate application (Reuveni et al. 1994). Similar enhancement of resistance of cucumber plants to powdery mildew pathogen *Podosphaera* (*fuliginea*) *xanthii* was also observed, following application of phosphates. The activities of peroxidase and ß-1,3-glucanase were increased in protected uninoculated top leaves of cucumber plants (Reuveni et al. 1997).

Nutrient solutions (at 5, 20 and 40 ppm) containing phosphorus (P) applied through a hydroponics system provided induced systemic resistance (ISR) against *Podosphaera fuliginea*, causing powdery mildew disease of cucumber. Protection was expressed as a significant reduction (up to 92 %), compared with control in the mildewed leaf area. ISR was also expressed as a 53–91 % reduction in the number of conidia of *P. fuliginea* per infected leaf area, as determined on first true leaf and on the next leaf at 9 days after inoculation. A concentration of 20 ppm P in the hydroponics solution was found to be optimum for inducing ISR. At this P concentration, uptake of Ca was enhanced in treated leaves by 50 %, compared with control. Foliar application of 1 % solution of monopotassium phosphate (MKP) effectively protected the foliage against powdery mildew, regardless of the concentration of P in the nutrient solutions. The effect of P application, as foliar spray, persisted for 21 days after inoculation with pathogens. The development of powdery mildews was reduced by 72.3 %, compared with the control (Reuveni et al. 2000). These results indicated the possibility of exploiting the phenomenon of induction of SAR for the protection of field crops against microbial pathogens by using inexpensive chemicals without any adverse effects on crop development. Phosphate-mediated resistance in cucumber was shown to be associated with localized cell death, preceded by a rapid generation of superoxide and H_2O_2. In addition, local and systemic resistance increases in levels of free and conjugated salicylic acid, following phosphate application, were also observed (Orober et al. 2002). When inorganic phosphate, an essential element for all organisms, was applied endogenously, a rejection reaction and superoxide generation were stimulated in pea tissues, but phytoalexins production was not induced. Phosphate-induced superoxide generation was sensitive to cycloheximide (CHX) and salicylhydroxamic acid (SHAM), indicating that part of the generation was dependent upon the expression of peroxidase gene(s). Peroxidases (POXs) are known not only to scavenge hydrogen peroxide with phenolics, but also generate superoxide via NADH oxidation in the presence of *p*-coumeric acid and manganese ion. Five pea POX cDNAs that were predicted to be located outside of the cells were cloned. The extent of accumulation of five POX mRNAs, NTPase mRNA and phenylalanine ammonia lyase mRNA was assessed by semi-quantitative RT-PCR assay. The expression of the five POX genes was induced by a fungal elicitor. On the other hand, inorganic phosphate induced the accumulation of POX11, POX14 and POX21 mRNAs, but not of POX13, POX29 and PsPAL1 mRNAs within 1–3 h after treatment of pea seedlings. The results suggested that inorganic phosphate might function as a signal transmitter inducing part of the plant defense responses (Kawahara et al. 2006).

Phosphites (Phi) are alkali metal salts of phosphorous acid with the ability to protect plants against microbial plant pathogens. Seed tubers and foliage of potato cultivars Shepody and Kennebec were treated with Phi to assess the effects on their ability to induce resistance against *Phytophthora infestans, Fusarium solani* and *Rhizoctonia solani*. Protection provided by seed tuber treatment with Phi was high against *P. infestans*, intermediate against *F. solani* and low against *R. solani.* In addition, seed tubers treated with calcium or potassium phosphites (CaPhi and KPhi) at 1 % commercial product, emerged earlier than untreated ones. When Phi sprays were applied on the foliage two to four times at different doses, high levels of protection against *P. infestans* were observed on both cultivars. Higher protection was recorded in Kennebec, when CaPhi was applied, whereas the protective effect of KPhi was greater in the cv. Shepody. Expression of ß-1,3-glucanases was induced at different times after treatment, but no correlation between ß-1,3-glucanases expression and level of foliar protection could be established. On the other hand, Phi positive protection effects did not produce negative effects on plant growth. Leaves in treated plants were darker green in color than those in untreated plants. Increase in Rubisco protein and a delay in setting of leaf senescence in Phi-treated leaves were also seen (Lobato et al. 2008). The protective potential of potassium phosphate (KPhi) was assessed in vitro and in vivo, in order to suppress the development of *Penicillium expansum*, causing blue mold disease in stored apples. Phi amended to malt extract agar medium at 2 and 4 mg/ml, completely inhibited the mycelial growth and conidial germination of the pathogen respectively. Infection in wounded and inoculated Elstar apples with a thiabendazole-resistant isolate of *P. expansum* was reduced significantly ($P = 0.01$), following a curative treatment with Phi at 2 mg/ml. When applied on freshly harvested unwounded Elstar apples, Phi (2 mg/ml) reduced blue mold incidence by about threefold, compared with control treatment. Phi treatment was equally effective as thiabendazole against natural blue mold infections, after 6 months of storage at 2 °C. The results indicated the possibility of employing potassium phosphate as part of general program for management of postharvest diseases caused by fungal pathogens (Amiri and Bompeix 2011).

A foliar fertilizer containing potassium phosphite, DL-3-aminobutyric acid (BABA) and benzothiadiazole (BTH) were evaluated for their ability to suppress the development of Fusarium head blight (FBH) disease of winter wheat caused by *Microdochium majus* (=*M. nivale* var. *majus*), using detached leaves for preliminary tests. Greenhouse-grown winter wheat plants were sprayed with aqueous solutions of the test chemicals at 7 days prior to inoculation of heads with the pathogen. Disease development was recorded, as number of bleached spikelets per inoculated spike. Spraying plants with the foliar fertilizer reduced the disease severity up to 40 %. Reduced disease development was observed in inoculated detached leaves pretreated with foliar fertilizer. The foliar fertilizer reduced mycelial growth, when incorporated in medium, indicating its antifungal activity. The resistance inducers BABA and BTH did not reduce disease development significantly. The results indicated that application of foliar fertilizer containing potassium phosphate might be an alternative strategy for reducing incidence of FBH in winter wheat (Hofgaard et al. 2010). The ability of phosphorous acid salts (PASs) to suppress the development of bacterial spot disease of tomato was assessed under greenhouse and field conditions.

Application of PAS was as effective as the standard copper bactericide treatment. PAS combined with the bactericide was more effective in reducing the disease incidence. Phytotoxicity due to PAS application was observed on tomato plants under greenhouse conditions. Pas affected multiplication of the pathogen *Xanthomonas perforans* only slightly in the in vitro assays and it was also ineffective as an inducer of disease resistance (Wen et al. 2009).

The effectiveness of induced resistance for protecting harvested produce against diseases in storage, following application of calcium (Ca) or silicon (Si) has been investigated. Vacuum infiltration of calcium (Ca) into apple fruits was found to be more effective than field application in protecting the fruits against gray mold disease (Conway and Sams 1983). Exogenous application of silicon (Si) as sodium metasilicate reduced the development of *Penicillium expansum* and *Monilinia fructicola*, infecting sweet cherry fruit at 20 °C. Treatment with Si induced significant increase in the activities of PAL, PPO and PO in sweet cherry fruit. In addition, the biocontrol efficacy of the yeast antagonist *Cryptococcus laurentii* was markedly increased, when it was combined with Si application (Qin and Tian 2005). In order to have a deep insight into the role of Si in protecting plants against microbial pathogens, a complete transcriptome analysis of both control and powdery mildew (*Erysiphe cichoracearum*) -infected *Arabidopsis* plants with or without Si application was performed, using a 44-K microarray. Inoculation of plants with or without Si treatments, altered the expression of a set of nearly 4,000 genes. Many of the up-regulated genes were involved in primary metabolism. Regulated defense genes included R genes, stress-related transcription factors, genes involved in signal transduction, biosynthesis of stress hormones (SA, JA, ET) and the metabolism of reactive oxygen species (ROS). This study provided evidence that contradicts the role of Si in passive resistance offered against plant pathogens evidenced by earlier reports (Fauteux et al. 2006).

Enhancement of levels of resistance of many monocot plant species to fungal pathogens has been observed, when the plants are grown in soils amended with Si. The resistance mediated by Si to rice blast disease (*Magnaporthe grisea*) was suggested to be due to a mechanical barrier produced from Si polymerization in planta. The mechanism of induction of resistance by silicon (Si) to rice blast disease has been the subject of research in different countries. Accumulation of the phytoalexins sakuranetin, momilactone A and oryzalexin S was considered to be associated with an increase in the resistance of rice cultivars susceptible and partially resistant to blast disease (Dillon et al. 1997). In a later investigation, rice plants amended with Si exhibited an enhanced level of resistance against infection by *M. grisea* and the increased resistance appeared to be strongly associated with the production of momilactones A and B. The leaf extracts from plants amended with Si^{+}, followed by inoculation with *M. grisea*, contained higher concentrations of momilactone phytoalexins, compared with those from unamended rice plants (Si^{-}). The more efficient stimulation of the terpenoid pathway in Si+ plants occurred. Consequently the increase in the levels of momilactones appeared to be a factor, contributing to enhanced rice resistance to blast. The low level of blast severity observed on leaves of Si^{+} plants at 96 h, after inoculation with *M. grisea* could be explained on the basis of stimulation of terpenoid pathway in Si^{+} plants. The results suggested that the

capacity of Si-amended rice plants to respond more effectively to *M. grisea* infection, through the production of momilactones, could be considered as a potentially important factor in the reduced blast disease severity (Rodrigues et al. 2005).

The cytological and molecular features associated with resistance to *Manaporthe grisea* in compatible and incompatible interactions with rice cultivars supplied with Si were studied. Differential accumulation of glucanase, peroxidase and PR-1 transcripts were associated with limited colonization by *M. grisea* in epidermal cells of Si^+ plants of the susceptible cultivar M201. On the other hand, Katty, a resistant cultivar responded to an avirulent race of *M. grisea* through development of a HR along with a strong induction of PR-1 and peroxidase transcripts independent of Si amendment. In Si^+ plants of M201, differential accumulation of transcripts from glucanase, peroxidase and PR-1 correlated with an inhibition of the spread of the fungus. The cytological observations indicated that the cytoplasmic granulation within epidermal cells appeared to be a reliable indicator of R gene-conditioned cell death associated with limited pathogen spread. The early autofluorescence in epidermal cells may be indicative of incompatible interaction. However, autofluorescence did not markedly differ at later stages in compatible and incompatible interactions. The results suggested an active participation distinct from single gene-defined resistance for Si in the defense of rice plants against *M. grisea* (Rodrigues et al. 2005). The importance of H_2O_2 accumulation and lipid peroxidation was investigated in the disease suppressive activity of silicon (Si) in rice blast-*M. grisea* pathosystem. Rice plants supplied with Si as a single dose immediately after pathogen inoculation (−/+ Si) exhibited the same high degree of protection against the blast disease, as plants treated continuously with Si for the whole growth period (+/+Si), with disease severity indices of 20.8 % and 19.6 % respectively which were significantly lower than that for the control treatment without Si (73.7 %). Silicon induced a rapid but transient burst of H_2O_2 at 24 h after inoculation. Application of Si to rice plants significantly altered the activity of catalase and lipoxygenase and the concentration of malodialdehyde (indicative of lipid peroxidation) in rice plants. The results suggested that rice plants might respond to Si by increased H_2O_2 accumulation and lipid peroxidation which in turn may be linked to host defense mechanisms such as lignin production, oxidative cross-linking in the cell wall, phytoalexins production and the hypersensitive reaction (Sun et al. 2010).

Foliar application of silicon (Si) was reported to effectively suppress the development of powdery mildew disease in cucumber, muskmelon and zucchini squash (Menzies et al. 1992) and in grapevine (Bowen et al. 1992). The effect of foliar- and root-application of Si on the resistance of cucumber to infection by *Podosphaera xanthii* (syn. *Sphaerotheca fuliginea*) was investigated, with respect to induced defense-related pathogenesis-related (PR) proteins (PRs). Two cucumber cultivars differing in their level of resistance to powdery mildew, Ningfeng No. 3 (susceptible) and Jinchun No. 4 (resistant) were treated with Si by foliar and root application. Root-applied Si significantly suppressed powdery mildew, the disease index being lower in Si-supplied than in control (Si-deprived) plants. The resistant cultivar showed a more consistent lower disease index than the susceptible cultivar, irrespective of Si treatment. Root application of Si resulted in increased activities of PRs (peroxidase, polyphenol oxidase and chitinase) in inoculated lower leaves of non-inoculated upper leaves in

inoculated plants of both cultivars. On the other hand, root applied Si significantly decreased the activity of phenylalanine ammonia lyase in inoculated leaves, but increase it in non-inoculated upper leaves. In contrast, foliar-applied Si had no influence either on the suppression of subsequent infection by the pathogen or on the activity of PRs, irrespective of inoculation. The results indicated that foliar application of Si might control the powdery mildew disease through the physical barrier of Si deposited on leaf surfaces, whereas root-applied Si might enhance SAR in response to infection by *P. xanthii* in cucumber (Liang et al. 2005).

The efficacy of soluble silicon (liquid potassium silicate) was assessed for suppressing the development of powdery mildew disease of strawberry caused by *Sphaerotheca aphanis* var. *aphanis* using highly susceptible cv. Toyonoka and less susceptible cv. Sachinoka. Soluble silicon, as soil drench suppressed the powdery mildew disease more effectively as a protective treatment than as curative treatment. Soluble silicon reduced the disease to a greater level (85.6 %) in Toyonoka than in Sachinoka (60.2 %). Silicate treatment did not alter leaf hardness significantly, indicating that the disease suppression might be due to factor(s) other than the physical action (Kanto et al. 2006, 2007). In a later investigation, to understand the mode of action of soluble silicon, the germination rates of conidia of *S. aphanis* var. *aphanis* on excised leaves from plants treated with Si and untreated control were determined in petridishes. The conidial germination was reduced (49.7 %) on Si+ leaves, compared with untreated control (67.2 %). Strawberries grown hydroponically with additional silicon in medium were inoculated with conidia and the leaves were observed under scanning electron microscope (SEM) at 1–2 days after inoculation. The germtubes and secondary hyphae were shorter and had fewer branches on Si+ leaves than on control. In addition, penetration of treated leaves appeared to be inhibited. The germination of conidia was markedly reduced on detached cuticles from Si+ leaves, compared with those from untreated leaves. The results suggested that soluble silicon induced physiological changes in the cuticle layer to direct inhibitory effect on conidial germination and formation of appressoria by the powdery mildew pathogen (Kanto et al. 2007). The effectiveness of silicon (Si) applied to roots of greenhouse potted roses in reducing the incidence and severity of powdery mildew disease caused by *Podosphaera pannosa* was assessed. Four genotypes of miniature potted roses, representing different genetic backgrounds and susceptibility to disease were included in this study. Plants were watered with a nutrient solution containing 3.6 mM Si (100 ppm) or supplied as K_2SiO_3 (Si^+) or no Si (Si^-), before challenge with the pathogen. Si application increased leaf Si content by two- or fourfolds, compared with the untreated control plants. Greater deposition of Si in the apoplast was observed in Si-treated plants, than in control plants, as revealed by confocal microscopic observations. Further, the onset of powdery mildew disease was delayed by 1–2 days and disease severity was reduced by up to 48.9 %, following Si application. The Si-induced protection was greater in resistant genotypes and it was accompanied by increased formation of papillae and fluorescent epidermal cells (FEC), as well as deposition of callose and production of hydrogen peroxide, especially at the sites of penetration by the pathogen and in FEC. Results indicate that treatment of rose plants with Si induced host defense responses, resulting in suppression of powdery mildew disease development (Shetty et al. 2012).

The effects of application of potassium silicate and calcium/magnesium silicate on the development of coffee leaf rust disease caused by *Hemileia vastatrix* and the growth of coffee plants were determined. Silicon (Si) was incorporated into the soil at 0, 0.25, 1.25, 2.5, 4 and 5 μm of Si for each source. The seedlings were inoculated with urediniospores of the pathogen (2 mg/l) at the seventh month after planting (six-pair-leaf stage). The number of rust lesions/leaf were counted and recorded for each treatment. The number of lesions was reduced up to 66 % in plants receiving the highest silicon dose, when compared to the controls. Infection intensity (based on number of lesions) was negatively correlated with the concentration of silicate. The results showed that application of silicon could be considered as an effective alternative for an ecological management system for coffee rust disease (Martinati et al. 2008). The effect of application of calcium silicate (Si+) or calcium carbonate (Si−) on the development of Phytophthora blight disease caused by *Phytophthora capsici* was investigated. Calcium silicate or calcium carbonate was incorporated in No. 2 peat mix and seedlings were planted after 6 weeks. The soil was infested with two isolates of *P. capsici* (Cp30 and Cp32). The roots and stem samples were examined for the presence of lesions on crowns and stems and wilting of plants was recorded up 9 days after planting the pepper seedlings. Data obtained, were used to calculate the area under disease progress curve (AUDPC) and area under wilting progress curve (AUWPC). Relative lesion extension (RLE) was deduced as the ratio of vertical lesion extension to stem length at 9 days after planting. An increase of 40 % in the Si concentration in the roots, was observed, but not in the stems of bell pepper plants in the Si+ treatment, compared to the S− treatment. The AUDPC was reduced by 15.4 and 37.5 %, while AUWPC was reduced by 29.1 and 33.3 % in two experiments respectively, compared with control treatments. RLE values showed reduction in Si+ treatment. The dry root weights and stem weight also registered increases as compared with control treatment, indicating growth promotion, due to Si application (French-Monar et al. 2010). The extent of protection of tomato plants provided by silicon (Si) against Fusarium crown and root rot disease caused by *Fusarium oxysporum* f.sp. *radicis-lycopersici* (FORL) was determined in sand culture system. The Hoagland's nutrient solution with (100 mg Si/l, Si+) or without Si (Si−) was used as nutrient source for tomato plants. The plants, 3 weeks after transplanting, were inoculated with three inoculum levels (0, 10^6 and 10^7 conidia/plant). Disease severity was assessed at 2, 3, 4 and 6 weeks, after inoculation and silicon concentration in the roots and shoots of plants of different treatments were determined. Disease severity was significantly reduced by Si treatment at 4 weeks after inoculation. Si contents of roots and stems of treated tomato plants were significantly higher, compared with control plants. The increase in the Si contents of the roots showed significant positive correlation with reduction in diversity of root, crown and stem. Application of Si to tomato plants exerted a beneficial effect by reducing diversity. The results suggested that decrease in disease severity, following Si application might be due to a delay in the onset in initial infection of roots and the movement of the pathogen from roots to stems (Huang et al. 2011).

The possibility of using sodium silicate (Si) as an alternative postharvest treatment to conventional fungicides for the control of Alternaria, Fusarium and pink rots of Hami melons caused by *A. alternata, Fusarium* spp. and *Trichothecium roseum* respectively, was explored. Application of Si significantly reduced the mycelial

growth of all three pathogens in vitro. Si at 100 mM was more effective in suppressing the disease development in fruits than the lower concentrations (25 or 50 mM), but phytotoxic symptoms were observed, due to higher concentrations of Si (200 mM). Preinoculation treatment with Si was more effective with respect to *T. roseum* than the postinoculation application. Suppression of pink rot disease was clearly correlated with induction of activities of two families of defense-related enzymes, peroxidase and chitinase. Accumulation of both enzymes was induced in fruits treated with Si and challenged with *T. roseum* 24 h later. The protection induced, was sustained for at least 9 days in the cultivar New Queen and for 10 days in 8601 at room temperature. Peroxidase enzyme is known to contribute to cell-wall reinforcement and it is involved in the final steps in lignin biosynthesis and in the cross-linking of cell wall proteins. The results indicated that Si could induce resistance to a broad spectrum of fungal pathogens, causing postharvest diseases in melon. As Si is inexpensive and readily available, use of Si for protecting fruits against postharvest pathogens holds promise for large scale application (Bi et al. 2006).

The potential of silicon (Si) in inducing resistance in banana (*Musa acuminata*) against *Cylindrocladium spathiphylli*, causative agent of banana toppling disease, was determined. Banana plantlets inoculated by dipping the root system in a conidial suspension of *C. spathiphylli* were grown on a desilicated ferralsol and amended with 2 mM of soluble Si under greenhouse conditions and control without Si amendment. The severity of root lesions was assessed, using the image analysis program WinRHIZO at 7, 14 and 21 days after inoculation. Root necrosis (lesions) was reduced by about 50 % at 14 days after inoculation in plants supplied with Si, compared with control plants. In addition, Si amendment exerted growth promotion effect resulting in the alleviation of the negative effects of the pathogen. The results provided evidence for the beneficial effects of Si application by reducing the negative effects of the pathogen by enhancing the level of resistance of plants supplied with Si. Application of Si appears to provide an ecofriendly alternative to chemicals (Vermeire et al. 2011). The effect of Si application on the development black Sigatoka disease caused by *Mycosphaerella fijiensis* infecting banana (*Musa acuminata*) was studied. Banana plants were grown in the presence of Si in pots filled with compost with comparable control plants without Si supply. The leaves were inoculated at 4 or 6 months of plant growth by spray inoculation with conidial suspensions of *M. fijiensis* or by brushing with mycelial fragments. Progress of disease at regular intervals was monitored and disease severity was also analyzed, using an image analysis software ASSESS. The disease progressed more rapidly and severely on banana plants without Si supply. The areas under disease progress curve (AUDPCs) in Si-supplied plants were significantly lower than that of control plants, irrespective of method of challenge inoculation with the pathogen, indicating that Si application could be adapted for the effective management of major diseases of banana (Kablan et al. 2012).

Silicon (Si) amendment has been reported to decrease incidence of banana Fusarium (Panama) wilt disease caused by *Fusarium oxysporum* f.sp. *cubense* (*Foc*). The physiological and biochemical mechanisms involved in enhancement of resistance to Fusarium wilt disease, following treatment with Si amendment were studied. Plants of the Grand Nain (resistant) and Maca (susceptible) cultivars were grown in plastic pots amended with Si at 0 or 0.39 g/kg of soil (Si− and Si+ respectively) and inoculated with

race 1 of *Foc*. Relative lesion length (RLL) and asymptomatic fungal colonization in tissue (AFCT) were evaluated at 40 days after inoculation. Root Si concentration was significantly increased by 35.3 % for the Si+ treatment, compared with Si− treatment, the resistant cultivar showing greater increase than the susceptible cultivar. Grand Nain and Maca plants in Si+ treatment showed significant reductions, 40 and 57.2 % respectively for RLL, compared with Si− treatment. Similar reduction in resistant and susceptible cultivars was observed for AFCT, compared with unamended treatments. The activities of PAL, PPO, PO, chitinases and ß-1,3-glucanases were enhanced in the roots of banana plants grown in Si amended soils. The results suggested that symptoms of Fusarium wilt on roots of banana plants supplied with Si decreased due to an increase in H_2O_2, total soluble phenolics (TSP) and lignin thioglycolic acid (LTGA) derivatives and enhanced activities of defense-relatedenzymes (Fortunato et al. 2012). Management of lettuce downy mildew disease caused by *Bremia lactucae* was found to be difficult in soilless systems by employing conventional methods. The effect of silicon salt (potassium silicate) and electrical conductivities on the development of downy mildew disease was investigated, using the susceptible cultivar Cobham Green. Silicon as potassium silicate was supplied at 100 mg/l of nutrient solution at three levels of electrical conductivity: 1.5–1.6 $mScm^{-1}$ (EC1), 3.0–3.5 $mScm^{-1}$ (EC2, 0.70 g/l NaCl) and 4.0–4.5 $mScm^{-1}$ (EC3, 0.95 g/l NaCl). EC and potassium silicate had significant influence on the incidence and severity of downy mildew. Addition of the standard nutrient solution (EC1) of potassium silicate resulted in significant reduction of downy mildew severity in two of the four trials, where plants were artificially inoculated at 15 and 20 days after transplanting. Maximum reduction in disease incidence and severity was achieved by the addition of potassium silicate to the EC3 solution. This combination of Si and EC enhanced the plant biomass also reflecting the growth promotional effect of the treatment (Garibaldi et al. 2012).

The efficacy of sodium nitrite (in citric acid buffer pH 2), chitosan and Tillekur (known elicitors of plant resistance) in suppressing seedborne infection by *Didymella lycopersici* in tomato seeds, was compared. Nitrite was highly effective, when applied at 300 mM/l for 20 min. Tillekur was also equally effective, but chitosan was significantly less effective in reducing the fungal infection of seeds. Tillekur was found to be phytotoxic. The results indicated that high efficacy and low cost of acidified sodium nitrite could be an effective alternative to the fungicide applied against seedborne fungal pathogens (Kasselaki et al. 2008). The protective potential of potassium nitrate (KNO_3) was assessed for the control of Phytophthora stem rot diseases of soybean caused by *Phytophthora sojae*. Application of KNO_3 (4–30 mM) prior to challenge inoculation with the pathogen, reduced the disease incidence in two soybean cultivars. The extent of disease suppression was related to increase in potassium concentration in plants of two cultivars. Scanning microscopic examination indicated marked accumulation of potassium at the penetration-stopping sites of *P. sojae* in the cortex layer of soybean plants treated with 30 mM KNO_3, compared with untreated control plants. Decreased release of zoospores of *P. sojae* was observed in the presence of 0.4–30 mM KNO_3, indicating some direct effect of the chemical on the pathogen development. The results revealed the potential of KNO_3 as a possible alternative in place of fungicides for reducing the incidence of Phytophthora stem rot disease of soybean (Sugimoto et al. 2009).

Sodium salts have been demonstrated to offer beneficial effects by reducing the incidence of postharvest diseases, when applied alone or in combination with other agents. The effects of treatment of oranges with sodium carbonate (3 %) at 24 and 45 °C in combination with *Pseudomonas* spp. was assessed for suppressing the development of *Penicillium digitatum*, causing blue mold disease. The efficacy of *Pseudomonas* isolates in controlling the blue mold disease was significantly improved, when combined with sodium carbonate (3 %). A further increase in biocontrol activity was achieved, when *Pseudomonas* isolates were applied in combination with hot sodium bicarbonate. The results revealed the practical utility of the combination of hot sodium bicarbonate with bacterial biocontrol agent for the effective control orange blue mold disease (Zamani et al. 2008). Gray mold disease caused by *Botrytis cinerea* drastically reduces the postharvest quality of rose flowers. Postharvest dipping of rose flowers in 200 µl sodium hypochlorite (NaOCl) for 10 s at 20 °C significantly suppressed the development of the gray mold disease on Akito and Gold Strike rose flowers. NaOCl derived from Clorox® Ultra household bleach solution was more effective than laboratory grade NaOCl in reducing the disease severity. Lowering the pH of the NaOCl solution from 9.7 (unadjusted) to pH7.0 improved the effectiveness of treatment with NaOCl. Treating Gold Strike rose flowers in the neutral NaOCl solution was more effective in reducing the level of infection on petals than postharvest dips in the conventional fungicides. Application of NaOCl prior to a 3- or 10-day commercial shipment provided the most consistent protection to a wide range of rose cultivars, as compared to conventional fungicides. The efficacy of NaOCl attained its peak, when Gold Strike rose flowers treated with NaOCl were incubated at 20 °C and 90 % RH for 6–9 h. This investigation demonstrated the usefulness of NaOCl treatment for effective protection of rose flowers against infection by *B. cinerea* (Macnish et al. 2010).

Appendix 8.1: Induction of Resistance to Apple Fire Blight Disease by Acibenzolar-S-Methyl (ASM) and DL-3-Aminobutyric Acid (BABA) (Hassan and Buchenauer 2008)

A. Application of Inducers of Disease Resistance

i. Prepare solutions of BABA at a concentration of 1.0 mg/ml and ASM at a concentration of 0.1 mg/ml; spray BABA at 4 days before inoculation (dbi) or ASM at 2 dbi separately or spray the inducers simultaneously and spray the control plants with sterile distilled water.
ii. Inoculate the apple seedlings treated with inducers/control with a suspension of the pathogen (1×10^8 CFU/ml).
iii. Spray the apple seedlings with BABA and ASM at different days, as determined to select the time interval for optimal expression of acquired resistance.

B. Evaluation of Disease Severity

i. Inoculate the plants using sterilized scissors dipped in bacterial suspension for 20 s and cut the leaf tips (about 1.5 cm from the tip of two young leaves beneath the two apical ones) of apple seedlings; place the inoculated seedlings in dew chamber at 100 % RH and 24 ± 2 °C for 24 h and transfer the plants to the greenhouse.
ii. Record the disease indices at 10 days after inoculation expressed as browning discoloration index (BDI) and stem bending index (SBI), as percentages as detailed below for treatments and controls.

Brown discoloration index (BDI) using 0–6 scale: 0 = no leaf symptom; 1 = 25 % or less; 3 = 50–75 %; 4 = >75 %; brown discoloration from the edge of the cutting to the midrib; 5 = >75 % brown discoloration from the edge of cutting to the midrib and/ or the petiole turns brown; 6 = petiole turns brown or black releasing bacterial ooze:

$$\mathrm{BDI}(\%) = \sum \frac{(\text{number of leaves} \times \text{symptom category}) \times 100 / \text{number of leaves evaluated}(2) \times \text{maximum score}\ (6)}{}$$

Stem bending index (SBI) using 0–5 scale: 0 = seedlings and leaves fully turgid; 1 = leaf lamina flaccid, stem turgid; 2 = stem bent 0–30° from vertical position; 3 = stem bent 30–60° from vertical position; 4 = stem bent 60–90° from vertical position and 5 = stem bent >90° from vertical position

$$\mathrm{SBI}(\%) = \text{Category of symptom} \times 100 / \text{maximum socre}\ (5)$$

Appendix 8.2: Induction of Systemic Acquired Resistance (SAR) by Acibenzolar-S-Methyl (ASM) in Tobacco Against *Tomato spotted wilt virus* (Mandal et al. 2008)

A. Treatment of Tobacco K326 Plants with ASM

i. Dissolve ASM (as 50 % active ingredient (a.i) in wettable powder formulation) in water; spray the solution of ASM with a suitable sprayer on K326 plants at 40–45 days after sowing (DAS) with different concentrations of ASM (0–4.0 g a.i./7,000 plants); wash the plants by spraying with 80 ml of water per flat, containing nine plants to move ASM in the root zone.
ii. To determine the time required for SAR activation, treat the tobacco plants with two concentrations of ASM at 2 and 4 g a.i./7,000 plants; to determine the effect of age of tobacco plants on SAR activation, treat the seedlings (47 DAS) and older plants (77 DAS) with 2 g a.i./7,000 plants.
iii. Spray the plants with distilled water for non-treated control plants.

B. Challenge Inoculation with TSWV

i. Challenge the ASM-treated plants with mechanical inoculation with TSWV inoculum; prepare the inoculum by grinding systemically infected leaves in 0.1 M phosphate buffer, pH 7.0, containing 0.2 % Na_2SO_4 and 0.01 M mercaptoethanol at the rate of 1:10 tissue and buffer ratio (w/v); remove the debris by squeezing the extract through a layer of nonabsorbent cotton; add 2 % carborundum 320 grit and 1 % celite 545 to the inoculum and maintain the inoculum on ice.
ii. Apply inoculum to two youngest fully expanded tobacco leaves and gently rub with cotton swab dipped in the inoculum; gently wash the inoculated leaves with water and maintain the plants in the greenhouse at 25–30 °C.

C. Assessment of TSWV Infection in ASM-Treated Plants

i. Observe the experimental plants for the development of symptoms of infection by TSWV; count the number of local lesions on two inoculated leaves of each plant at 6 days post-inoculation (DPI); confirm the infection of TSWV by performing ELISA tests, using tenfold dilution of the sap to relative levels of TSWV in the inoculated leaves of different treatments.
ii. Allow the plants to grow for a longer period for the development of systemic symptoms induced by TSWV.
iii. Determine the presence and concentration of TSWV in the roots and newly emerged leaves by ELISA tests.
iv. Measure the plant height and root length to determine the effect of ASM on plant growth and also record the phytotoxicity symptoms, if any.

Appendix 8.3: Induction of Systemic Acquired Resistance in Pea Against Rust Disease by Abiotic Chemical Inducers (Barilli et al. 2010)

A. Application of Inducers of Disease Resistance

i. Prepare predetermined concentrations of the abiotic chemical inducers [SA (5, 7, 8.5 and 10 mM), BTH (1, 5 and 10 mM) and BABA (5, 10, 20 and 50 mM)] in sterile water; add Tween 20 (0.03 % v/v); apply three droplets (15 μl each) of each inducer on the first leaflets and treat control plant leaves similarly with sterile water + Tween 20.
ii. Inoculate plants at 5 days after treatment with inducers by dusting freshly collected urediospores of *Uromyces pisi* (2 mg of spores/plant) mixed with talc (1:10) using a spore settling tower; incubate the plants for 24 h at 20 ± 2 °C in complete darkness and 100 % RH and place the plants in growth chamber for development of disease symptoms.

B. Examination Under Light Microscope

i. At 24 h after inoculation, cut one leaf per plant at first node; lay them individually adaxial surface up, on filter paper dipped on fixative acetic acid/ethanol (1:3, v/v) mixture to remove chlorophyll from the chloroplast membranes; bleach the leaf segments using several changes of the fixative and transfer to filter paper moistened with tap water for at least 2 h, to soften the tissues.
ii. Transfer the leaf segments to lactoglycerol (1:1:1, lactic acid/glycerol/water, v/v/v) for at least for 2 h; add a drop of Trypan blue in lactoglycerol (0.1 %, w/v) on a cover glass; place the leaf segment carefully and mount in lactoglycerol on microscope slide.
iii. Examine approximately 150 urediospores per leaf sample under the microscope (×40 magnification) and count the number of germinated spores (with germtubes at least as long as the diameter of the spore).

C. Assessment of Disease Severity

i. Record the infection frequency (IF) on first, second and third pair of leaves separately by counting the number of pustules/cm^2 in a marked area in each leaflet, using a hand lens (×7); convert the number of pustules in the control as equivalent to 100 % and calculate the relative values for the treatments, in relation to that of the control.

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