Microorganisms for Sustainability 40
Series Editor: Naveen Kumar Arora

Udai B. Singh · Pramod K. Sahu ·
Harsh V. Singh · Pawan K. Sharma ·
Sushil K. Sharma *Editors*

Rhizosphere Microbes

Biotic Stress Management

Springer

Microorganisms for Sustainability

Volume 40

Series Editor

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Preface

For sustainable food production and food security at global level in the line of Sustainable Development Goals (SDGs), food supplies must keep pace with increasing population that can partially be accomplished by reducing the yield losses caused by the devastating pest and pathogens. Doubling in global food demand by 2050, changing climatic conditions impose a number of challenges towards agricultural sustainability. Today, crop production to fulfil food demands is being enhanced by the increasing application of agrochemical inputs, which act as plant growth regulators, plant nutrients supplier and plant protectors. Apart from increasing cost of production, excessive use of agrochemicals increases the possibilities of residual effects in agricultural commodities, land degradation and deterioration of environmental health. With the increasing world population and global demand for food, there is an urgent need to adopt sustainable approaches to ensure perpetual agricultural production with less or no use of agrochemicals. Besides urbanization, reduction in arable land and land degradation, numerous biotic stresses cause significant crop loss from field to storage. The biotic factors include insect-pest, pathogens, weeds and others including both vertebrates and invertebrates. The average yield losses due to biotic stress factors, i.e. insect-pest and disease, have been reported to be as high as 40% every year at global level (FAO 2015). Management of biotic stresses mainly relies on the use of toxic chemical pesticides and resistant plant varieties. The use of resistant plant varieties is an important approach for conferring agricultural sustainability. However, non-availability of suitable donor parents and the breakdown of resistance have still remained a great concern. Further, negative impact of plant protection agrochemicals on the non-target microflora and fauna, environment, animal and human health has forced researchers to explore alternative measures for management of biotic stresses of important crop plants. Among the more recent strategies, stress tolerance/resistance induced by inducers of microbial origin and/or rhizosphere microorganisms has emerged as a promising approach in crop protection. The multidimensional factors involved in microbial communities present in the ecosystems which can provide the answers to the current agricultural problems. Microbial communities play a significant role in microbe-microbe,

microbe-insect/pest and plant-microbe interactions which have not yet been fully exploited to harness their potential benefits to achieve agricultural sustainability. There are numerous microorganisms comprising fungi, bacteria, actinomycetes and cyanobacteria having mechanisms of plant growth promotion and biological control properties.

The rhizosphere is a micro-environment contrastingly different from non-rhizosphere. Plant rhizosphere is the battlefield for beneficial and harmful organisms. Microorganisms in the rhizosphere co-exist in perfect communities which show division of labour and different functions for microbe-plant interactions. The significance of microbe-plant interactions in the rhizosphere ecosystem is enormous for agricultural sustainability. The positive interactive effect of the beneficial rhizosphere microorganisms on plants is induction of plant growth, conferment of abiotic and biotic stress tolerance and modulation in several pathways of the plants for the proper establishment in all kinds of environments including degraded and contaminated soils. Moreover, interactions among microbes, plants, soil and insects play a crucial role in the rhizosphere ecosystem functioning and modulate the physico-biochemical properties of the rhizosphere soil. Further, the plant secretome influences the rhizospheric microbial communities by recruiting the specific microflora around the root system and interacting with them. However, rhizospheric interactions are quite complex and dynamic. It is rather difficult to elucidate as they take place under different circumstances and at different interfaces such as endosphere, rhizoplane and rhizosphere. In view of the above facts, large-scale exploitation of rhizospheric interactions is crucial for enhancing the agro-ecosystems resilience to biotic stresses by adopting novel microbe-based strategies for maximizing the sustainable food production under changing climatic conditions. Therefore, strategic and applied researches are essential to explore and exploit all root-associated microorganisms for harnessing benefits from all kinds of interactions for biotic stress management in low-input sustainable agriculture under changing climatic conditions. In this context, the book *Rhizosphere Microbes: Biotic Stress Management* edited by Udai B. Singh, Pramod K. Sahu, Harsh V. Singh, Pawan K. Sharma and Sushil K. Sharma is a topical and timely contribution on plant-microbe interactions and offers a great scope for harnessing the beneficial interactions for biotic stress tolerance and agricultural sustainability. The objective of the present book is to furnish a broad-based review on updated critical developments on the management of biotic stresses by using rhizospheric microbes. Chapters which provide a consolidated state-of-the-art work in this area have been incorporated in this book. This much awaited book is aimed to impart a vision for the advancement of science with a special focus on the development of biological control researches worldwide. The book contains critical reviews, mini-reviews, case studies and success stories within the ambit of its title. It covers the complete knowledge on all spheres of stress tolerance, i.e. diverse role of microbes and microbial communities in biotic stress tolerance, diversity, ecology and population dynamics of biocontrol agents, exploring the microbial resources for antimicrobial bioactive compounds, microbe-mediated mitigation of biotic stresses in many crop plants, microbial signalling in the rhizosphere, biofilm formation, plant-microbe

interactions under biotic stresses, role of microorganisms in ecosystems functioning under various biotic stress conditions, development of sustainable techniques/bioformulation, increased agricultural productivity through the application of microbial bio-pesticides, molecular studies using microbial systems, etc. Further, the present book volume *Rhizosphere Microbes: Biotic Stress Management* is very particular to rhizosphere microbe-mediated management of biotic stress with special reference to disease management. This book does not deal with the management of insect-pests, weeds and invertebrates-vertebrates. This book has 16 contributory chapters from well-experienced researchers in plant pathology, microbiology and biotechnology working on different aspects and issues of detection of plant pathogens and characterization of biological control agents for the management of diseases in plants of agricultural importance. This book is unique with complete knowledge about rhizosphere microbe-mediated biotic stresses in major crop plants. Last but not least, this book highlights the role of microbial technologies in sustainable crop protection that may help increase food production for food security to achieve targets of SDGs by the year 2030.

Maunath Bhanjan, India — Udai B. Singh
Maunath Bhanjan, India — Pramod K. Sahu
Maunath Bhanjan, India — Harsh V. Singh
Maunath Bhanjan, India — Pawan K. Sharma
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Pramod K. Sahu has completed his B.Sc. from IGKVV and M.Sc. (Gold-Medalist) and Ph.D. (Agricultural Microbiology) from UAS, Bengaluru, India. He was a DST-Inspire fellow and was selected in various national level exams including ICAR-JRF, ICAR-SRF, ICAR-NET, ARS and GATE. Being a Scientist of Agricultural Microbiology at ICAR-NBAIM, Mau, India, working on plant-endophyte interaction, consortium of bioinoculants, biological control and salinity stress alleviation, he has to his credit more than 48 research/review papers, 1 book, 11 book chapters, 6 training manuals, 13 extension folders, 5 popular articles and 30 abstracts. He has won NAAS Young Scientist Award in Plant Protection 2021, Dr. B. P. Pal Gold Medal award, Dr. G. Rangaswamy Gold Medal, Willium Rigo award, several best posters and best oral presentation awards along with Young Scientist in Agricultural Microbiology-2018 award.

Harsh V. Singh is currently working as a Principal Scientist (Plant Pathology) at ICAR-National Bureau of Agriculturally Important Microorganisms, Kushmaur, Maunath Bhanjan, Uttar Pradesh, India. He is specialized in the areas of plant-microbe interactions, biological control, biotic and abiotic stress management. He has vast research experience in the field of plant pathology with special reference to management strategies of biotic stress with application of microbes-based technologies. He has also experience of biotic stresses of temperate crops of Jammu and Kashmir and grasses and fodder crops. He has worked on microbial role in grassland productivity and nutrient cycling. Studies on microbial diversity and seasonal dynamics in different horti- and silvopastoral systems are his significant contributions towards grassland productivity. He served as a Junior Scientist-cum-Assistant Professor in Plant Pathology (2002–2007) at RARSS, SKUAST-K, Kargil, Programme Coordinator (2007–2009) at KVK, Poonch, SKUAST-J and Senior Scientist (Plant Pathology) at ICAR-IGFRI, Jhansi, from 2009 to 2018. So for he has published more than 40 publications as research articles, 4 books and 6 reviews and 15 book chapters both in national and international journals. He is an editor and reviewer of journals of reputed societies. He has delivered several expert talks and has been an invited speaker for different national and international symposium and conferences.

Pawan K. Sharma did his M.Sc. and Ph.D. in Plant Pathology from DR YSP University of Horticulture & Forestry, Solan, HP. He is working as Principal Scientist at ICAR-NBAIM at Mau, UP, since September 2013. He had been associated with 14 projects as PI or Co-PI sponsored by various agencies. Currently, he is working on biological control of wilt and collar rot diseases through liquid-based formulation of *Trichoderma* and is also in charge of National Agriculturally Important Microbial Culture Collection. He has published 46 research papers in national and international journals, edited 4 books and authored 1 book.

Sushil K. Sharma is currently working as a Principal Scientist (Agricultural Microbiology) at ICAR-National Institute of Biotic Stress Management, Raipur, Chhattisgarh, India, in the area of microbial resource conservation, secondary metabolites and antimicrobial peptides for biotic stress management of crops. So far he had published 110 articles (research, review, book chapter, book, etc.) in both national and international journals/book/conferences. His team has developed (1) a simple novel method for the detection of operation of tryptophan-independent pathway in *Micrococcus aloeverae* DCB-20 and 20 low-cost preservation method of *Chromobacterium violaceum* using natural gums at room temperature. He has been decorated with *Best Scientist* award by PEARL Foundation, Madurai, India, and *Excellence in PGPR Research* by Asian PGPR Society. Recently, he has been nominated as a member of *Biosafety and Biosecurity Cell* (*B2Cell*) of Indian Council of Medical Research (ICMR), New Delhi. He has also delivered talks in PGPR conference organized in Columbia, Uzbekistan and Indonesia.

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Chapter 1
Detection and Identification of Soil-Borne Pathogens: Classical to Recent Updates

Manjunath Hubballi, I. Johnson, V. A. Anjali, T. S. Archana, and S. Nakkeeran

Abstract Soil being biologically complex atmosphere offers shelter to diverse microbes. The survival of microbes in soil is greatly influenced by both edaphic and atmospheric factors. In addition, microbiome dwelling in soil competes with each other for space nutrients and other essential elements. The microbes in soil causing disease in crop plants are called soil-borne pathogens. They mainly encompass actinomycetes, bacteria, fungi, and viruses. These pathogens, though represent a very small portion of total microbial biomass in soil, are responsible for yield losses of varying dimensions in a range of crops. The fact that they reside and cause damage underground remains unnoticed many a times. The presence of a favorable environment for the establishment of host–pathogen relationship and delayed diagnosis of the interaction of soil-borne pathogens contributed to a huge loss in many crops. However, proper detection and diagnosis of the diseases at an early stage can aid in saving the losses caused by these pathogens. There has been an enormous number of methodologies for a diverse group of pathogens. The traditional methods of detecting soil-borne pathogens using direct quantification of pathogens from soil, enumeration of fungal and bacterial pathogens present in soil, and use of selective media for culturing desired pathogen are all laborious and time consuming. Recent advances in science have led to the development of immunological and molecular techniques for the detection of pathogens in soil. These improved methods are not only quick and efficient but are also reliable in detecting particular pathogens.

Keywords Soil · Bacteria · Fungi · Transient visitor · Resident visitors · Inoculum · PCR · LAMP · Immunoassays

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1.1 Introduction

Soil is a biologically active, complex environment developed on the uppermost layer of the earth's crust. It is more porous in nature and has an immense role in the existence of life on earth as it forms a major reservoir of water and nutrients. It is mostly dominated by a multitude of invertebrates, microbial organisms, and a highly complex animal biota. It is said that one gram of soil is composed of innumerable microbes having immense ecological significance. According to the study, approximately 10^8–10^9 bacteria, 10^5–10^8 actinomycetes, 10^1–10^2 nematodes, and 10^3–10^5 invertebrates are present in one gram of soil (Trevors 2010). In addition, soil fertility is greatly contributed by a large number of earthworms present in soil. It is estimated that around 300 earthworms are present in one square meter area. Thus, soil is a microbial biochemical gene library (Dindal 1990). The enormous population of microbes present in soil can be broadly grouped into beneficial, neutral, or harmful to plants. The harmful category of microbes causing harm to plants is considered soil-borne plant pathogens. In other words, microbes residing in soil and causing economic damage to the plants growing in soil are considered soil-borne pathogens. According to Stevens et al. (2003), the term soil-borne pathogens can be defined as pathogens that cause plant diseases via an inoculum that comes to the plant by way of the soil.

1.2 Classification of Soil-Borne Pathogens

Soil is complex in nature, harboring a large number of microbes in it. This has created complexity in its ecology, and hence, it is very essential to establish the role of each microbe in soil as they largely determine the growth and establishment of plants. Based on their ecological role, the soil-borne pathogens are divided into three major categories: transient visitors, resident visitors, and residents (Schuster and Coyne 1974).

1. Transient visitors: The pathogens that in their life cycle spend very less time in soil and commonly don't perpetuate in soil are grouped under this category. These pathogens are more specialized parasites, and the presence of such pathogens is usually associated with a particular host. A prolonged absence of the host in particular soil eliminates these pathogens from the soil owing to their inability to compete with general soil saprobes for existence on nonliving matter. This intimate relation of host and pathogen is conditioned by general soil microflora. These are also called soil invaders, soil transients, root inhabitants, root-specific pathogens, or short-lived exotics. Most bacteria infecting plants fall in this group. The typical examples of this are *Verticillium*, *Rhizoctonia solani*, *Pythium debaryanum*, *Erwinia stewartii*, *E. amylovora*, *E. tracheiphila*, *X. citri*, *X. vesicatoria*, *X. vasculorum*, *E. rubrifaciens*, *X. malvacearum*, *X. juglandis*, *X. vesicatoria*, *X. pruni*, *P. syringae*, *P. pisi*, *P. phaseolicola*, *P. solanacearum*

race 2, *P. tabaci*, *P. glycinea*, *P. lachrymans*, *P. mors-prunorum*, and *P. savastanoi* (Buddenhagen 1965).

2. Resident visitors: These pathogens are typified by a gradual decline of population in soil, and populations of these pathogens largely depend on host or cropping practices followed in soil. The examples of this category would include *Agrobacterium tumefaciens*, *Ralstonia solanacearum*, and *Erwinia carotovora*.
3. Residents: These pathogens are primitive types and are general or unspecialized parasites having a large host range. These pathogens are distributed throughout the soil, and their parasitism appears to be incidental to their saprophytic existence as members of the general soil microflora. Unlike the previous group, these pathogens survive in soil for a longer period, and their relation with plants is ephemeral in nature (Stevens et al. 2003). In general, the competitive saprophytic ability of these pathogens is very high. The species of the genera *Pythium*, *Rhizoctonia*, and *Sclerotium* and bacteria species of *Erwinia* and *Pseudomonas* fall in this group (Veena et al. 2014).

1.3 Significance of Soil-Borne Diseases

The losses incurred due to soil-borne diseases in many agriculture and horticultural crops are largely underestimated because they appear underground. It is estimated that more than 50 different species of fungi, a large number of bacteria, nematodes, and a few viral species and also a few parasitic plants are reported to be soil-borne (Acuf 1988). According to Papavizas (1985), the loss incurred due to soil-borne diseases alone in annual crops is tolling 50% in total. The damage incurred by these diseases is considered the major factor limiting the growth, establishment, and health of plants ultimately influencing negatively on yield both quantitatively and qualitatively (Buchenauer 1998). In a study, the major pathogenic species belonging to *Sclerotinia*, *Pythium*, and *Phytophthora*, *Fusarium*, *Verticillium*, and *Rhizoctonia* inflict yield losses of 50–75% in selected agricultural crops like maize, cotton, wheat, and horticultural crops viz., ornamental crops and fruits (Lewis and Papavizas 1991; Mokhtar and El-Mougy 2014; Baysal-Gurel and Kabir 2018). Furthermore, in the USA, the loss caused by soil-borne diseases was assessed, and it was inferred that around $ 4 billion was lost due to these diseases. Mokhtar and El-Mougy (2014) reported 90% yield losses in about 2000 diseases infecting major crops in the USA (Table 1.1).

1.4 Soil-Borne Pathogens Vs. Foliar Pathogens

The line of difference between soil-borne pathogens and foliar pathogens cannot be always demarcated. The diseases caused by foliar pathogens and soil-borne pathogens differ greatly in the way of spread. The foliar diseases are polycyclic whereas

Table 1.1 Yield loss due to soil-borne diseases in major crops

Crop	Disease	Pathogen	Yield loss (%)	Reference
Rice	Sheath blight	*Rhizoctonia solani*	50	Zhao et al. (2021)
Wheat	Soil-borne wheat mosaic virus disease	*Soil-borne wheat mosaic virus*	10–80	Liu et al. (2020)
Maize	Late wilt	*Magnaporthiopsis maydis*	100	Degani and Dor (2021)
Pigeon pea	Fungal wilt	*Fusarium udam*	50	Kumar et al. (2020)
	Dry root rot	*Rhizoctonia bataticola*	10–100	Vamsikrishna et al. (2021)
	Stem canker	*Macrophomina phaseolina*		
Ground nut	Bacterial wilt	*Ralstonia solanacearum*	20	Yuliar et al. (2015)
	Stem rot	*Sclerotium rolfsii*	25–30	Acharya et al. (2021)
Cotton	Verticillium wilt	*Verticillium dahliae*	10–35	Song et al. (2020)
Tobacco	Bacterial wilt	*Ralstonia solanacearum*	10–30	Yuliar et al. (2015)
Potato	Root knot nematode	*Meloidogyne incognita*	35	Mardhiana et al. (2017)
	Bacterial wilt	*Ralstonia solanacearum*	33–90	Yuliar et al. (2015)
Tomato	Root knot nematode	*Meloidogyne incognita*	24–38	Mukhtar (2018)
	Fusarium wilt	*Fusarium oxysporum*	10–80	Patil et al. (2011)
	Early blight	*Alternaria solani*	79	Dhaval et al. (2021)
	Bacterial wilt	*Ralstonia solanacearum*	90.62	He et al. (2020)
Brinjal	Damping-off	*Pythium* sp.	60	Mahadevakumar and Sridhar (2020)
	Dry root	*Macrophomina phasiolina*	10	Pugalendhi et al. (2019)
Bean	Root knot nematode	*Meloidogyne incognita*	20	Mardhiana et al. (2017)
Cucumber	Root knot nematode	*Meloidogyne incognita*	69.2	Singh and Balodi (2021)
	Fusarium wilt	*Fusarium oxysporum*	70–100	
	Root rot	*Rhizoctonia solani*	5–80	
Banana		*Ralstonia solanacearum*	80–100	

(continued)

Table 1.1 (continued)

Crop	Disease	Pathogen	Yield loss (%)	Reference
	Bacterial wilt			Yuliar et al. (2015)
	Fusarium wilt	*Furaruim* sp.	30	Bubici et al. (2019)
Pomegranate	Root knot nematode	*Meloidogyne incognita*	17.3	Tulika et al. (2019)
	Fungal wilt	*Fusarium oxysporum*	36	Das et al. (2021)
		Ceratocystis fimbriata	30	Shruthi et al. (2019)
Water melon	Root knot nematode	*Meloidogyne inconita*	24–50	García-Mendívil and Sorribas (2021)
Guava	Wilt	*Fusarium oxysporum* f. sp. *psidii*, *Fusarium solani*, *Gliocladium roseum*, *Cephlosporium* sp., *Nalanthamala psidii*, and *Gliocladium roseum*	5–60	Singh et al. (2021)
Wheat, cotton, maize, vegetables, fruit, and ornamentals	–	*Rhizoctonia* spp., *Fusarium* spp., *Verticillium* spp., *Sclerotinia* spp., *Pythium* spp., and *Phytophthora* spp.	50–75	Panth et al. (2020)

soil-borne diseases are monocyclic in nature (Katan 2017). The fluctuations of climatic conditions greatly influence foliar pathogens. For example, a change in temperature and relative humidity will directly influence the growth and development of pathogens and their spread in the case of foliar diseases as the pathogens are directly exposed. On the other hand, such fluctuation is masked in the soil-borne pathogens due to soil mass (Garrett 1970). The research progress in the case of soil-borne diseases is hindered by various factors.

1. The opaque nature of soil prevents in situ examination of pathogens (Cytryn and Minz 2012).
2. Surviving structures of pathogens such as sclerotia, conidia, mycelia, rhizomorph, oospores, and chlamydospores exhibit difference in their resistance to hostile environment and also their survival capacity. These differences contribute to the quantity and quality of inoculum present in soil, thereby influencing pathogenicity.
3. The heterogeneous nature of soil conditioned by a huge microbial population leads to uneven distribution of pathogens in soil, especially in the rhizosphere region (Campbell and Van der Gaag 1993).
4. A large number of microbial species present in soil mask the population of disease-causing organisms in soil.

1.5 Groups of Soil-Borne Pathogens

Streptomyces These are filamentous prokaryotes having the capacity to produce mycelium colonizing the organic matter present in soil. Similar to fungi, these also have an immobile lifestyle, and they also produce spores for dispersal. The species of *Streptomyces* are well known for the production of metabolically active antibiotics, and these compounds improve the fitness in soil. It is interesting to note that only a small proportion of the described *Streptomyces* species are known to be plant pathogens (Table 1.2).

Bacteria These are single-celled microscopic organisms lacking a true nucleus. The structure of bacteria is simple as they do not possess nucleus and membrane-bound organelles. Their genetic information is placed in a loop of DNA. One gram of soil contains approximately 40 million bacterial cells. Among this huge population, a very minute portion of bacteria cause plant diseases, and the important genera reported to be plant pathogenic and reside in soil are *Erwinia*, *Streptomyces*, *Rhizomonas*, *Pseudomonas*, and *Xanthomonas* (https://ausveg.com.au/biosecurity-agrichemical/crop-protection/overview-pests-diseases-disorders/bacterial-diseases/) (Table 1.3).

Fungi These are eukaryotic organisms having well-defined nuclei and membrane-bound organelles. These organisms grow from the tips of hyphae that make up mycelia. They are very successful inhabitants in soil owing to their high adaptive nature in adverse conditions (Sun et al. 2005). According to Gardi and Jeffery (2009), the soil fungi can be grouped into fungi involved in biological control activity, fungi involved in the regulation of ecosystem, and fungi involved in the decomposition organic matter and transformation of compounds. Apart from this, a small group of fungi cause diseases in different crops. The predominant soil-borne pathogenic fungi are *Sclerotium rolfsii*, *Rhizoctonia solani*, *Fusarium* sp., *Pythium*, and *Phytophthora* with diseases (Table 1.4).

Table 1.2 *Streptomyces* spp. associated with different diseases

Streptomyces spp.	Disease name	Reference
S. scabies or *S. scabiei*, *S. acidiscabies*, *S. stelliscabiei*, and *S. turgidiscabies*	Common scab disease	Lerat et al. (2009)
S. aureofaciens and *S. griseus*	Potato superficial scab	Loria et al. (1997)
S. europaeiscabiei, *S. niveiscabiei*, *S. microflavus* or *S. luridiscabiei*, and *S. puniciscabiei*	Common scab disease in Korea	Park (2003)
S. reticuliscabiei	Netted scab of potato	Bouchek-Mechiche et al. (2000)
S. ipomoeae	Soil rot of sweet potato	Zhang et al. (2003)
Streptomyces sp.	Root tumor of cucurbits	Loria et al. (1997)

Table 1.3 Bacterial species associated with various diseases in different crops

Bacterial species	Disease name	Crop	Reference
Xanthomonas campestris pv. *campestris*	Black rot	Brassicas	Ignatov et al. (1998), Vicente and Holub (2013)
Clavibacter michiganensis pv. *michiganensis*	Bacterial canker	Tomato, capsicum, and chilli	Chang et al. (1992), Nandi et al. (2018)
Pseudomonas spp. and *Erwinia* spp.	Bacterial soft rot	Wide range of vegetables, including lettuce, brassicas, cucurbits, tomato, capsicum, potato, sweet potato, carrots, and herbs	Charkowski (2018), Sławiak et al. (2009), Charron et al. (2002)
Xanthomonas campestris	Bacterial leaf spot/bacterial spot	Range of vegetables including lettuce, cucurbits, tomato, and capsicum	Batista et al. (2021)
Ralstonia solanacearum	Bacterial wilt	Potato, tomato, capsicum, and eggplant	Sharma et al. (2021)
Pseudomonas syringae	Bacterial leaf spot/bacterial spot/bacterial blight	Beet, spring onions, leeks, rocket, and coriander	Fonseca-Guerra et al. (2021)

Table 1.4 Different diseases caused by fungal pathogens

Pathogen	Diseases	Reference
Cylindrocladium, *Pythium*, *Phytophthora*, and *Rhizoctonia*	Root rot	https://www.thespruce.com/what-are-soilborne-diseases-1402990
Phytophthora, *Rhizoctonia*, *Sclerotinia*, and *Sclerotium*	Stem, collar, and crown rots	https://www.thespruce.com/what-are-soilborne-diseases-1402990
Fusarium oxysporum and *Verticillium* spp.	Wilt	https://www.thespruce.com/what-are-soilborne-diseases-1402990
Pythium, *Phytophthora*, *Rhizoctonia*, and *Sclerotium rolfsii*	Damping-off	https://www.thespruce.com/what-are-soilborne-diseases-1402990
Ganoderma sp.	Stem rots and root rots	

Viruses These are obligate parasites that require living hosts for their multiplication and survival. They usually require vectors insects, nematodes, or fungi for transmission and spread. However, these vectors contribute to local movement within the field or adjacent fields. The long-distance movement of soil-borne viruses is due to the movement of infected planting materials and shifting of soils. Soil-borne viruses typically infect plant roots or other underground parts, causing significant losses in different crops (Roberts and Alison 2014) (Table 1.5).

Nematodes These are unsegmented worms with round bodies and pointed ends, otherwise called roundworms. The wide adaptability of these worms has made them as most abundant animals on earth. These occur as both free and parasites in nature

Table 1.5 Soil-borne viruses and their vectors

Virus name	Vectors	References
Barley mild mosaic virus	*Polymyxa*	Kanyuka et al. (2003)
Cherry rasp leaf virus	*Xiphinema*	Griffin and Epstein (1964)
Strawberry latent ringspot virus	*Xiphinema*	Griffin and Epstein (1964)
Arabis mosaic virus	*Xiphinema* and *Longidorus*	Griffin and Epstein (1964)
Freesia sneak virus	*Olpidium*	Sekimoto et al. (2011)
Cucumber soil-borne virus	*Abiotic transfer*	Kakani et al. (2003)
Melon necrotic spot virus	*Olpidium*	Sekimoto et al. (2011)
Carnation ringspot virus	*Olpidium*	Sekimoto et al. (2011)
Cucumber necrosis virus	*Olpidium*	Sekimoto et al. (2011)
Chinese wheat mosaic virus	*Polymyxa*	Kanyuka et al. (2003)
Peanut clump virus	*Polymyxa*	Kanyuka et al. (2003)
Beet soil-borne virus	*Polymyxa*	Kanyuka et al. (2003)
Beet virus Q	*Spongospora*	Falloon et al. (1996)
Potato mop-top virus		Santala et al. (2010)
Pea early-browning virus	*Paratrichodorus* and *Trichodorus*	Karanastasi et al. (1999)
Beet necrotic yellow vein virus	*Polymyxa*	Kanyuka et al. (2003)
Tobacco rattle virus		Karanastasi et al. (1999)
Lettuce big-vein virus	*Olpidium*	Lot et al. (2002)
Watercress yellow spot virus	*Spongospora*	Falloon et al. (1996)

(https://www.britannica.com/animal/nematode). A very minute portion of nematodes has been identified to be pathogenic to different crops. The major genera of nematode infecting plants include *Meloidogyne*, *Globodera*, *Heterodera*, *Pratylenchus*, *Ditylenchus*, *Rotylenchulus*, *Xiphinema*, *Aphelenchoides*, and *Bursaphelenchus* (https://ohioline.osu.edu/factsheet/plpath-gen-8). About 90% of nematodes reside in the top 15 cm of soil. The plant parasitic nematodes are reported to consume 10% of global agricultural production, tolling to 125 billion loss every year (Chitwood 2003). In addition to acting as pathogens, they also act as vectors of plant viruses (Table 1.6).

1.6 Detection Methods for Major Soil-Borne Pathogens

Soil-borne diseases represent a major share of the reported 80,000 plant diseases across the globe. Most of the diseases are fatal to crops reflecting huge yield loss as indicated in the tables earlier. Furthermore, they cause 10–20% more diseases compared to airborne and seed-borne pathogens. As they reside in soil, they cause their initial damage to crops, which is underestimated many times. Thus, the early detection of these microorganisms in the soil could help farmers to optimize their

Table 1.6 Nematodes acting as vectors of different virus

Nematode vector	Virus
Xiphinema diversicaudatum	Arabis mosaic virus
X. index and *X. italiae*	Grapevine fanleaf virus
X. americanum and *X. rivesi*	Peach rosette mosaic virus
X. americanum, *X. californicum*, *X. intermedium*, *X. rivesi*, and *X. tarjanense*	Tobacco ringspot virus
X. americanum, *X. californicum*, and *X. rivesi*	Cherry rasp leaf virus
X. diversicaudatum	Strawberry latent ringspot virus
Longidorus apulus and *L. fasciatus*	Artichoke Italian latent virus
L. elongatus	Beet ringspot virus
L. martini	Mulberry ringspot virus
L. elongatus, and *L. macrosoma*	Raspberry ringspot virus
L. attenuates and *L. elongatus*	Tomato black ring virus

crop yield by suppressing pathogens and avoiding disease development. The detection of major pathogens is discussed hereunder.

1.7 Detection of Soil-Borne Pathogens

1.7.1 Traditional Methods

1.7.1.1 Direct Quantification

The method of estimating soil-borne fungi mainly depends on the direct counting of resting structures of pathogens, and it is more precisely applicable to fungi producing sclerotial bodies such as *Sclerotium rolfsii* and *Rhizoctonia solani*. In this approach, the number of sclerotial bodies present in a known quantity of soil is estimated by sieving soil through a sieve of 250 mesh. The viable count of sclerotial bodies can be estimated after moistening the 50 g of soil with 12.5 ml of 1% methanol (Rodriguez-Kabana et al. 1980).

1.7.1.2 Enumeration of Pathogens

Soil is a complex environment, and the presence of microbial pathogens is influenced by various biotic and abiotic factors. In order to assess the load of particular pathogens, enumeration of pathogenic propagules (cells and spores) from soil is one of the basic and primeval methods for detection and quantification of soil-borne plant pathogens. Conventional enumeration techniques prerequire sample preparation where the bacterial cells/fungal spores from the soil sample

matrix are dispersed in a suitable diluent (Foght and Aislabie 2005). Sterile distilled water, phosphate-buffered saline, potassium phosphate, or mineral salts medium devoid of carbon source are the most commonly used diluents (Atlas 1995). After the cells/spores are congruously dispersed in these diluents, serial dilutions are performed and the individual cells/spores are then enumerated by microscopic visualization or cultivation methods. The dilution factor employed for the detection varies with the technique used (Foght and Aislabie 2005). The two major enumeration techniques used for the detection and quantification of pathogens are the direct or microscopic visualization method and the culture-based enumeration method.

1.7.1.2.1 Enumeration of Bacteria

Direct or Microscopic Visualization of Bacteria

This technique enables to count the total number of cells present in the sample by staining with a fluorescent dye and subsequently visualizing the cells through epifluorescence microscopy. The most common fluorescent dyes used are acridine orange and 4,6-diamino-2-phenylindole (DAPI) (Bölter et al. 2002). One of the shortcomings of this method is that enumeration takes into account both dead and live cells. However, the recent development of certain new dyes such as 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) (Créach et al. 2003) and propidium iodide + thiadazole orange (Foght and Aislabie 2005) has resulted in detecting metabolically active cells, thus discriminating live and dead cells. Autofluorescence of soil matrix components and occlusion of bacterial cells by soil particles can interfere with detection techniques, thus reducing its efficacy. Recently confocal laser scanning microscopy has been employed to improve the detection and visualization of cells over conventional microscopy.

Culture or Cultivation-Based Enumeration of Bacteria

The viable cells present in the soil suspension can be detected and enumerated using this technique, but it is limited by the fact that only culturable bacterial populations can be detected by this method. As compared to highly sophisticated molecular techniques, this method is relatively simple, inexpensive, and easier to interpret. Culture-based enumeration techniques are of two types: the most probable number method (MPN) and plate count method.

Most Probable Number Technique (MPN)

This method involves the addition of serially diluted soil suspensions to a liquid medium, which is then incubated under required conditions to yield a series of cultures that is scored in accordance with a predetermined criterion (Alef and Nannipieri 1995; Atlas 1995). The cell population can be identified by employing various methods such as turbidimetry or screening the production of certain

metabolites. The data obtained is finally evaluated by statistical tools to infer the MPN of viable cells in the undiluted sample (Eaton et al. 1995; Alef and Nannipieri 1995; Koch 1994). This method can be used to detect and enumerate certain selective bacterial pathogens by providing suitable selective cultivation media. Although it gives only a statistical estimate of bacterial cells in the given suspension, it is more suitable for particulate samples and can detect pathogens that do not grow well in a solid medium.

Plate Count Technique

This is a relatively rapid and inexpensive technique that enables the detection of viable bacterial pathogens present in a soil sample by enumerating colonies formed over a solid growth media inoculated with sample dilutions. This method is based on the speculation that each bacterial colony on the growth media has originated from a single cell or endospore, thus referring to them as colony forming unit (cfu). Although the method is biased as it only allows the detection and counting of culturable cells, it yields well-separated colonies of bacterial pathogens, which can be subsequently purified and characterized (Foght and Aislabie 2005).

1.7.1.2.2 Enumeration of Fungal Pathogens

Enumeration of fungal pathogenic propagules from the soil can be done by using a common technique known as serial dilution. Serial dilution is a step-by-step dilution technique, where the soil dilution factor remains constant with a geometric progression. Tenfold serial dilutions result in 1M, 0.1M, 0.01M, 0.001M, and subsequent concentrations and are plated on specific media to count the number of viable pathogens (Aneja 2005). Mitsuboshi et al. (2016) enumerated *Fusarium* sp. present in soil by plate count technique.

This count gives the colony forming units and not the count of individual microbes. However, these counts are considered very accurate for estimating the number of microbes in original samples. Drawbacks of this test are time- and space-consuming and require specialized equipment that must be prepared correctly. The other important drawback with the enumeration is that only viable pathogenic structures can be assessed (Wetzel 2001).

1.7.1.3 Use of Selective Media

Isolation of pathogen residing in soil in pure form is an important step in the diagnosis of disease. There has been huge amount of literature on the use of specific media that supports the growth of desired organisms. The media supporting the growth of desired organisms by preventing undesired microbes in it through inhibitory chemicals are referred to as selective media. These types of media generally contain an inhibitory chemical that will selectively inhibit all microbes except the

desired group of microbes. The classical example of selective media for fungal pathogens is peptone-pentachloro nitro benzene (PCNB) medium (Papavizas 1967). PCNB was earlier used to prevent the contamination of zygomycetes in cultures. However, due to its hazardous nature and carcinogenic ability, it was banned from usage. Boknam Jung et al. (2013) developed a selective media for the isolation of *Fusarium graminearum.* Furthermore, acidified weak potato-dextrose agar (AWPDA) along with thiabendazole was developed as the selective media for the isolation of *Alternaria* species from samples of soil (Hong and Pryor 2004).

Different pathogenic phytobacteria utilize different metabolic pathways, and this nutritional diversity can be used in the development of selective agar media (Schaad 1987). Kado and Heskett (1970) developed five selective plating media for the detection of pathogenic bacteria in the genera *Xanthomonas*, *Pseudomonas*, *Erwinia*, *Corynebacterium*, and *Agrobacterium*. A major constraint faced in the development of selective media for plant pathogenic bacteria is that most of them have a very narrow nutritional demand. However, it is a relatively easy and rapid method once the growth media specific to a particular pathogen is standardized (Table 1.7).

1.7.1.4 Indicator Plants

The use of indicator plants or bio-indicators can aid in determining whether or not a field is contaminated with a bacterial pathogen. The detection of the pathogen is based on the symptoms observed and the time taken for symptom development. Tomatoes and potatoes are the most common indicator plants used for the detection of *Ralstonia solanacearum* race 3. More particularly, potato seedlings bearing small tubers can serve as a rapid diagnostic tool for the detection of *R. solanacearum* race 3 (Graham and Lloyd 1978). Also Paret et al. (2009) evaluated three different varieties of ginger and found that tissue-cultured edible ginger was most suitable for the detection of *R. solanacearum* race 4. Similarly in fungi, the presence of *Ganoderma* in coconut gardens was detected through the use of pigeon pea as indicator plants (Snehalatharani et al. 2016). The use of indicator plants for detection is time consuming, labor intensive, and not widely preferred.

1.7.1.5 Baiting or Trapping Techniques

Bait is any substance that is preferred by an organism for its growth, and in the presence of such substance, the growth of the organism is enhanced. The small piece of plant parts/substance is placed near soil for a known period of time so as to allow the desired organisms to grow into the bait. The baiting material will be afterward placed into selective culture media. The material used for the growth of the pathogen is called the bait and the method is referred to as baiting. In this method, the parasitic nature of the pathogen will be exploited to separate the pathogen from a diverse

Table 1.7 Selective media for isolation and enumeration of fungi and bacteria

Media	Pathogen	References
Fungi		
PARP (pimaricin, ampicillin, rifampicin, pentachloronitrobenzene) medium	*Pythium* spp.	Tojo (2017)
3P medium and PV medium	*Phytophthora*	Eckert and Tsao (1962)
DCPA (dichloran-chloramphenicol peptone agar) medium	*F. oxysporum*	Bragulat et al. (2004)
NS medium (Nash and Snyder medium)	*F. oxysporum*	Bragulat et al. (2004)
PDID medium (potato dextrose iprodione dichloran agar)	*F. oxysporum*	Bragulat et al. (2004)
CZID (Czapek Dox iprodione dichloran agar) medium	*F. oxysporum*	Bragulat et al. (2004)
PSAA (potato sucrose acidified agar) medium	*Sclerotinia Sclerotiarum*	Steadman et al. (1994)
Neon agar medium	*Sclerotinia sclerotiorum*	Peres et al. (2002)
TB-CEN medium	*Thielaviopsis basicola*	Specht and Griffin (1985)
Bacteria		
Kritzman's selective medium	*Xanthomonas campestris* pv. *campestris*	Kritzman and Ben-Yephet (1990)
MMG medium (maltose, methyl green, and antibiotics)	*Xanthomonas campestris* pv. *vitians*	Toussaint et al. (2001)
Modified Miller–Schroth medium	Pectolytic *Erwinia*	Pierce and McCain (1992)
MSCFF	*Curtobacterium flaccumfacciens* pv. *flaccumfaciens*	Maringoni et al. (2006)
Crystal violet pectate (CVP)	Pectolytic *Erwinia*	Cuppels and Kelman (1974)
Tetrazolium medium	*Ralstonia solanacearum*	Kelman (1954)
XAS medium	*Xanthomonas albilineans*	Davis et al. (1994)
SMSA	*Ralstonia solanacearum*	Elphinstone et al. (1996)

organism present in soil. In the earlier studies, many soil-borne pathogens were isolated and purified using this simple method. The common examples of baits are dead insects, boiled seeds, pollen grains, and nails (Shew and Meyer 1992). *Thielaviopsis basicola* is a soil-borne pathogen that is reported to be a pathogen on 200 plant species that produce two kinds of spores known as cylindrical endoconidia and as aleuriospores. This pathogen was proved to be isolated by carrot disc in soil (Yarwood 1946). Sharadraj and Chandra Mohanan (2016) identified leaves of the badam tree as baiting agents for the isolation of *Phytophthora palmivora*. Anandaraj and Sarma (1990) reported that *Albizia falcafaria* (L.) leaflets

could be used as baits for the isolation of black pepper *Phytophthora* from soil. Hussain et al. (2015) developed a baiting technique for the easy isolation and detection of *Phytophthora* from infected soil samples. This method included dipping of sliced potato tubers in distilled water containing Bemlat, Nystatin, and Rifampicin and placing them in soil for four days. Furthermore, carrot was used as one of the important baits for isolating *Ceretocystis* sp. from the soil (Zhang et al. 2019).

1.7.1.6 Bioassay Tests

It is one of the simple tests to assess the inoculum level in a given soil. This method involves collection of soil samples from a target field in small quantities. This soil sample will be used for raising a cultivar/variety that is most susceptible to a particular disease in the soil. All the conditions required for disease expression are created. The disease severity will be measured at an appropriate time to the risk index for a given soil. This method particularly correlates inoculum potential with disease level. It is found to be very effective as it takes into account the natural inoculum and soil environment on disease (Gatch and Du Toit 2015). Bioassay studies have been very successful in assessing the risk of *Aphanomyces euteiches* and *Fusarium solani* f. sp. *pisi* causing root of peas in the USA and Europe (Malvick et al. 1994). Bioassay studies were conducted to identify the causal agents of sugar beet diseases in southern Sweden. It was inferred that damping-off pathogens, *Aphanomyces cochlioides*, *Pythium* sp., and *Rhizoctonia solani*, were found to be dominating pathogens in soil (Amein 2006). Neher and Weicht (2018) reported the plate assay usage for the estimation of *Rhizoctonia solani* in soil. Dignam et al. (2015) compared the disease suppressive capacity of pasture soil through bioassay of *R. solani* AG 2-1 using *Brassica oleracea.*

1.7.2 Biochemical Methods for Bacteria

The use of various biochemical methods for bacterial identification is a widely used technique as each bacterium has distinct biochemical characteristics. A set of biochemical tests can be performed to detect and differentiate bacteria based on their ability to metabolize certain substrates. More recently, techniques such as FAME (fatty acid methyl ester) analysis (Sharma et al. 2021) and Biolog-based bacterial identification have resulted in rapid and easier diagnostics.

1.7.2.1 Basic Biochemical Tests

A combination of biochemical tests are performed, which can distinguish different pathogenic bacteria based on their nutritional and metabolic capabilities. The ability of bacteria to produce certain enzymes, degrade certain metabolites, or survive in the

presence of certain inhibitors is taken into account to distinguish and characterize different bacterial species. Thus, different soil-borne plant pathogenic bacteria can be characterized based on their ability to enzymatic capabilities, carbohydrate oxidation and fermentation, amino acid degradation, single substrate utilization, and inhibitor profiles. Although biochemical tests are inexpensive and don't require much expertise to perform, they are time consuming and the results can be unstable.

1.7.2.2 Biolog

The Biolog Omnilog identification system, introduced in 1989, is an automated system that can distinguish pathogenic bacteria based on its ability to oxidize a panel of 95 carbon sources. When the bacteria respire, the exchange of electrons leads to the reduction of tetrazolium dye present, turning it purple. Thus, the chemical sensitivity and ability to utilize carbon sources confers each bacterium with a unique metabolic fingerprint. These are then analyzed and compared with the available database to identify the bacteria (Miller and Rhoden 1991). The phytopathogenic bacteria can be identified up to genus and species level within an incubation time period ranging from 4 to 24 h. Massomo et al. (2003) were able to identify all the evaluated *Xanthomonas campestris* pv *campestris* strains up to genus level, 47% of strains to the pathovar and 43% to strain level. About 1000 strains of *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, and *Xanthomonas* were identified up to genus, species, and pathovar level using a Biolog GN microplate system. It has also been successfully employed for the identification of *Ralstonia solanacearum*, which offered more rapid and accurate results as compared to traditional methods (Tawfik et al. 2008). Wang et al. (2020) used the Biolog system to observe the effect of temperature on metabolic phenotypes of *Ralstonia solanacearum* and observed that temperature did have an effect on the characterization of the pathogen. The automated technology has ensured rapid and accurate diagnostics as compared to traditional biochemical tests. But the procedure still requires pure culture and growth of bacteria in the specified media, which becomes challenging when it comes to slow-growing or non-culturable bacteria.

1.7.2.3 Fatty Acid Methyl Ester (FAME) Analysis

FAME analysis is employed for the identification of soil-borne phytopathogens based on the principle that each bacteria has a unique fatty acid profile, both in terms of quality and quantity. After the bacteria is isolated and cultured in agar media, their fatty acids are extracted, saponified, and esterified for analysis. The fatty acid profile is then determined through gas chromatography, which is then compared with the profiles of a predetermined library of known isolates with pattern recognition software. Rajan et al. (2011) ascertained that FAME analysis is equally reliable and cost effective as compared to bacterial identification through 16S rDNA sequencing. Norman et al. (2009) employed FAME analysis to identify and confirm

several strains of *Ralstonia solanacearum* collected from different geographical locations. Although the method was successful in identifying the strains as *R. solanacearum*, it could not be distinguished based on biovar, original host, or geographical location. Apart from detection and identification, FAME analysis can also be used to delineate the relationship between different bacterial species based on their fatty acid profile (Jarvis et al. 1996). Bouzar et al. (1993) employed FAME analysis to differentially characterize four different species of *Agrobacterium*, namely, *A. tumifaciens*, *A. rhizogenes*, *A. vitis*, and *A. rubi*, based on their fats acid composition and metabolic fingerprinting. A fatty acid methyl ester-based identification system was developed for *Pectobacterium* sp. based on its ability to produce ten different fatty acids. The identification of unknown pectinolytic bacterial strains can be achieved by comparing ratios between different fatty acids (Dawyndt et al. 2006). The method offers a more accurate identification compared to biochemical tests and can differentiate bacterial strains based on their fatty acid composition. Moreover, the analysis is faster, simple, easier to perform, and highly automated. The only limitation is that the analysis is limited to fatty acid profiles available in the database.

1.7.3 Bacteriophage Typing

Although viruses have acquired a negative public perception in plant pathology, bacteriophages (virus-infecting bacteria) have been found resourceful in many instances. Ranging from phage therapy to phage typing, bacteriophages have found numerous applications in phytobacteriology. Bacteriophage typing (phage typing) is based on the specificity of each bacteriophage to attack its host bacterium (Clark and March 2006). Each strain of bacterial pathogen will be attacked by a specific bacteriophage. For the detection and identification of soil-borne pathogens, the bacterial samples in question are screened with specific phages. A bacterial lawn is prepared along with different phages, and the formation of plague indicates that the phage had lysed the bacterial cell, which facilitates the identification of the specific bacterial strain. Gross et al. (1991) utilized phage typing to detect the diversity and distribution of soil-borne pathogens *Erwinia carotovora* pv *carotovora* and *Erwinia carotovora* pv *atroseptica*. The phages isolated were specific to the pathogens, even facilitated the differential detection of *Erwinia carotovora* pv *carotovora* and *E. cvarotovora* pv *atroseptica.* Although isolation and characterization of specific bacteriophages are laborious and time consuming, the extreme specificity of the technique coupled with the possibility of mass multiplication of phages and its resistance to adverse conditions makes it a widely accepted diagnostic approach. Moreover, the sensitivity of the technique can be further improved if the bacteriophages attacking the bacteria are detected through specific antibodies (Watson and Eveland 1965). With the advent of genetic engineering techniques, bacteriophages can be genetically modified to express green fluorescent proteins when it infects the target bacteria (Funatsu et al. 2002). Bacteriophages can also be

genetically engineered to transfer reporter genes into target bacteria, and the expression of such genes can serve as a marker for bacterial detection (Farooq et al. 2018). Moreover, integration of phage typing with nucleic acid amplification methods, such as q-PCR, is a much more sensitive and rapid approach in diagnostics of soil phytopathogens. A phage-based indirect assay was employed where primers were developed for detecting bacteriophages specific to *R. solanacearum* from different substrates including soil. The combined technique was able to overcome the limitations of both conventional PCR and bacteriophage typing for pathogen detection. From soil, *R. solanacearum* was detected approximately to 10^2 cfu/g (Kutin et al. 2009).

1.7.4 Immunological Methods

Immunoassays or protein-based detection techniques make use of antigen–antibody interaction to detect soil-borne bacteria. This method prerequires the synthesis of monoclonal or polyclonal antibodies specific to bacterial antigens. Bacterial antigens can be proteins, complex carbohydrates, polynucleotides, or lipopolysaccharides, which can elicit the production of antibodies in the mammalian body. Despite the fact that the productions of these antibodies are time consuming, once they are synthesized, the diagnostic procedure can yield results quickly. Moreover, they can be employed for laboratory as well as field level detection, and the sensitivity can go up to nanogram level. Immunoassay techniques have also got their own share of demerits. These techniques cannot detect previously undescribed bacteria as only those bacterial pathogens against which antibodies have been synthesized can be detected. Furthermore, there is an occurrence of cross-reactions, and the degree of relatedness between cross-reacted isolates cannot be determined. The most commonly available immunological techniques for the detection of soil-borne pathogens are ELISA, dip-stick immunoassay, lateral flow devices, tissue blot, western blot, immunofluorescence assay, and immunoelectron microscopy (Mancini et al. 2016; Luchi et al. 2020; Afouda et al. 2009).

1.7.4.1 ELISA

Enzyme-linked immune sorbent assay is a rapid immunochemical test that detects the soil-borne fungi based on antigenic properties and color change. In this method, target antigens from the fungi are made to specifically bind with the antibodies conjugated to the enzyme. The detection of the pathogen is carried out by the visualization with the color change. It is a very specific and highly sensitive test mainly used for detecting various plant pathogens. The performance of ELISA can be increased with specific monoclonal and recombinant antibodies. For more specific and sensitive detection, monoclonal antibodies are used and the detection limits are in the range of 10^5–10^6 (López et al. 2003). Various ELISA techniques can be

used for the detection of soil-borne fungi. Indirect ELISA techniques can be used for detecting soil-borne *Phytophthora*, which shows a strong positive reaction with the sporangia, mycelium, and oospores. The detection was possible through infected leaves as well as tubers, which help in the accurate detection, diagnosis, and isolation of the pathogen (Hussain et al. 2016).

Tsuchiya et al. (1991) compared both direct and indirect ELISA in detecting *Pseudomonas cepacia* present in soil. Both variants of ELISA were equally sensitive in detecting the pathogen from soil, with detection limits going up to 10^4 cfu/ml. Also, the detection limits could be further enhanced to 10^2 cfu/ml by heating the samples at 100 °C for 15 min. Enrichment ELISA is an improvement over the conventional ELISA to detect soil-borne bacteria as it increases the sensitivity of ELISA by 10^4-fold, by multiplying metabolically active cells. *Ralstonia solanacearum* in soil samples was detected by this technique by enriching soil suspension in a semi-selective broth containing potato tuber infusion (Priou et al. 2006). Serial dilution of soil suspensions before enriching also allowed the quantification of bacteria in soil, thus enabling analysis of its field soil population. Also, by developing more specific antibodies, the different biovars of *R. solanacearum* could be distinguished using ELISA.

1.7.4.2 Lateral Flow Immunoassays

Lateral flow tests are otherwise called lateral flow immunochromatographic assays and are the devices made to detect a target substance in a given sample (Koczula and Gallotta 2016). These devices work on the same principle as the ELISA does. The specimens in the lateral flow device flow through capillary action along the test strip. Positive and control tests are visible in different colors. It is very rapid and easy, does not require sophisticated instruments for the detection, is low cost, and can be performed with basic training. LFA results are immediate and typically available between 10 and 60 min (Lindsley 2013, 2016; Pfeiffer and Wong 2015). Safenkova et al. (2017) developed a lateral flow assay method for the detection of *Dickeya dianthicola* and *Dickeya solani*, causing black leg disease in potatoes. The polyclonal antibodies developed after immunization of rabbits with two bacterial strains were collected and tested for their efficacy in detecting the bacteria. It was confirmed that the polyclonal antibodies could only detect the related two species, and there was no cross-reactivity. Similarly, lateral flow dipsticks were developed for the detection of *P. sojae* from soil. The test could detect pathogens within 5 min with good specificity (Dai et al. 2019). Harrison et al. (1990) developed a polyclonal antiserum, which reacted with crushed mycelial extracts of *Phytophthora* spp. but did not cross-react with other pathogens of potatoes.

1.7.4.3 Immunofluorescence Assay

Immunofluorescence assay (IFA) is another important technique used for the detection mainly based on the presence of antibodies by their specific ability to react with antigens. Bound antibodies are visualized with fluorescently labeled antibodies. A more number of immunological assays have been developed for oomycetes. A fluorescently tagged immunofluorescence assay has been developed for the detection of *Phytophthora cinnamomi* from infected soils (which made early, easy detection of the pathogen from soil. Hansen (2006) developed a surface plasmon resonance (SPR) immunosensor for the detection of *P. infestans* sporangia in soil where potatoes are grown. Immunofluroscent assay has been used for the detection of a few bacterial pathogens such as *Ralstonia* (*Pseudomonas*) *solanacearum* Biovar 2 (Race 3). The success rate was 92–96% (Machmud and Yadi 2008; van der Wolf et al. 2000). Other protein-based techniques include immunomagnetic separation, immunofluorescence, lateral flow devices, and immunostrips. Though the principle is the same as that of ELISA, these methods offer more sensitivity and reduce the loss of bacterial cells due to washing in ELISA microtiter plates. Paret et al. (2009) evaluated the efficacy of immunostrips over conventional ELISA to detect *R. solanacearum* from soil as well as water samples. Immunostrips trap the antigen more efficiently and offer more sensitive detection within the limits of 10^2–10^1 cfu/ml. Although different serological methods offer highly sensitive detection of soil-borne pathogens, the cost involved in the development of monoclonal antibodies is a major limitation.

1.7.4.4 Western Blot

The western blot, also known as western blotting, is a commonly used technique in molecular biology and immunogenetics for the detection of specific proteins in a sample of tissues (Yang et al. 2012). It mainly consists of three elements for separating specific proteins. Generally, separation is based on size, transfer of protein to a solid support, and marking the protein by using a primary and secondary antibody. The primary antibody binds to the specific target protein. A secondary antibody is added, which recognizes and binds to the primary antibody. Visualization of secondary antibodies is carried out through various means like immunofluorescence and staining techniques.

Monoclonal antibodies were raised against antigens from *Pythium sulcatum* species in order to detect. The monoclonal antibodies showed high specificity to seven *P. sulcatum* isolates among the total 26 species of various soil-borne fungi. Weak cross-reactivities were recorded in various *Pythium* species such as *Pythium aristosporum*, *Pythium myriotylum*, and *Pythium zingiberum* with indirect enzyme-linked immunosorbent assay (ELISA) techniques. However, no such weak cross-reaction was observed in western blot analysis. The monoclonal antibodies recognized glycoproteins present in the cell wall. By using these techniques,

Pythium sulcatum was easily detected in carrot tissues and also from soil (Kageyama et al. 2002).

1.7.5 Molecular Methods

Soil-borne plant pathogens exhibit a great diversity with respect to morphology and cultural characteristics. Traditional identification mainly relied on the morphology and cultural characteristics of pathogens. However, in many pathogens, the morphological characters are overlapping to each other. The accurate identification of these pathogens based on these conventional methods is little difficult and needs taxonomic expertise. Furthermore, these methods are time consuming and laborious. It is at this juncture that more advanced molecular techniques have gained importance for the detection of plant pathogens. Furthermore, the sensitivity of these techniques is very high. For example, the sensitivity of the molecular techniques for detecting soil bacteria ranged from 10 to 10^6 colony-forming units/mL (López et al. 2003). The most commonly used molecular methods for soil-borne pathogens are discussed hereunder.

1.7.5.1 Fluorescent Microscopy

Fluorescent microscopic observations are an important tool for observing and detecting various microbes from different habitats. Cationic and anionic dyes such as fluorochromes are used in the case of fluorescent microscopes based on the ability to bind with specific cellular components. The common fluorochromes used for imaging soil fungi are aminofluorescein, 8-anilino-1-naphthalene sulfonic acid, calcofluor white M2R, fluorescein isothiocyanate, acridine orange, and ethidium bromide. Fluorescence in situ hybridization, also known as FISH, is one of the important cytogenetic techniques and works based on fluorescent probes, which bind to a specific part of nucleic acid. Fluorescence microscopy can be utilized to find where the probe has been bound. It is an important technique mainly used for the species-level identification of microorganisms (Amann et al. 2008). This technique was used to view resting structures and spores in soil (Tsao and Ocana 1969).

1.7.5.2 DNA/RNA Sequencing

The literal meaning of sequencing is determining the primary structure of a biopolymer. It results in the depiction of sequences that summarizes the atomic structure of the molecule sequenced. The process of DNA sequencing involves determining the nucleotide sequence order in a given fragment of DNA. This sequence encodes specific amino acids that make up proteins, and these proteins determine the characters of organisms (Wheeler et al. 2008). The sequencing of nucleotides

gives information on how and why organisms live in the environment. Therefore, it is necessary to understand the sequence of nucleotides in organisms. Sequencing of DNA gives the genetic profile of an organism, whereas sequencing RNA gives us information on sequences expressed in cells. In order to sequence RNA, first RNA has to be reverse transcribed to generate cDNA fragments, and these fragments can be later sequenced like DNA.

For bacterial identification, the 16S RNA gene has been regarded as the universally conserved region and is widely used in the molecular characterization of plant pathogenic bacteria (Song et al. 2004). The 16Sr RNA gene sequence is also used for the phylogenetic classification and taxonomic characterization of a new bacterial pathogen based on its similarity to sequences existing in the database (Mizrahi-Man et al. 2013). Nowadays, 16S rRNA gene-based metagenomic analysis has also enabled researchers to detect and quantify nonculturable bacteria from soil (Tshikhudo et al. 2013). The causal agent of new bacterial wilt of *Cucurbita maxima* in China was identified as *R. solanacearum* species complex through sequencing of ITS regions of bacteria (She et al. 2017). The sequencing of other housekeeping genes such as genes for virulence, pathogenicity, or plasmid can also aid in the identification of specific plant pathogenic bacteria (Tewari and Sharma 2019).

In the case of fungi, 18S ribosomal RNA (rRNA) gene sequencing is commonly used for identifying and comparing species present within a given sample (Banos et al. 2018). The universal regions ITS1 and ITS2 sub-regions have been applied as metabarcoding markers and are the widely sequenced regions. B-tubulin, actin, GADPH, Ef1a, Cox1, and Cox2 are the common sequencing genes for the identification of fungal pathogens. The genetic relations of two stem rot pathogens, namely *S. rolfsii* and *S. delphinii*, were evaluated by sequencing ITS regions. It was concluded that two different ITS types exist within *S. rolfsii* and *S. delphinii* strains (Okabe and Matsumoto 2003).

Sequencing technologies have also seen major evolutions in the last few years. The basic and commonly used technologies like Sanger sequencing are now being replaced by high-throughput sequencing, which offers a large amount of genetic information within a limited time period. Several next-generation sequencing technologies such as sequencing by hybridization, sequencing by synthesis, 454 pyrosequencing, ion torrent, and Illumina technology have revolutionized bacterial genome sequencing and identification (Slatko et al. 2018). High -throughput sequencing employing an Illumina MiSeq-2500 sequencer was used to compare and characterize the genomes of *R. solanacearum* moko ecotypes of different sequevars (Pais et al. 2021). These techniques offer rapid identification of plant pathogenic bacteria with increased accuracy and efficiency with reduced expense.

1.7.5.3 DNA Fingerprinting

DNA fingerprinting refers to the method of detecting unique DNA patterns, which allow the identification of individuals with a probability of error similar to (or lower than) that obtained by comparing fingerprints in humans. These unique, individual

patterns of DNA are the result of Mendelian inheritance of polymorphic, hypervariable loci of repetitive DNA. The most useful loci are those consisting of tandem repeats of short (15–60 bp) or very short (3–5 bp) specific base sequences. DNA fingerprinting is otherwise called by various names: DNA typing, genetic fingerprinting, genotyping, or identity testing, and DNA profiling (https://www.britannica.com/science/DNA-fingerprinting). Fingerprinting allows the screening of random regions of the pathogen genome mainly for recognizing species-specific sequences when conserved genes are not enough to identify species (Patil 2018). It is commonly used to understand the phylogenic structure of fungal populations. It is also used for identifying specific sequences to detect the pathogen at a low taxonomic level, such as for differentiating at the species level (Ghosh et al. 2019). This technique was originally developed by British scientist *Alec Jeffreys* in 1984. However, Dr. *Lalji Singh* is known as the father of DNA fingerprinting in India. There are various methods for fingerprinting, viz., RFLP, AFLP, RAPD, etc.

RAPD markers were the oldest marker system for developing the genetic structure of pathogens (Nasir and Hoppe 1991). They have been used in a number of cases for assessing the genetic structure of many fungal and bacterial pathogens owing to their ease in handling and cost-effectiveness (Belabid et al. 2004; Kini et al. 2002; Sayeda et al. 2015). RAPD analysis can be used to detect genetic variation and relationships among different isolates of *R. solanacearum* (Nishat et al. 2015). However, due to its non-reproducibility and dominance nature, its usage in the recent era has been almost limited.

Restriction fragment length polymorphism (RFLP) is another type of fingerprinting method in which the polymorphism is obtained by cutting the DNA with restriction enzymes. The sites at which DNA are cut are known as restriction sites (Varshney et al. 2004). There have been a number of studies demonstrating the use of RFLP in identifying plant pathogens (Camele et al. 2005; Drenth et al. 2006). Gómez-Alpízar et al. (2011) used PCR-RFLP for the identification and detection of *Pythium myriotylum*, the causal agent of the cocoyam root rot disease. Detection and characterization of *Erwinia carotovora* species from soil were performed by PCR-RFLP test targeting pectate lyase encoding genes. The test enabled detection of *Erwinia carotovora* up to subspecies level and perception of the molecular diversity of the pathogen (Hélias et al. 1998).

Amplified fragment length polymorphism (AFLP) is a fingerprint technique that is based on selective amplification of a subset of digested DNA fragments to generate and compare unique fingerprints for genomes of interest (Paun and Schönswetter 2012). AFLP markers have been found to have the widest application in studying the genetic structure of pathogens. Garzón et al. (2005) used AFLP to diagnose and analyze the population of *Pythium* sp. causing root rot and damping off in different crops.

1.7.5.4 Polymerase Chain Reaction (PCR)-Based Detection

It is a simple thermal reaction to amplify the piece of DNA and was developed by Kary Mullis in the 1980s. It was a magnificent invention in the field of molecular biology, and the inventor was awarded Nobel Prize in the field of chemistry in 1993. It was otherwise called molecular photocopying. The three basic steps in this are denaturation, annealing, and extension. Any piece of DNA can be amplified with suitable primers. Since the discovery of the PCR technique by Kary Mullis in 1983, a wide variety of applications in plant pathology have been found, the most important being the detection of plant pathogens. Over the years, many variants of PCR have emerged, namely multiplex PCR, nested PCR, colony PCR, real-time PCR, etc. A semi-nested PCR was developed to detect tumor-inducing *Agrobacterium* in soil using three novel primers targeting the tms2 gene. The technique enabled the detection of pathogens to the range of 1–2 bacterial cells in one gram of soil when the soil suspension was pre-incubated in a selective medium prior to PCR reaction (Puławska and Sobiczewski 2005). Faster identification of *Agrobacterium* biovars 1 and 2, *A. rubi*, and *A. vitis* was facilitated by the development of multiplex PCR based on the difference in sequences of the 23S rRNA gene. Apart from identification, the technique also aided in ecological studies of soil-borne bacteria (Puławska and Sobiczewski 2005). Recently, real-time PCR has evolved as a high-throughput technology, which allows quick detection and quantification of soil-borne phytopathogens. Compared to conventional PCR, the chances of false positives are reduced in this technique. A combination of real-time PCR with modified soil DNA extraction protocol resulted in a specific and sensitive method for the detection and quantification of *R. solanacearum* from soil with a degree of sensitivity of 100 fg/ul (Huang et al. 2009). One of the major problems encountered in real-time PCR detection assays is the presence of inhibitors from soil and plant samples. To overcome this, immunomagnetic separation and magnetic capture hybridization are coupled with RT-PCR, which permitted the detection of *R. solanacearum* race 3 biovar 2 at 500 cells/ml (Ha et al. 2012). PCR techniques are one of the most accurate and sensitive techniques for the detection and quantification of soil-borne pathogens. More recently, portable real-time PCR detection systems are being developed for the on-site detection of pathogen inoculum in agricultural plots.

1.7.5.5 Isothermal Amplification Techniques

Loop-mediated isothermal amplification (LAMP) is yet another nucleic acid amplification technique that offers specificity, sensitivity, and accuracy without the use of expensive thermal cyclers. It makes use of a set of four primers, that is, two long outer and two short inner primers, which recognize six specific sequences in target DNA. It is regarded as an ideal point of care detection method for bacterial pathogens in the field, and the results of the reaction could be interpreted by the naked eye through a change in color or turbidity. Moreover, the detection tests can be

carried out and interpreted by any person without any technical or taxonomic expertise. Kubota et al. (2008) have developed a quick and precise technique for the detection of *R. solanacearum* from soil and water samples. The set of four primers designed amplified the gene encoding flagellar subunit, fliC, and the amplification was detected by the turbidity developed. This technique could detect *R. solanacearum* cells in the range of 10^4–10^6 cfu/ml. Shen et al. (2017) developed the isothermal amplification (LAMP) method for the rapid detection of *P. ultimum.* A target gene coding the spore cell wall protein was identified and used for the detection. Katoh et al. (2021) developed loop-mediated isothermal amplification (LAMP) techniques for detecting *Fusarium oxysporum* f. sp. *fragariae*, causing wilt disease in strawberries. The assay was based on the genomic region between the transposable elements. The assay allowed the efficient detection of the pathogen by DNA visual inspection without gel electrophoresis. The detection limit was 100 pg of genomic DNA as compared with conventional PCR.

1.7.6 Recent Techniques

1.7.6.1 Real-Time PCR

Real-time PCR work is based on the specific fluorescent signal detected by an integrated fluorometer to provide real-time analysis as well as allows the quantification of specific DNA targets. It is highly specific and reduces the false positives because of cross-contamination of reaction mixtures. Real-time PCR uses SYBR Green dye and TaqMan-labeled probes, which shows high sensitivity for the detection and quantification of soil-borne fungi. For example, real-time qPCR assays are used for the detection of various soil-borne fungi, *Verticillium dahlia*, *Pythium* spp., *Phytophthora*, *Fusarium*, *Sclerotium*, *Rhizoctonia*, etc. Multiple real-time assays are also available for the detection of different pathogens simultaneously with low cost and labor.

Landa et al. (2021) developed a TaqMan real-time PCR assay for the detection of *Fusarium solani* from soil and plant parts causing wilt disease in strawberries. The assay was specially designed based on the sequences from the EF-1α gene (translation elongation factor 1 alpha). It specifically detected *F. solani* with a detection limit of 50 fg for genomic DNA and 10^2 conidia/g of soil. Furthermore, Salamone and Okubara (2020) used SYBR green dye-based PCR assay to quantify *R. solani* from the soil. Primers for the real-time assay were specially designed based on the internal transcribed spacer (ITS) regions of fungal isolates. The present assay is mainly used for the detection and quantification of the pathogen from infected soil.

1.7.6.2 DNA Microarrays (Gene Chip Technology)

A DNA microarray, also known as a DNA chip/ biochip, is a collection of microscopic DNA spots mainly attached to a solid surface. Each DNA spot contains a specific DNA sequence of 10–12 picomoles, also known as probes, which hybridize with the complementary sequence. The technology can be used in various applications such as measurement of gene expression and detection of pathogens (Simon et al. 2003). Lievens et al. (2005) developed a DNA array technique for the identification of multiple soil-borne fungi simultaneously. The microarray was specially designed and optimized for the detection of vascular wilt pathogens *Verticillium albo-atrum*, *Verticillium dahliae*, and other tomato pathogens such as *Fusarium oxysporum*, *Fusarium solani*, *Pythium ultimum*, and *R. solani*. All the arrays showed good quantification as well as a very high degree of correlation and reliability for the detection.

1.7.6.3 Metagenomics

Metagenomics is the study of genetic material recovered from various environmental samples. It is the combination of two words "meta" and "genomics," where genomics describes the DNA sequence and the term meta implies that of many organisms together. This technology is mainly used in the study of microbial communities. Globalization led to the generalized movement of planting materials and introduction of new pathogens and a corresponding increase in economic loss. The current diagnosis provides a precise and useful tool to enact surveillance protocols by metagenomics, which helps in the high-throughput diagnosis of fungal plant pathogens. Given the full form of HTS (HTS) techniques such as metagenomics, metabarcoding is an efficient tool for surveillance of soil-borne pathogens and identifying the outbreaks. Advance in bioinformatics accelerates the use of the metagenomics approach in the detection of plant pathogens (Xu et al. 2015; Piombo et al. 2021).

Taheri et al. (2017) used Illumina MiSeq to estimate the abundance of oomycetes in agroecosystems. The oomycetes communities were characterized by a metagenomics approach by using the ITS1 region. Among the 105 identified OTUs (operational taxonomic units), 45 and 16 oomycetes were identified based on the genus and species level. Multivariate analysis revealed the abundance, composition, and diversity of oomycete communities present in agricultural soils. Landa et al. (2021) used high-throughput Illumina sequencing technology by using the Internal Transcribed Spacer 1 (ITS1) region of rRNA barcoding and comparing it with the mitochondrial cytochrome c oxidase I (COI) gene to understand the diversity at disturbed and undisturbed soil. By using this technology, the diversity and distribution of several *Phytophthora* spp. were evaluated under disturbed and nondisturbed soil conditions.

1.7.6.4 Whole-Genome Sequencing

This technique is also known as complete genome sequencing. Full genome sequencing is the process of determining the complete or nearly complete DNA sequence of an organism (Ng and Kirkness 2010). It provides the most comprehensive collection of an individual's genetic variation. Hane et al. (2014) conducted studies on genome sequencing and comparative genomics of the *Rhizoctonia solani* causing various soil-borne diseases and observed that DNA sequences originate from multiple nuclei with a high frequency of SNPs (single nucleotide polymorphisms) and more SNP diversity than most fungal populations.

1.7.6.5 Electronic Nose System

It consists of gas sensors that can detect many organic compounds. Each sensor has a sensitivity that is specific to it, and the sensitivity of many sensors could be used to differentiate many compounds in the atmosphere. This technology has been used in many fields such as determining the food quality, human disease diagnosis, and detection of microbes in food (Di Natale et al. 2001; Zhang et al. 2008; Balasubramanian et al. 2008). However, the application of this technology in plant pathogen detection is relatively new. However, its usage in the field of detection and diagnosis of plant disease is gaining importance. An experiment was conducted to detect basal stem rot disease infected oil palm. It was observed that the system could detect healthy and infected plants with high accuracy (Markom et al. 2009). Spinelli et al. (2006) used this technology to detect fire blight-infected asymptomatic plants of pear. The system could yield a distinct olfactory signature required to identify the disease. As a result, the pear plants infected with fire blight could be identified as early as six days after infection. The study indicated that an electronic nose system could be used as an effective tool for the early diagnosis of plant disease under natural conditions.

1.7.6.6 Loop-Mediated Isothermal Amplification (LAMP)

Detection of plant pathogens using PCR-based techniques fairly need multifaceted thermocyclers and the fluorescence detection of the end product is quite expensive in spite of their efficiency in the quantification of plant pathogens, whereas in LAMP, a simplified heating procedure with minimal incubation period facilitates the results in a shorter period. The LAMP technique was developed and standardized by Notomi et al. (2000), which is a sensitive, simplistic, and time-saving method. A strand displacement reaction is used to carry out the LAMP reaction at a constant temperature. This can be done in a water bath or on a heating block, without the need for a thermocycler. In contrast to PCR, LAMP uses strand displacing *Bst* DNA polymerase (a large fraction of *Bacillus stearothermophilus* DNA polymerase) to do auto-

cyclic isothermal amplification (60–65 °C) of target DNA. Gel electrophoresis is required to observe the results in PCR, while the final products in LAMP can be seen as turbidity or color change in the reaction tube, which saves time. As a result, LAMP has emerged as a promising technology for detecting human infections in the field, and it is now widely utilized as a quick, specific, sensitive, and cost-effective plant pathogen detection method. However, for the detection of low levels of microbes, a single overnight enrichment step is still required (Notomi et al. 2000).

Internal primers introduce self-complementarity into the amplification result, creating loops to develop on which primers can bind, and extension of the external primers promotes displacement of the internal primer-primed products (Gandelman et al. 2011). The insertion of loop or stem primers speeds up amplification by utilizing more primer binding sites within the LAMP amplification product, allowing for faster amplification and increased sensitivity. LAMP can typically achieve similar levels of specificity and sensitivity to PCR-based assays, with a sensitivity that is comparable to real-time PCR (Tomlinson et al. 2007).

Besides practicing real-time PCR for quantitative detection of soil-borne pathogens, loop-mediated isothermal amplification (LAMP) has also been exploited in recent days. The LAMP assay uses a DNA polymerase with strand-displacement activity and is carried out in isothermal conditions. The product is amplified using a set of four specially designed primers that recognize a total of six different sequences on the target DNA. The single-stranded loops in the amplicons allow primers to bind without the requirement for multiple cycles of heat denaturation (Nagamine et al. 2001). The by-product pyrophosphate ion forms a white precipitate of magnesium pyrophosphate as the LAMP reaction develops. The amount of DNA produced coincides with the rise in turbidity caused by the creation of white precipitate. There are a variety of different detection formats that can be employed as well. Positive LAMP reaction can be visualized with the naked eye by adding DNA-intercalating dyes such as ethidium bromide, SYBR Green I, propidium iodide and Quant-iT PicoGreen, or metal-ion indicators such as hydroxynaphthol blue (HNB), $CuSO_4$, and calcein (Nagamine et al. 2001; Zoheir and Allam 2011; Tomita et al. 2008). The reaction can also be monitored in real-time, allowing quantitative detection of the target (Tomlinson et al. 2010; Bekele et al. 2011). The ESEQuant tube scanner using fluorescent dye is a simple and cost-effective system for a real-time detection of parasite DNA (Lucchi et al. 2010; Njiru et al. 2012).

The production of LAMP products can also be tracked in real time by detecting the rise in turbidity caused by the creation of magnesium pyrophosphate to infer increases in amplified DNA concentration, allowing for quantitative detection of the target (Mori et al. 2001, 2004; Tomlinson et al. 2010; Bekele et al. 2011). An ESE-Quant tube scanner has recently been developed to detect amplified products using fluorescent dye. In comparison to other DNA-based tests, the method requires no expensive equipment or reagents and is a more simple and cost-effective technology (Lucchi et al. 2010; Njiru et al. 2012). *Fusarium oxysporum* f. sp. *cubense* (Foc), the causal agent of Fusarium wilt (Panama disease), is one of the most devastating diseases of bananas (*Musa* spp.). The Foc tropical race 4 (TR4) is currently known as a major concern in global banana production. Zhang et al.

(2013) developed a real-time fluorescence loop-mediated isothermal amplification assay (RealAmp) for the rapid and quantitative detection of Foc TR4 in soil. The detection limit of the RealAmp assay was approximately 0.4 pg/ml plasmid DNA when mixed with extracted soil DNA or 103 spores/g of artificially infested soil, and no cross-reaction with other relative pathogens was observed. Quantification of the soil-borne pathogen DNA of Foc TR4 in naturally infested samples showed no significant difference compared to classic real-time PCR (P.0.05). Additionally, the RealAmp assay was visual with an improved closed-tube visual detection system by adding SYBR Green I fluorescent dye to the inside of the lid prior to amplification, which avoided the inhibitory effects of the stain on DNA amplification and made the assay more convenient in the field.

The RealAmp assay is highly specific because it uses four primers that recognize six regions on the target DNA. The LAMP reaction is considered to progress through two steps by DNA polymerase with strand displacement activity: the starting structure producing step and the cycling amplification step. The outer primers, F3 and B3, recognize one of the six sites each and prime amplification of the entire region in a noncycling manner. The inner primers, FIP and BIP, each recognize two of the six sites within the amplified sequence of the primer pair and form a dumbbell-like DNA structure used for subsequent cycling amplification. The LAMP primer set used in this study is a compromising consideration between detection specificity and amplification efficiency. On the one hand, the higher SNP frequency of the IGS region provides a rich source of genetic diversity in Foc, which was successfully exploited to develop a Foc TR4-specific PCR detection method by Dita et al. (2010), and the designed FocTR4-F/FocTR4-R prime set was used as an outer prime in this study for the consideration of specificity.

An improved closed-tube visual inspection was achieved by the addition of 1 ml of SYBR Green I to the inside of the lid of the amplification tube prior to start of the reaction. After reaction, the SYBR Green I was added to the LAMP reaction solution by gentle centrifugation at about 500 g for 10 s. Furthermore, the risk of cross-contamination is minimal using the improved closed-tube visual detection system, which facilitates rapid screening of samples without the use of gel electrophoresis or a fluorescence reader and would be helpful for high-throughput application. Moreover, the RealAmp assay had a high tolerance to inhibitors of DNA from soil samples. It would be a simple and effective approach for the quantitative detection and monitoring of Foc TR4 in soil, avoiding further dissemination of Foc TR4, and would be useful for a routine soil-borne detection service. The detection limit of real-time PCR was about 100-fold higher than that of RealAmp assay in pure spores. However, the RealAmp assay with nearly the same detection limit as real-time PCR for artificially infested soil indicates that the LAMP-based assay has an increased tolerance of inhibitory substances, compared with PCR-based methods (Kaneko et al. 2007).

Phytophthora ramorum, a causal agent of sudden oak death disease of tanoak (*Lithocarpus densiflorus*) and *Quercus* spp., has destroyed large numbers of trees in the forests on the west coast of the USA. A simple and rapid method of extracting DNA on the nitrocellulose membranes of LFD was used. LAMP of target DNA,

using labeled primers, and detection of the generically labeled amplification products by a sandwich immunoassay in a lateral-flow-device format were applied. Each step in the procedure could be performed without special equipment and applied for on-site testing. The results were obtained in about one hour. The LAMP assay for the detection of plant DNA (cytochrome oxidase, *COX* gene) was employed in conjunction with pathogen-specific assays to confirm negative results. Labeled LAMP could be used to increase the specificity of pathogen detection when sufficiently specific antibodies are not available, as in the case of *P. ramorum* and *P. kernoviae*, but LFDs are not available for detecting all *Phytophthora* spp. The lowest amount of DNA of *P. kernoviae* and *P. ramorum* detected by LAMP assay was ~17 pg. The *P. ramorum* LAMP assay was used in multiplex with COX LAMP assay to test CTAB DNA extracts from healthy and P. *ramorum*-infected rhododendron and an extract from *P. ramorum* culture. The multiplex products were run on DIG and FITC LFDs, demonstrating the detection of single products (*P. ramorum* or *COX*) and mixed products. This procedure was sufficiently sensitive to detect *Phytophthora* spp. in symptomatic rhododendron (mixed 1:10 or 1:5 infected/healthy material (Tomlinson et al. 2010).

Similarly, a real-time fluorescence loop-mediated isothermal amplification (RealAmp) assay was developed by Peng et al. (2013) for the rapid and quantitative detection of *Fusarium oxysporum* f. sp. *niveum* (Fon) in soil, which causes wilt disease on watermelons. No significant differences were found between the results tested by the RealAmp and real-time PCR assays. The application of this colorimetric assay using visual observation systems, particularly HNB, SYBR Green I, and GeneFinder™, seems to be more effective as a new fungi diagnostic method for epidemiological studies of *F. oxysporum* f. sp. *lycopersici* even without DNA purification (direct-LAMP), particularly in less well-equipped laboratories (Almasi et al. 2013).

Pythium myriotylum Drechsler is a causal agent of root rot in economically important crops, including peanuts, tomatoes, rye, wheat, oats, cucumbers, soya beans, sorghum, tobacco, cabbage, and maize (Wang et al. 2020). Early infection by *Pythium* species causes yield losses in many vegetables and ornamental crops. Fukuta et al. (2014) designed the primer set targeting the ITS sequence of *P. myriotylum* that worked most efficiently at 60 °C and allowed the detection of *P. myriotylum* DNA within 30 min by fluorescence monitoring using a real-time PCR instrument. In specificity tests using eight *P. myriotylum* strains, 59 strains from 39 species of *Pythium*, 11 *Phytophthora* strains, and 8 other soil-borne pathogens, LAMP gave no cross-reactions. The LAMP assay provided reliable results in the range of 1 ng to 100 fg genomic DNA within 30 min. It could also detect *P. myriotylum* in hydroponic solution samples, and the results coincided with those of the conventional plating method in almost all cases. The LAMP reaction with intercalating dyes such as EvaGreen has another advantage. As the amplification efficiency of the LAMP reaction is extremely high, any contamination of reagents and instruments can easily result in a false-positive result (Peng et al. 2011). Enzyme reactions and electrophoresis using LAMP products raise the danger of contamination, so LAMP products should not be handled directly if possible.

Rhizoctonia solani causes root rot diseases in several crops, and it persists in soil for many years, surviving as a saprophyte on plant debris or other organic matter. Various kinds of techniques have been applied for the detection and quantification of *R. solani* populations in plants and soils. LAMP procedure can be performed inexpensively, using a water bath or heating block. LAMP primers based on ITS DNA sequence were designed for detecting most AGs of *R. solani*. The LAMP reactions were performed for the detection of four isolates of *R. solani* from tomato and other host plants, with 5 mM Mg^{2+} at 65 °C for 60 min. The LAMP test detected very low levels of DNA (10 fg, equivalent to approximately one copy of 87.1 Mb of *R. solani* genome) for *R. solani* and *R. zeae*, but not for *R. oryzae*. The LAMP products were simultaneously assayed using a generic LFD, turbidity, and CYBR green staining. These analyses also showed that 10 fg of DNA was the lowest limit that could be detected using LFD, but not with turbidity and CYBR Green staining. The detection limit for *R. zeae* was 1.0 pg, which was two orders of magnitude higher than the *R. solani* detection limit. The LAMP procedure was specific for the detection of *R. solani*, as no amplicon was generated from DNA of *F. oxysporum* and *P. parasitica* and several host plant species. The LAMP had a detection limit of 5 μg of *R. solani* DNA/g of soil. For on-site application in the field, the LAMP procedure was performed with a generic anti-biotin and anti-fluorescein antibody-based LFD. The LAMP reactions were performed using biotin-labeled primers, which were hybridized with a fluorescein amidite (FAM)-labeled hybridization probe and detected with the LFD (Fig. 1.1).

This LAMP-LFD procedure could detect *R. solani* in infected plant tissues. The LAMP-LFD assay and real-time quantitative PCR (qPCR) format had similar detection limits of 10 fg DNA of *R. solani* with no false-positive or -negative results. But LAMP-LFD procedure is simple, rapid, and equally sensitive and specific as the qPCR format for the detection and quantification of *R. solani* in different plant species and soil, with the distinct possibility of being amenable for on-site testing (Patel et al. 2015). *R. solani*, a causal agent of seedling blight, and *M. phaseolina*, an incitant of charcoal rot diseases of soybean, were directly detected by LAMP assay. The primers were designed and screened using ITS sequences as targets of both pathogens. An ITS-Rs-LAMP assay for *R. solani* and an ITS-Mp- LAMP assay for *M. phaseolina* were employed to detect these pathogens in diseased soybean plant tissues in the field. Verticillium wilt is one of the most important biotic constraints for olive (*Olea europaea* L.) cultivation in Iran, leading to significant yield losses and the death of the trees (Sanei and Razavi 2012). The collection of 32 *V. dahliae* naturally infested soil samples from olive orchards was analyzed by nested-PCR, LAMP, and direct-LAMP methods.

The results showed that the direct-LAMP assay could directly detect the presence of *V. dahliae* in the soil samples (Moradi et al. 2014). These results indicated that LAMP (run by purified DNA from soil samples) yielded a better detection rate (26/32) compared to other methods. Direct-LAMP yielded slightly better results (24/32) than nested-PCR (simultaneous detection of D and ND pathotypes by purified DNA from soil samples, Mercado-Blanco et al. 2003) (22/32, Table 1.8). All soil samples positive for the nested-PCR method were also positive for the

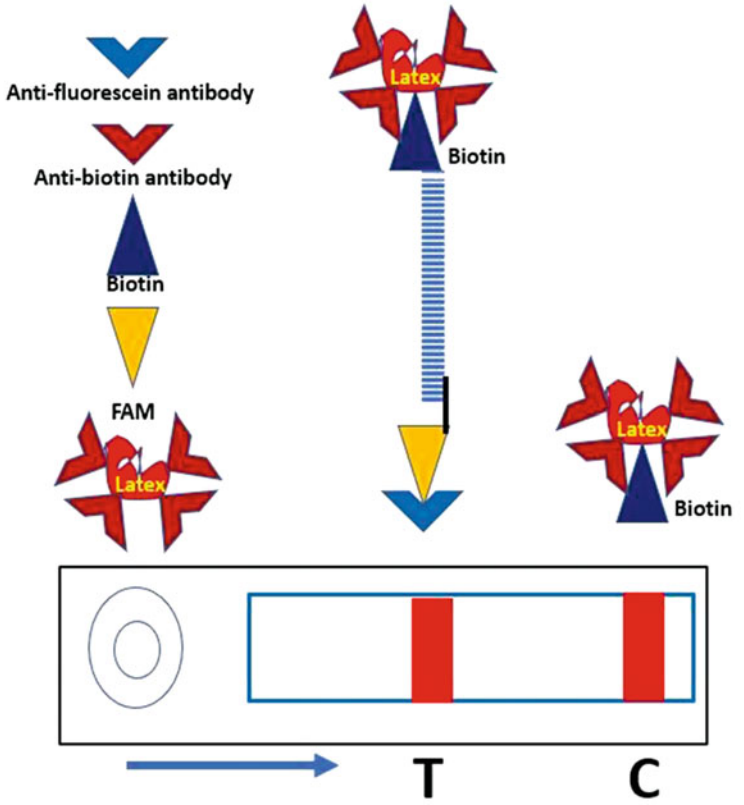

Fig. 1.1 Schematic illustration of chromatographic loop-mediated isothermal amplification combined with lateral flow devices (LAMP-LFD). Using biotin-labeled primers, LAMP amplicons are labeled with biotin and then hybridized to a target-specific ssDNA probe labeled with a fluorescein-containing label, in this case fluorescein amidite. When loaded on the LFD device, these amplicons capture colored latex beads coated with anti-biotin antibodies. These latex beads/LAMP complexes when passing through the LFD device are selectively captured and enriched by an anti-fluorescein antibody at the test line "T" showing a strong color, in this case blue. Unbound excess latex beads/anti-biotin antibody complexes are captured and enriched at the control line "C" by biotin (Patel et al. 2015)

Table 1.8 Detection of *Verticillium dahliae* in 32 agricultural soil samples using nested-PCR, LAMP, and direct-LAMP methods and their comparative results

Methods	Nested-PCR[a]	LAMP	Direct-LAMP
No. of positive samples	ND 22/32 (68.75%) D 0/32 (0.00%)	26/32 (81.25%)	24/32 (75.00%)
Detection time[b]	7–10 h	3 h	60–80 min

[a] The detected ND pathotype showed the C pattern (824 bp amplified, which is associated with ND isolates) for previously described markers

[b] DNA extraction was considered in detection time

LAMP and direct-LAMP assays. On the other hand, amplification was never observed in autoclaved soil samples, which always failed to yield detectable amounts of DNA. The results of the three methods were verified by the medium NP-10. These experiments were carried out using three biological replicates, and the same results were obtained for all of them. The detection methods compared in terms of time and results indicated that the direct-LAMP could detect *V. dahliae* within a total of 60–80 min without the need for any DNA purification procedures (Table 1.8). The results of simultaneous detection of the D (highly virulent, defoliating) and ND (moderately virulent, non-defoliating) *V. dahlia* pathotypes by duplex, nested-PCR (Mercado-Blanco et al. 2003) exhibited that the ND pathotypes were only detected in the inspected olive orchards, while D pathotypes were nonexistent. Moreover, the detected ND pathotype in soil samples was examined for the PCR pattern, and it yielded the PCR pattern.

Ghosh et al. (2015) did the LAMP assay with *F. oxysporum* f. sp. *ciceris* DNA as the template, and the best results were obtained in a 25-μL volume containing a 2.0-μ l primer mixture (20 μM each of FIP, BIP, Loop F, and Loop B primers, and 2.5 μM each of F3 and B3 primers), 1.28 M betaine, 1 mM dNTPs, 4 mM $MgCl_2$, 20 mM Tris–HCl (pH 8.8), 10 mM KCl, 10 mM $(NH_4)_2SO_4$, 2 mM $MgSO_4$, 0.1% Triton X-100, 8 U of Bst DNA polymerase, 150 μM HNB, and 1 μL of target DNA. As noted in the methods, the reactions were performed in a 0.2-mL microcentrifuge in a water bath for temperature control. When the tubes were examined before gel electrophoresis, a positive LAMP reaction was indicated by a sky-blue color; the color remained violet for negative reactions. After the tubes were visually assessed for the color change, the samples were subjected to agarose gel electrophoresis; characteristic bands were evident in the gel if the product was present but not if the product was absent. The results showed that the primers were effective and that the same result was obtained with HNB visualization and gel electrophoresis.

For the LAMP specificity assay, the assay was performed with template fungal DNA from six other fungal cultures (*Fusarium acuminatum*, *F. udum*, *F. solani*, *Rhizoctonia bataticola*, *Alternaria alternata*, and *Phytophthora cajani*) as well as DNA isolated from infected field samples of chickpea (black root rot caused by *F. solani*, dry root rot caused by *R. bataticola*, and *Alternaria* blight caused by *A. alternata*). Under optimum conditions, no positive amplification was observed in the case of other fungal DNA samples. The same result was obtained when products were assessed by gel electrophoresis or by HNB-visualization. After incubation at 63 °C for 60 min, the LAMP assay was positive only for Foc; i.e., no positive DNA products were observed when other plant pathogenic fungi were used as a template. This was true whether assessments were based on gel electrophoresis or HNB visualization. Similarly, in the case of infected plant samples, DNA isolated from *Fusarium* wilt-infected chickpea plants showed positive reaction. These results indicated that the LAMP technique developed in this study is highly specific for Foc and has distinguished between Foc and six above-mentioned common plant pathogenic fungi.

Ras-related protein (Ypt1) is a promising target gene for the design of *Phytophthora*-specific detection (Schena and Cooke 2006). The introns of the *Ypt1*

gene are sufficiently polymorphic for the development of molecular markers for almost all *Phytophthora* species, with more conserved flanking coding regions appropriate for the design of *Phytophthora* genus-specific primers (Schena and Cooke 2006; Meng and Wang 2010). A set of six primers for LAMP amplification was designed using the standard default parameters of the online Primer Explorer V4 software tool (Zhao et al. 2015). The *PsYpt1*-LAMP assay amplified the target in 60 min at 65 °C with high specificity and sensitivity. Hydroxynaphthol blue was evaluated as an endpoint detection method for LAMP. A portable NaOH lysis method suitable for DNA extraction from infected plant tissue was effective in conjunction with LAMP. Thus, the *PsYpt1*-LAMP assay shows potential as a reliable, rapid, and cost-effective method for the visual detection of *P. sojae* pathogen in field-grown soybean plants and production fields.

Dong et al. (2015) developed a novel, highly sensitive loop-mediated isothermal amplification (LAMP) assay for the specific detection of *P. capsici* using calcein as a fluorescent indicator. They designed four LAMP primers based on the ITS sequence of *P. capsici*. A total of 23 isolates of *P. capsici* from geographically distinct counties in China yielded positive results in the LAMP assay. No cross-reaction was observed with other oomycetes or fungal pathogens. The detection limit of *P. capsici* by LAMP was 100 fg genomic DNA per 25 μL reaction. The LAMP assay developed in this study is simple, fast, sensitive, and specific and can be used in the field to detect *P. capsici* in infected plant tissue.

Duan et al. (2016) developed a LAMP assay for the rapid detection of *S. sclerotiorum* mutant genotype (F200Y) resistant to carbendazim due to the point mutation at codon 200 (TTC → TAC). Specific LAMP primers were designed, and concentrations of LAMP components were optimized. The optimal reaction conditions were 62–63 °C for 45 min. There was no requirement for any special equipment for the highly sensitive and specific LAMP assay for the detection of the F200Y mutant genotype. Inclusion of the loop backward (LB) primer reduced the reaction time to 15 min. The results of the LAMP assay corroborated those of MIC determinations. The advantages of the LB-accelerated LAMP assay for the detection of the F200Y mutant genotype were revealed by assaying sclerotia produced on rape stems artificially inoculated in the field. The LAMP assay could be employed for specific detection of the target genotype of *S. sclerotiorum*.

Ralstonia solanacearum is metabolically versatile and can survive not only in soil but also in latently infected plants and water (Xue et al. 2011). The species complex has been subdivided into four phylotypes (phylotypes I, II, III, and IV) corresponding to the four genetic groups identified via sequence analysis (Fegan and Prior 2005). Twenty-five microliters of the LAMP reactions mixture contained 1.6 mM FIP and BIP, 0.2 mM F3 and B3, 0.4 mM LF and LB, 1.4 mM dNTPs, 1.0M betaine (Sigma–Aldrich Corp, St. Louis, MO, USA), 20 mM Tris-HCl (pH 8.8), 50 mM KCl, 10 mM $(NH_4)_2SO_4$, 8 mM $MgSO_4$, 0.1% Tween-20, 8 U of the *Bst* DNA polymerase large fragment (New England Biolabs, USA), and 1 ml of the template DNA. Sterile deionized water was used as the template for the negative control. These reactions were carried out in 0.2-ml microtubes, and 1 ml of 1/10 diluted original SYBR Green I (Molecular Probes Inc.) was added to the inner lid of

every microtube before incubating the reactions in a Veriti96 well thermal cycler (Applied Biosystems, USA). The LAMP method also showed high sensitivity over its PCR counterpart with a detection limit of 160 fg genomic DNA, which is 10 times higher than conventional PCR, and 2.2×10^2 CFU/ml of bacterial cells, which is 100 times higher than conventional PCR (Huang et al. 2017).

Using this method, *R. solanacearum* was detected in the stem extracts of manually inoculated eggplants, tomatoes, peanuts, sesame, potatoes, sweet potatoes, and *Amaranthus lividus*; the detection rate by LAMP was much higher than that by PCR method (Xin-shen et al. 2021). A rapid, visual, and sensitive LAMP assay was developed for the detection of *M. phaseolina*, causing root rot in common bean (*P. vulgaris*) seeds, based on SCAR (GU018142.1). The assay was performed by providing amplification conditions of 65 °C for 45 min. The results of the LAMP assay could be visually recorded by the addition of HNB dye. Sky blue color developed to indicate positive reaction, while controls remained violet under ambient light, indicating negative reaction. The assay had a detection limit of 1 pg of *M. phaseolina* genomic DNA per reaction. No cross-reaction was noted with DNA isolated from five other pathogens infecting bean seeds. *M. phaseolina* was detected both in naturally infected and artificially contaminated bean seeds. The detection limit was one infected seed per seed lot of 400 seeds, without the need for isolation of the pathogen DNA prior to the LAMP assay. The results indicated the applicability of the LAMP assay for the sensitive detection of *M. phaseolina* in seeds from which the pathogen could reach the soil (Rocha et al. 2017).

In less than a decade, the loop-mediated amplification (LAMP) approach has grown in importance as a diagnostic tool for a variety of plant diseases, and it has enormous potential in plant disease management. Because LAMP is unaffected by inhibitors, it can be used to analyze raw samples. Due to the amplification of nucleic acids using up to six primers, this tool is exceptionally sensitive and specific (Becherer et al. 2020). LAMP technology's isothermal and energy-efficient intensification requirements make it an ideal contender for quick and low-cost alternative tests. Thus, LAMP is well established in several areas, including medicine, agriculture, and food industries. On the other hand, the short size of target gene fragments, the use of six primers, which might cause issues in experimental design, and a large amount of indicator and other reaction ingredients, which inhibit polymerase and carryover contaminations, are considered to be the limitations of LAMP (Tanner et al. 2015; Hariharan and Prasannath 2021).

1.8 Conclusion

Detection and diagnosis of soil-borne pathogens are crucial to determine the presence and quantity of pathogen inoculum present in the soil. Timely and accurate detection of inoculum in soil can aid farmers in taking appropriate decisions regarding control measures and preventing its further proliferation in soil. Over the years, the conventional methods have been replaced by more accurate and specific

techniques, which ensure rapid results without compromising accuracy. It would be better to enlist the latest methods for pathogen diagnostic for the knowledge of readers in this section. Yet, there is no single technique that fulfills all the necessary criteria of a detection technique, and hence further research is essential to fill in the knowledge gap in this area.

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Chapter 2
Microarray-Based Detection and Identification of Bacterial and Viral Plant Pathogens

Aastha Sinha, Jobin John, Sachidanand Singh, and Parul Johri

Abstract The development and application of diagnostic procedures are influenced by a variety of circumstances. Plant viruses, unlike cellular diseases, have a naturally huge population with no nucleotide sequence type in common (e.g., ribosomal RNA sequences). Plant virus detection is going to be incredibly difficult as globalization of commerce, particularly in agriculture and ornamentals, and the potential consequences of climate change increase viral and vector mobility, changing the diagnostic environment. DNA microarrays can simultaneously offer data on the expression patterns of thousands of genes. Microarray techniques are still underexploited in phytopathology, despite their vast potential compared to RT-PCR or ELISA-based procedures. The processes that govern the result of a microbial pathogen's contact with a host plant appear to be complicated. The molecular intricacies of this interaction, such as pathogen genes essential for infection, host defense responses, and mechanisms regulating host and pathogen signaling networks, can be used to develop new plant protection measures. Knowing how both the host's and pathogen's genes are transcribed during an encounter is a crucial step in identifying and characterizing the main pathways that determine eventual compatibility and incompatibility. Large-scale genomic sequencing projects in plant and pathogen species have already yielded information on the basic gene and genome organization

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templates of these organisms. Microarray technology's current and future state is presented and explored, covering probe design, array fabrication, assay target preparation, hybridization, washing, scanning, and interpretation.

Keywords Microarray · ELISA · RT-PCR · Probe designing · Transcriptome · DNA sequencing

2.1 Introduction

Since the beginning of the study of plant virology over 100 years ago, detecting and identifying pathogens has been difficult, and a wide range of methodologies have been developed to allow the discrimination of viral and microbial diseases. Plant health monitoring and early pathogen detection are critical for reducing disease propagation and determining the optimal strategy (Buja et al. 2021). A fast and reliable detection approach in combination with decision support systems is vital to reduce the damage caused by existing and new pathogens, as well as to speed up the management and reduction of crop loss by reducing yield and quality (Raza et al. 2019). Viruses have a very simple genetic structure, but the specific mechanisms of their interaction with host plants, including how they influence a plant's physiology to meet their needs and activate antiviral responses in hosts, are yet unknown (Mandadi and Scholthof 2013). If plants can effectively fight illnesses using inherited genetic weapons such as resistance (R) genes, which are found in abundance in all plant species, they start broad resistance pathways, which lead to a hypersensitive reaction (HR). To prime and escalate their infections, viruses induce some responses in vulnerable plants lacking R genes to a specific viral pathogen (Balint-Kurti 2019). Identifying the up-and down-regulation of defense-related genes, as well as developing a plant defense network, has proven to be a significant difficulty for biologists and scientists. Initially recognizing the diverse biological properties of different viruses, they were quickly augmented with light or electron microscopy and serology. Antibody-based detection involves enzyme-linked immunosorbent assay (ELISA), electron microscopy, and biological indexing (PCR), which is a highly sensitive technique that revolutionized molecular biology and diagnostic methods (Rubio et al. 2020). Multiplex primers can be used to detect more than one virus; however, due to the difficulties in recognizing precisely related viral sequences present in the same sample and the creation of suitable sets of primers, the number of detectable viruses in a single assay is low whereas microarray detection is performed by some of the techniques used to test seed, other propagation materials, and field samples for the presence of specific viruses (Engel et al. 2010).

Extensive research with the dicotyledonous model plant *Arabidopsis thaliana* has led to the identification of numerous critical components and pathways involved in defensive signaling in the last decade (Provart et al. 2016). cDNA or oligonucleotide microarray techniques have been utilized to simultaneously monitor large-scale or genome-wide gene expression and provide a massive amount of molecular data that could aid in elucidating the defensive response network. Various microarray or gene chip technologies have been used to explore global gene expression profiles

(transcriptome) in *Arabidopsis* during biotic and abiotic stresses (Atkinson et al. 2013; Gul et al. 2016). Microarray technology has been widely used to investigate global gene expression in a variety of biological activities, leading to the discovery of a large number of DR (defense-related) genes and a better understanding of the defense signaling network (Lodha and Basak 2012).

A microarray is a technique for simultaneously detecting the expression of multiple numbers of genes. It is a microscopic slide with thousands of small spots printed in predetermined places, each spot relating to a known DNA sequence or gene (Adams et al. 2015). These slides are referred to as DNA chips. For sensitive and high-throughput transcriptome (cDNA or oligonucleotide microarray) and DNA sequence variation studies, DNA microarray has become a vital tool (oligonucleotide microarray). Probes for different genes can be deposited or directly produced on a solid substrate in a patterned fashion in an oligonucleotide microarray (Bumgarner 2013). On a genomic level, microarrays can be utilized for expression analysis, polymorphism identification, genotyping, and DNA resequencing (Gul et al. 2016). Advanced arraying technologies like micro spotting, ink jetting, and photolithography, in combination with advanced fluorescence detection systems and bioinformatics, allow for unparalleled molecular data collection. Because of their ability to overcome the shortcomings of existing approaches, microarrays are rapidly being considered a viable alternative for detecting many targets (Du et al. 2021; Kumari et al. 2021; Singh et al. 2021). Successful outcomes have been established in plant–pathogen detections by microarray (Abdullahi and Rott 2009; Boonham et al. 2003; Agindotan and Perry 2008; Pasquini et al. 2008). These technologies are capable of increasing the density of array elements to even higher levels. The core concept of microarrays is that each element generates a distinct signal as a result of the hybridization of targets to probes and that when each signal is analyzed in parallel, effective data capture across many genes and even entire genomes is conceivable. They have recently been used to hybridize targets obtained from genomic DNA, providing information on changes in transcriptional activity sites, promoter binding, chromatin state, and overall polymorphism across genotypes. This approach can detect bacteria, viruses, parasites, fungus, viroids, and phytoplasmas (Hadidi and Barba 2008). Postnikova and Nemchinov (2012) employed microarray-based analysis to compare the several viruses found in Arabidopsis. Using microarrays, Leborgne-Castel and Bouhidel (2014) investigated plant–microbe interactions. Microarray analysis was utilized by Osmani et al. (2021) to detect responses of several potato viruses.

2.2 Influence of Plant Disease on Global Agriculture

Plant diseases are one of the most serious challenges faced in agriculture around the world (Narayanasamy 2010; McDonald and Stukenbrock 2016; Rubio et al. 2020). By 2050, the projected growth of the human population will necessitate a 60% increase in staple food crop production. Protecting crops from pathogens will almost

certainly necessitate a major re-engineering of the global agroecosystems to make them more disease-resistant and less conducive to the emergence of new pathogens or the rapid evolution of more damaging traits (e.g., higher virulence and fungicide resistance) in existing pathogens. Pathogens are thought to be responsible for 12.5% of global agricultural losses, putting many commercially and socially important commodities, including coffee, cassava, oranges, olives, wheat, and rice, in jeopardy. Bacteria, viruses, and fungus can diminish agricultural yields, affect crop quality, and even kill their hosts in some situations (Velásquez et al. 2018).

Plant diseases can reduce agricultural output significantly. The losses associated with viruses and pests in five important crops (maize, rice, wheat, potatoes, and soybean) were found to range between 17% and 30% yearly on a global scale (Savary et al. 2019). Furthermore, infection distribution ranges are expanded as a result of human-mediated activities, which allows hybridization and horizontal gene transfer, resulting in the creation of new pathogens (McDonald and Stukenbrock 2016). A very well-known example is *Pyricularia graminis-tritici*, a wheat blast disease that may have originated in South America, and made its first appearance in Asia recently, destroying more than 15,000 acres of crops in Bangladesh (Castroagudín et al. 2016). Since 2013, *Xylellafastidiosa* has been causing serious damage to olive trees in Italy's southern region (Frem et al. 2021).

Climate change is another factor that influences plant–pathogen interactions. Increased temperatures, climate extremes, and changes in the quantity and pattern of yearly precipitation can help plant diseases spread in farms and forests (Schmidhuber and Tubiello 2007). It is critical to develop fast, efficient, and economical technologies for early detection of pathogens to manage, and where feasible, prevent, plant diseases and the spread of plant pathogens into new areas (Rubio et al. 2020). Symptom observation and culture-based procedures are used in traditional methods for fungal and bacteria identification (Alvarez 2004; Lievens and Thomma 2005). Plant genetics and gene functions are currently being studied using microarray technology (analysis of gene expression). The first DNA chip for determining the safety of genetically modified foods has been created. The chip screens and identifies GMOs in raw materials, processed food, and animal feed. It can perform the standard detection of viral DNA (CaMV), selection genes (bar, resistance to antibiotics), and gene fragments (Nos-terminator), as well as specific gene fragments (Bt, EPSPS) (Fraiture et al. 2015; Salisu et al. 2017).

2.3 Plant–Pathogen Interaction and Defense Mechanism

Plant viruses produce significant economic losses and pose a threat to agriculture's long-term viability. International trade, climate change, and viruses' ability to evolve quickly contribute to the frequent appearance of novel viral illnesses (Rubio et al. 2020). To defend against infections, plants have evolved two techniques. Pattern recognition receptors (PRRs) recognize conserved microbial elicitors called pathogen-associated molecular patterns (PAMPs) on the host cell's exterior face,

and stimulation of PRRs leads to PAMP-triggered immunity (PTI) (Amarante-Mendes et al. 2018). The second line of plant defense involves resistance (R-) genes inside the plant recognizing certain effectors (now referred to as virulence (Avr) proteins), generating what is typically regarded as a higher resistance response and referred to as effector-triggered immunity (ETI) (Andersen et al. 2018). These pathogen-derived "effector" proteins include various avirulence (Avr) proteins like Slp1 of *Magnoporthe oryzae* and TALEs of *Xanthomonas oryzae* (Liu et al. 2014). ETI is more powerful and quicker than PTI, and it can cause localized cell death (hypersensitive reaction) in both pathogen and pathogen-infected plant cells. The PTI and ETI highlight the advanced key gap identification to understand the interactions enabling plants to recognize and battle against the pathogen attack. Our growing understanding of pathogen effectors and their involvement in inhibiting PTI responses, the mechanism of effector recognition, and the downstream responses to pathogen perception. Amps include the bacterial flagellin and elongation factor (EF)-Tu peptide surrogates, flg22 and elf18, as well as chitin, which are recognized by the plant PRRs flagellin-sensitive22 (FLS2), EF-Tu receptor (EFR), and chitin elicitor receptor kinase1 (CERK1) (Liu et al. 2013). During plant–pathogen interaction in the apoplast, secreted proteins are relatively simple to isolate from suspension-cultured cells. In vitro interaction systems using suspension-cultured cells were largely employed to study secreted proteins in response to pathogen infection (Agrawal et al. 2010). Only six proteins were found to be common in both in vitro and planta-secreted proteins in rice, out of a total of 222 proteins examined (Jung et al. 2008).

2.4 Microarray-Based Study of Plant Defense Mechanism

Plant viruses, viroids, and phytoplasmas have been linked to significant reductions in crop output, plant quality, and plant products (Sastry and Zitter 2014; Rao et al. 2018; Rubio et al. 2020). The plant defense system is diverse and complex, and the study would undoubtedly help by DNA microarray technology after several treatments with the fungal pathogen *Cochliobolus carbonum*, an Affymetrix chip containing oligonucleotide probes for 1500 maize genes revealed 117 genes that showed a consistent alteration (Kazan et al. 2001). The interaction between the incompatible fungal pathogen *Alternaria brassicicola* and *Arabidopsis* was examined with microarrays containing 2375 selected genes. During the treatment with the defense-related signaling molecules salicylic acid (SA), methyl jasmonate (MJ), or ethylene, changes in the expression patterns of 2375 selected genes were investigated simultaneously by cDNA microarray analysis in *Arabidopsis thaliana* (Schenk et al. 2000). Currently, microarray technology is being utilized to better understand plant genetics and gene functions (analysis of gene expression). A schematic diagram detailing the various steps involved in virus identification is shown in Fig. 2.1.

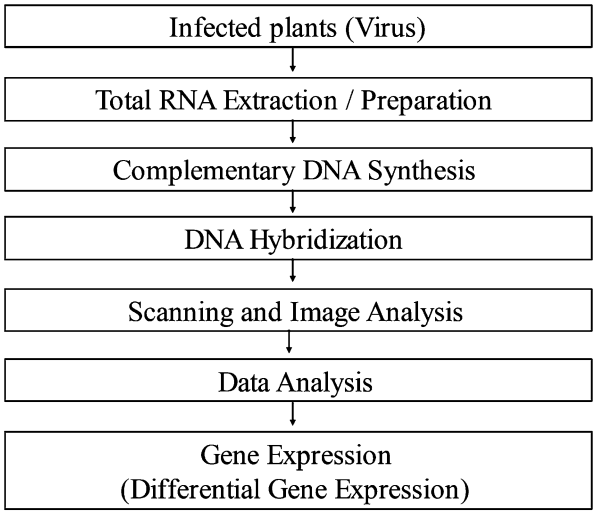

Fig. 2.1 A schematic flow chart detailing the steps involved for virus-infected plants

Evolution in Microarray Concept

The simultaneous hybridization of a single sample extract (RNA, complementary DNA cDNA, or protein) to thousands of immobilized targets on a microarray surface has become the center of genomics research in almost all situations.

Early History of Microarray

Grunstein and Hogness' colony hybridization approach was used to produce the initial DNA array (Grunstein and Hogness 1975). DNA of interest was cloned at random into *E. coli* plasmids and plated onto nitrocellulose-filtered agar Petri dishes. Additional agar plates were made using replica plating. The colonies on the filters were lysed, and their DNA was denatured and fixed to the filter, resulting in a random and disorderly collection of DNA spots representing the cloned fragments. Hybridization of a radiolabeled probe of an interesting DNA or RNA was used to quickly screen 1000s of colonies for clones with complementary DNA to the probe.

In 1979, Gergen et al. used this strategy to build ordered arrays into 144 well microplates. They used a mechanical 144-pin device and a jig to replicate numerous microtiter plates on agar and grow 1728 distinct colonies in a 26–38-cm area. Filter-based arrays and protocols similar to these were used in a variety of applications over the next decade, including cloning genes of specific interest, identifying SNPs (Miller and Barnes 1986), cloning differentially expressed genes between two samples (Crampton et al. 1980), and physical mapping (Carig et al. 1990).

Hans Lehrach's group mechanized these operations in the late 1980s and early 1990s by using robotic devices to swiftly array clones from microtiter plates onto filters (Carig et al. 1990; Lennon and Lehrach 1991). In the late 1970s and early 1980s, the development of cDNA cloning (Auffray et al. 1980; Auffray and Rougeon 1980a; Auffray and Rougeon 1980b) coincided with multinational projects to fully sequence both the human genome and the human genome. Sets of nonredundant cDNAs became commonly available in the late 1990s and early

2000s, and entire genome sequences of some organisms allowed for sets of PRC products representing all known open reading frames (ORFs) in small genomes (Lashkari et al. 1997; Richmond et al. 1999).

Modern DNA Array
The transition from spotting relatively long DNA on arrays to producing arrays with 25–60-bp oligos happened gradually. Edwin M. Southern has offered an early history and overview of the microarray discipline. DNA arrays already existed in the 1970s as dot blot DNA and slot blots, allowing homology determination or expression study on a series of samples with radioactive labeling (Kafatos et al. 1979; Brown 1993). cDNA arrays were developed and utilized for expression analysis employing radioactive labeling and high-density membranes in the early 1990s, primarily for efficient library screening. Early in the human genome project, matrix arrays comprising multiple DNA sequences were designed to automate DNA sequence by sequence by hybridization, and later on, his technology was applied to the simultaneous expression of thousands of genes (Southern 2001).

Spotted Array In 1996, DeRisi et al. published a method that allowed very high-density DNA arrays to be made on glass substrates (DeRisi et al. 1996). The basic approaches were created on the principles of enzymatic nucleic acid labeling and DNA hybridization. The non-isotopic methods were exclusively used for gene expression and profiling; the first cDNA arrays were employed in 1990s when Mark Schena in Patrik Brown's laboratory at Stanford University (CA, USA) produced cDNA microarray measurements (Schena et al. 1995). To produce, two enormous collections of 70-mer probes were utilized to identify oligonucleotide arrays, which were then used to assess gene expression in two distinct human RNA samples. A two-color approach in which the ratio of signals on the same array is measured is much more reproducible (Barczak et al. 2003).

In Situ, Synthesized Array
In 1991, Fodor et al. published a method for light-directed, spatially addressable chemical synthesis on a solid substrate that combined photolabile protecting groups with photolithography. Affymetrix arrays were used to discover mutations in the HIV-1 reverse transcriptase and protease genes, as well as to quantify variation in the human mitochondrial genome (Lipshutz et al. 1995; Chee et al. 1996). Affymetrix was able to create high-density DNA arrays by combining photolithography techniques with experimentation on glass slides. DNA microarray chips are smaller arrays with an ever-increasing number of DNA sequences (Ewis et al. 2005).

Because the DNA sequences are directly generated on the surface, only a minimal number of reagents are required to construct an arbitrarily complex array (the four modified nucleotides plus a handful of reagents for the de-blocking and coupling operations). Each model of the array needed the creation of a unique set of photolithographic masks to direct light to the array at each phase of the synthesis process; it was limited in versatility. The photo-deprotection process of Fodor et al. (1991) and Lipshutz et al. (1999) is performed using micro-mirrors (similar to those used in video computer projectors) to guide light at the pixels on the array in a method

described in 2002 by Nimblegen Systems Inc. (Nuwaysir et al. 2002). This allows for the fabrication of customized arrays in small quantities at a considerably lower cost than photolithographic technologies that use masks to direct light (which are cheaper for large volume production). The total number of addressable pixels (e.g., unit oligos that can be synthesized) is restricted in this technology due to the number of addressable places in the micro-mirror device (of order 1M).

Blanchard et al. suggested a method for producing oligo arrays using inkjet printing technology and normal oligo synthesis chemistry in 1996. The four distinct nucleotide phosphoramidites were delivered on a glass slide that was pre-patterned to contain regions containing hydrophilic regions (with exposed hydroxyl groups) surrounded by hydrophobic regions using inkjet printer heads. The surrounding hydrophobic sections contained the droplet(s) released by the inkjets to defined locations, while the hydroxylated regions provided a surface to which the phosphoramidites could couple, while the hydroxylated regions supplied a surface to which the phosphoramidites could couple (Blanchard et al. 1996).

Self-Assemble Array

The Tufts University group of David Walt developed an alternate strategy to array creation (Ferguson et al. 2000; Michael et al. 1998; Steemers et al. 2000; Walt 2000) that was eventually licensed to Illumina. Their method required manufacturing DNA on little polystyrene beads and putting them on the end of a fiber-optic array with the fiber ends etched to create a well slightly larger than one bead. Different forms of DNA would be produced on various beads, and a mixture of beads would be applied to the fiber optic cable to produce a randomly constructed array. The total number of distinct beads that could be recognized was limited by optical decoding by fluorescent labeling. As a result, later and current approaches for decoding the beads entail a series of stages that include hybridizing and detecting several short, fluorescently tagged oligos (Gunderson et al. 2006).

The first DNA chip aimed at testing the integrity of genetically modified food has already been developed. The chip screens and identifies GMOs in raw materials, processed food, and animal feed. It can detect viral DNA (CaMV), selection genes (bar, resistance to antibiotics), gene fragments (Nos-terminator), and specific gene fragments (Bt, EPSP) (Hadidi et al. 2004). Citrus greening or huanglongbing (HLB) is a devastating disease of citrus. HLB is associated with the phloem-limited fastidious prokaryotic α-proteobacterium "*Candidatus Liberibacter* spp." (Dala-Paula et al. 2019). Leaf tissue from sweet orange (*Citrus sinensis*) infected with "*Ca. Liberibacter* asiaticus" was compared to healthy controls. Citrus microarray hybridization was used to investigate the host response using 33,879 expressed sequence tag sequences from various citrus species and hybrids. HLB infection had a substantial effect on the expression of 624 genes whose encoded proteins were classified according to function based on the microarray study. Genes involved with sugar metabolism, plant defense, phytohormone, and cell wall metabolism, as well as 14 additional gene categories, were included in the categories. Thus, the HLB pathogen changes the expression of host genes, causing symptoms to appear (Kim et al. 2009).

Through a microfluidic approach, a study was conducted to detect three greenhouse pathogens (*Botrytis cinerea*, *Botrytis squamosa*, and *Didymella bryoniae*) that do not require pin-spotting and allows for quick hybridization on the same microarray chip (Wang and Li 2007). Sixteen samples (21-mer complementary oligonucleotides and 260-bp PCR products) were successfully hybridized at the channel-probe line intersections in a short amount of time (minutes). It detected and discriminated between two 260-bp PCR products with a one-base-pair difference from closely related greenhouse plant fungal pathogens. There were 38 probes developed for 16S rDNA, housekeeping genes were used to identify several EPPO quarantine microorganisms (rpoB, groEL, ftsZ), and the ARB library was made possible by a tool that generates probes from large sequencing databases (Ludwig et al. 2004).

The most disastrous viral pathogen of stone fruits like plum, peach, and apricot is plum pox virus (PPV) (Levy and Hadidi 1994; Olmos et al. 2006). As a result, plant quarantine and certification procedures rely heavily on the detection and identification of its strains (Barba and Hadidi 2012). A genomic technique for PPV screening based on the viral nucleotide sequence was developed for the identification and genotyping of the virus from infected plant tissue or biological materials. This method employs a long 70-mer oligonucleotide DNA microarray capable of simultaneously detecting and genotyping PPV strains (Wang et al. 2002). All distinct strains of PPV were detected by a single probe (universal), which was derived from the genome's highly conserved three nontranslated regions. Without the use of PCR amplification, indirect fluorescent tagging of cDNA with cyanine following cDNA synthesis improved the sensitivity of virus detection. The PPV microarray successfully detected and identified PPV strains in peach, apricot, and *Nicotiana benthamiana* leaves infected with the virus. This PPV detection method is versatile and allows for the detection of multiple plant diseases at the same time (Pasquini et al. 2008).

2.4.1 Principle of Microarray

The fundamental premise of DNA microarray is the base-pairing of complementary sequences via hybridization. The specific binding of DNA allows a target DNA or RNA to hybridize into a specific complementary DNA probe on the array (Barba and Hadidi 2008). Each probe is made of thousands of cDNAs or oligonucleotides, each specific for a gene, DNA sequence, or RNA sequence of interest. An array is an orderly arrangement of samples; it provides a medium for matching known and unknown nucleic acid samples based on base-pairing rules (A–T and G–C for DNA; A–U and G–C for RNA) and automating the process of identifying the unknown (Miller and Tang 2009). Each array is generated by depositing a few nanoliters of DNA probes on a solid support. The printing is performed by a robot that allows identical spotting serially (Krawczyk et al. 2017). High-density microscopic array components, planar glass substrates, low reaction volumes, multicolor fluorescent

labeling, high binding specificity, high-speed apparatus for manufacturing and detection, and sophisticated software for data processing and modeling are all used in microarrays (Boonham et al. 2007).

Microarray technology works on the premise of large-scale hybridization of fluorescently labeled nucleic acid molecules from biological samples to complementary single-stranded DNA sequences immobilized on a solid surface (Bumgarner 2013). Total RNA from infected plants is turned to cDNA and amplified through PCR using pathogen-specific primers, which are then labeled and randomly primed with appropriate molecules for detection (Bystricka et al. 2005). It has been essential in identifying the underlying networks of gene regulation in plants that contribute to a wide range of defense responses. Microarray technology has been utilized to identify regulatory genes and end-point defense genes, as well as to better understand the signal transduction processes that underpin disease resistance and their close connections to other physiological networks. The approach in cancer biology (Cole et al. 1999). The hybridization of fluorescently labeled sequences (targets) to their complementary sequences is spotted on a solid surface, acting as probes. The main advantage of this method is the opportunity to detect many pathogens simultaneously.

A method was illustrated using *Arabidopsis thaliana* and *Oryza sativa* as proof of concept, and the process is designed for identifying and analyzing unknown genetic changes in the direct hybridization of whole genomic DNA to high-density microarrays with probes tiled throughout a set of reference sequences used. Crop potatoes can be attacked by about 50 viruses and viroids (Brunt et al. 1996). With some particular amplicons from six potato viruses, different primer combinations were created or adopted: potato virus A (PVA), potato virus S (PVS), potato virus X (PVX), potato virus Y (PVY), potato mop-top virus (PMTV), and potato leaf-roll virus (PLRV). Also, other potato viruses (Bystricka et al. 2005), cucurbit-infecting tobamoviruses (Lee et al. 2003), plum pox virus (Pasquini et al. 2008; Barba and Hadidi 2012), grapevine viruses (Nicolaisen 2011), and several plant viruses in multiple other microarray investigations include both complicated detection of a broad spectrum of pathogens and targeted identification of diseases in a specific plant host, such as tomatoes (Tiberini et al. 2010) or potatoes (Zhang et al. 2013). Experiments were all detected using DNA microarrays (Engel et al. 2010).

Rapid identification of pathogenic microorganisms is required for effective disease control. Xu et al. and Bordoni et al., for example, used oligonucleotide microarrays to identify genetically modified soybeans and maize. Warren et al. and Ronning et al. used microarray technology to distinguish between fish pathogens or closely related crops, respectively. Plant pathogens in potatoes, tomatoes, and apples have been identified using DNA microarrays, which have also been used to differentiate between bacterial phytopathogens and *Fusarium* and *Pythium*s species.

2.4.2 Construction of Microarray

In a single experiment, microarray provides an appropriate platform for measuring the expression levels of thousands of genes in a sample, resulting in the creation of an expression profile or transcriptome for the sample under study for a global picture of cellular function (Southern 2001). Although there are other DNA microarray techniques apart from the one shown in Fig. 2.2, the basic methodology requires extracting mRNA from two biological samples, one of which is a control sample and the other an experimental sample. Reverse transcriptase-polymerase chain reaction (RT-PCR) is used to convert the isolated mRNAs to cDNA. Each of the two cDNA

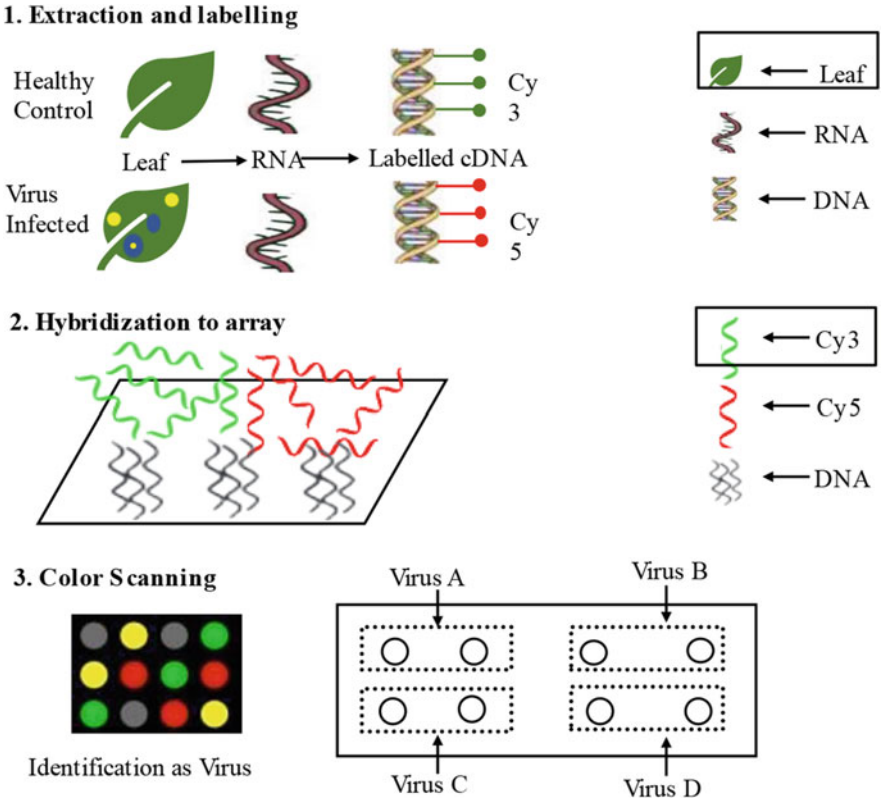

Fig. 2.2 A schematic diagram showing virus detection using a microarray. From the test material and known healthy leaf material, RNA is extracted and reverse transcribed into cDNA through the use of fluorescently labeled nucleotides. Cy 3 is applied for the healthy and Cy 5 for the test material. The cDNAs are then pooled and hybridized to the array (2). Following washing and scanning of the slides, the results show specific hybridization of Cy5-labeled (red) target to the probe spots that belong to virus A. As a part of internal positive control, probes that are homologous to plant genes are present on the array; these hybridize to cDNA from both the test (labeled with Cy5) and healthy (labeled with Cy3) plants. Therefore, these spots appear yellow

pools is fluorescently labeled with two separate fluorochromes, mixed, and hybridized to a large number of gene sequences put as individual spots on a microarray slide for a length of time. Excess cDNA is rinsed away after hybridization. Hybridization results are analyzed by using a laser scanner to determine the relative intensity of fluorescence at each gene location. Spots that fluoresce predominantly with one of the labels indicate a gene that is differentially upregulated or downregulated in the sample under the study's circumstances (Lenz et al. 2008).

2.4.3 Types of Microarrays

cDNA Microarray
The spotted arrays are made by robotic pins depositing a concentrated solution of double-stranded DNA onto a solid surface, as shown in Fig. 2.2.

Oligonucleotide Microarray
They are usually 16–20 bp in length. Oligonucleotide microarrays are made up of certain oligonucleotides generated in a predetermined spatial orientation on a solid surface using a technology called photolithography (Pasquini et al. 2008). Spotting or small instruments like inkjet printers are sometimes used to deposit oligonucleotides onto glass slides. Modern arrays have represented 12,000 sequences at 16–20 oligomers per sequence, resulting in a total of 192,000–240,000 oligonucleotides per chip (Tiberini et al. 2010). A schematic diagram of the construction of oligonucleotides is shown in Fig. 2.3.

2.5 Databases and Tools for Studying Plant–Pathogen Interaction

Most of the viral sequence data came from GenBank's curated library of fully sequenced viral genomes. Each completely sequenced genome was segmented into overlapping 70 nucleotide (70-nt) segments offset by 25 nt for a specific family of viruses, and a pairwise BLASTN alignment was performed between each 70-mer and each viral genome in the family. The best BLAST hit (if any) for each segment–viral genome pair was used to tabulate the results of these alignments. The 70-mers were then sorted according to the number of viral genomes with which they shared strong similarities (>20-nt identity) (Chou et al. 2004, 2006). In most cases, the top five oligonucleotides for each virus, as well as the reverse complement oligonucleotides, were chosen.

Many free and commercial software tools are now available to analyze microarray data sets but finding a single comprehensive software package that solves all functional-genomics issues remains difficult (Mehta and Rani 2011). Many bioinformatics firms provide microarray analysis software, but there are also free software

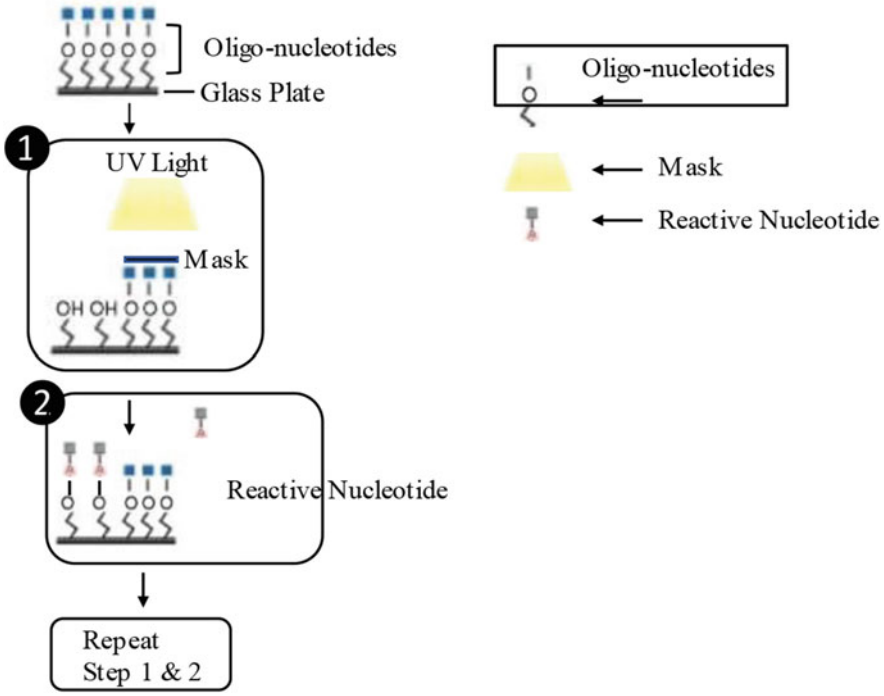

Fig. 2.3 A schematic diagram showing the photolithographic process of oligonucleotides being built directly onto the glass slide's surface that is developed by Affymetrix (Santa Clara, CA). A mask (1) is applied to the array to allow UV light to activate only the sites where the next nucleotide is to be added. The reactive nucleotides are then added (2). The process is then repeated by applying the mask from the next nucleotide (1). www.annualreviews.org. Microarrays for Plant Viruses 3

packages that can be used to execute the analytical approaches. Some important ones are listed in Table 2.1.

2.5.1 Bacterial and Viral Pathogen Identification by Bioinformatics Tools

Viral diseases are a major threat to sustainable and productive agriculture worldwide, resulting in losses of several billion dollars every year (McDonald and Stukenbrock 2016). Due to their quick replication and creation of vast populations, viruses have tremendous potential for genetic variety (Rubio et al. 2020). There are various databases used to retrieve the pathogen sequences. Researchers must unravel the complicated biological pathways underlying pathogen infection to develop innovative plant disease control measures (Köhl et al. 2019). A growing number of pathogens have been sequenced thanks to whole-genome sequencing technology,

Table 2.1 Tools and databases for expression data analysis

S. No.	Software	Function performed	Url
1.	TM4 (MeV)	Multi experiment viewer (MeV) is a Java application designed to allow the analysis of microarray data to identify patterns of gene expression and differentially expressed genes	https://bioinformaticshome.com/tools/rna-seq/descriptions/MeV.html
2.	EDGE	EDGE (extraction of differential gene expression) is a point-and-click open-source software tool for analyzing DNA microarray studies. Standard and time course differential expression analysis are both possible with EDGE	https://edgebioinformatics.org
3.	R	R is a statistical computing and graphics language and environment	https://www.r-project.org
4.	CYBER-T	Web interface for the test, regularized test, etc.	http://cybert.ics.uci.edu
5.	FiRe	FiRe (Find Regulons) is an Excel macro that quickly scans microarray data for "interesting" candidate genes that follow a specific mRNA accumulation pattern. Genes are chosen based on their fold-change ratios under a variety of experimental circumstances	http://www.unifr.ch/plantbio/FiRe/main.html
6.	Cluster, TreeView	The standard for hierarchical clustering and dendrogram visualization, as well as the creation of self-organizing maps and principal component analysis	http://jtreeview.sourceforge.net
7.	GeneCluster2.0	Self-organizing maps are constructed with this software. The most recent version now performs supervised approaches such as finding the nearest neighbors. This application, which is written in Java, can run on almost any computer operating system	https://genecluster2.software.informer.com
8.	MultiExpression Viewer	Creates self-organizing maps and performs hierarchical clustering and principal component analysis. This package also provides a support vector machine component, but there is currently minimal documentation available. The software is developed in Java, and it also comes with a license for the source code	http://www.tm4.org/mev_webstart.html
9.	MAExplorer	Many parts of microarray processing are performed, including raw picture analysis. There are a few analytical approaches in it, such as hierarchical clustering. The software is written in	https://swmath.org/software/8354

(continued)

Table 2.1 (continued)

S. No.	Software	Function performed	Url
		Java, and the source code can be modified at any time	
10.	TELNET	Creates networks of relevance. The software is written in Java, and it comes with a source code license	https://datatracker.ietf.org/doc/html/rfc854

and a vast amount of genetic data has been gathered (Land et al. 2015; Xu and Wang 2019; Piombo et al. 2021). To better understand disease infection mechanisms and pathogenic targets, bioinformatics techniques for examining pathogen genomes, effectors, and interspecies interactions have been developed (Table 2.2).

As the RNA polymerases lack a proofreading activity, viruses with RNA genomes, such as most plant viruses and viroids, have the highest mutation rate of any category of replicons (Domingo et al. 1996; Drake and Holland 1999; Gago et al. 2009). For example, a survey of seven tomato viruses in Sicily, Italy (Panno et al. 2012) found that most plants (75.5%) had multiple infections, 17.8% had a single virus infection, and only 6.7% were virus-free. Recombination, which is an addition to mutation, is another source of genetic variation and the creation of new viruses is the result of mixed infections of two viruses. Recombinants have been described between different species of plant viruses (Padidam et al. 1999; Chare and Holmes 2006; Codoñer and Elena 2008; Davino et al. 2012) or divergent viral strains (Rubio et al. 2013; Lian et al. 2013).

The identification of potato viruses PVY, PVA, PVX, and PVS in single and mixed infections in the detection of plant viruses using short synthetic single-stranded oligomers (40 nt) instead of PCR products as capture probes. A microchip was designed and tested to detect the potato viruses PVA, PVS, PVM, PVX, PVY, and PLRV in both single and mixed infections as reported by Boonham et al. (2003). The chip was also created to discriminate between the two main PVY and PVS strains. The results of preliminary experiments utilizing various probes for one virus with PVYNTN and PVYO strains were provided. Also, the cDNA chip was created to identify and differentiate four species of cucurbit-infecting tobamoviruses [target viruses: cucumber green mottle mosaic virus (CGMMV); cucumber fruit mottle mosaic virus (CFMMV); Kyuri green mottle mosaic virus (KGMMV); and cucumber green mottle mosaic virus (CGMMV)] and ZGMMV (zucchini green mottle mosaic virus)]. Based on scatter diagrams, the signal strength of all combinations of probe and target was highly linked with nucleotide sequence identified between the probes and target viruses (Lee et al. 2003).

DNA microarray was used for simultaneous detection and identification of five microbial pathogens of maize: *Pantoea ananatis*, *P. agglomerans*, *Enterobacter cloaceae* subsp. *dissolvens*, maize dwarf mosaic virus (MDMV), and sugarcane mosaic virus (SCMV) (Krawczyk et al. 2017). Bacterial secreted effectors play vital roles in pathogen–host interactions. Computational approaches have accelerated the process of identifying secreted effector proteins in bacteria. Informative

Table 2.2 Bacterial and viral pathogen bioinformatics tools with their annotations

Plant pathogen databases	Url	Function	Pathogen target
PHYTOPATH	http://www.phi-base.org	Database that contains all sequenced and annotated plant pathogen genomes	Bacteria, fungi, and protists
NIASGBdb	http://www.gene.affrc.go.jp/databases_en.php	Genetic resource database and a plant diseases database, linked by a web retrieval database. The genetic resources database has plant and microorganism search systems to provide information on research materials. A database of plant diseases in Japan has been developed based on the listing of common names of plant diseases compiled by the Phytopathological Society of Japan	Bacteria, fungi, and viruses
Microbial genome databases			
FungiDB	https://fungidb.org/fungidb/app	Free online resource for data mining and functional genomics analysis for fungal and oomycete species	Fungi and oomycetes
PATRIC	https://www.patricbrc.org	The Pathosystems Resource Integration Center provides integrated data and analysis tools to support biomedical research on bacterial infectious diseases	Bacteria, viruses, and archaea
MBGD	https://mbgd.nibb.ac.jp	A database for comparative analysis of fully sequenced microbial genomes, which is continually increasing in number. MBGD's goal is to make comparative genomics easier from a variety of perspectives, including ortholog identification, paralog grouping, motif analysis, and gene order comparison	Bacteria, eukaryote, and archaea
PathoPlant (Bülow et al. 2004)	http://www.pathoplant.de	PathoPlant is a database that contains information on plant–pathogen interactions and signals transduction pathway components relevant to plant pathogenesis. PathoPlant also has gene expression data from *Arabidopsis thaliana* microarray experiments, which can be used to find individual genes that are influenced by stimuli such as pathogen infection, elicitor administration, or abiotic stress. PathoPlant may also be used to validate small DNA sequences as cis-elements that respond to various stimuli. For noncommercial users, PathoPlant is a free resource	Bacteria, viruses, and nematodes
PHI base	http://www.phi-base.org	From mutant genes to phenotypes! The mission of the PHI-base is to provide expertly curated molecular and biological information on genes proven to affect the	Bacteria, fungi, and protists

(continued)

Table 2.2 (continued)

Plant pathogen databases	Url	Function	Pathogen target
		outcome of pathogen–host interactions. Information is also given	
HPIDB	https://hpidb.igbb.msstate.edu	A resource that helps annotate, predict, and display host–pathogen interactions (HPIs). HPI that underpins infectious diseases is critical for developing novel intervention strategies	Bacteria, fungi, and viruses
VirusMentha	https://virusmentha.uniroma2.it/about.php	A website that provides a set of tools for analyzing proteins in the context of network interactions	Virus
PCPPI	http://bdg.hfut.edu.cn/pcppi/index.html	*Penicillium*-Crop Protein–Protein Interactions database, which is constructed based on the experimentally determined orthologous interactions in pathogen–plant systems and available domain–domain interactions (DDIs) in each PPI	Fungi

features of known effectors were first reviewed, which contribute to their identification, composed of the sequential, structural, and genomic information and others of the effectors (Zeng and Zou 2019).

2.6 Conclusion

Additional probes can be constructed to cover more viral genes as more viral sequences become available, thereby increasing the likelihood of identifying a virus (Rubio et al. 2020). Another advantage of probes against different genomic regions over PCR technology, where primers frequently target only one specific section of viral genomes, is the availability of probes against different genomic regions. Because microarrays are simple to upgrade, efforts are made for a modern version with additional probes against recently completed or newly reported viral genomes (Zhang et al. 2013; Liu et al. 2017, 2020). Including available resources and then carefully studying the strengths and weaknesses of multiple types of machine learning algorithms and statistical methods for predicting secreted effectors. This is demonstrated by implementing a benchmark of available ones based on our curated data sets. We propose a future where the fidelity of identifying secreted effectors in silico will be much more persuasive and beneficial. This may be owing to the construction of a more balanced number of known effectors without taxonomic, characteristic, numerical, and functional biases; more informative and discriminative features and more efficient methods of feature extraction/representation are desired,

with the improved reliability of the bioinformatic prediction tools and a better interpretation of the mechanisms behind the molecular pathogen–host interactions.

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Chapter 3
Application of Molecular Ecology Approaches in Sustainable Agriculture for a Better Understanding of Plant–Microbiome Interactions

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Abstract Plants are exposed to a natural habitat where the plants interact in diverse ways with their natural environment. To study the mechanisms of defense in the plants, microbes and their communities are given positive responses on the host. These beneficial responses include nutrient acquisition, resistance against plant pathogens, abiotic stress like drought, salinity, heat, and accelerated plant growth. Incredibly, in beneficial and pathogenic microbes, most of the other signals that induce plant immune responses are remarkably molecularly similar and sometimes identical. Therefore, it is uncertain which influences the results of direct interactions

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between the microbe and host, and the variables that allow plants to differentiate benefits from pathogens. Comprehensive approaches to empirical systems biology would be required to uncover the dynamic network of microbial, genetic, and metabolic interactions, such as the signaling pathways resolving microbe–host interactions.

Keywords Metagenomics · Molecular ecology · CRISPR · Rhizosphere engineering · Host–pathogen interaction

3.1 Introduction

The microbial environment has been improved in recent years, and a thorough study of microbiota function and structure associated with the host was made possible due to the low cost of sequencing. In future precision agriculture, the applications of beneficial bioformulations alleviate the abiotic and biotic stress and increase the yield. It is currently uncertain how plants behave and differentiate between the pathogenic agents and beneficial microbes, mitigate the infection from the former, and encourage colonization by the latter. In the pioneering work, the microbiologists like Winogradski, Pasteur, Koch, and Beijerinck primarily depend on the viable microbe counts under laboratory conditions. It later became clear that it is hard to re-culture or reviving microbes that exist in the stagnant or dormant phase. Based on Colwell and coworkers' observations on the occurrence of nonculturable *E. coli* strains (Xu et al. 1982) and the abundance of nonculturable human pathogenic strains, the drawbacks of traditional microbiological methods have been highlighted, e.g., *Salmonella* (Roszak et al. 1984), *Vibrio* (Colwell et al. 1985), *Aeromonas* (Allen-Austin et al. 1984), *Legionella* (Hussong et al. 1987), *Campylobacter* (Rollins and Colwell 1986), and *Shigella* (Colwell et al. 1985). These results have significant consequences on the field of plant–pathogen or beneficial microbial ecology (Colwell et al. 1989; Byrd et al. 1901). The identification and detection techniques of microbes have also increased with the application of recombinant DNA technology (Sussman et al. 1988; Fry and Day 1990). The use of recombinant DNA techniques and other existing molecular methods in microbial ecology has been abandoned by the new field of "Molecular Microbial Ecology." In DNA testing technology, the use of various methods enables research in this field to classify, on a molecular basis, ecological processes such as nucleic acid adaptation, selection, and genetic variation, i.e., gene transfer, gene stability, and expression of genes. 16S rRNA-targeted oligonucleotide probes have been used successfully to detect both cultured and uncultivated microorganisms. The development of these probes is relatively simple since PCR techniques could quickly gain the appropriate sequence information. The PCR technique is beneficial because the 16S rRNA gene could be amplified by using primers from different groups of bacteria against preserved regions in the 16S rRNA (Drahos 1991; Kashyap et al. 2021). The identification of *Frankia* strains, actinomycetes endophytes of nonleguminous (i.e., actinorhizal) plant nitrogen-fixing root nodules, is greatly facilitated by the use of oligonucleotide

probes. There have been established genus-specific and many species/strain-specific probes for pure strain cultures and those that have never been isolated from root nodules. Here we discuss the current status of the application of omics technologies to understand the molecular aspects of host–pathogen interactions in different crops.

3.2 Understanding Molecular Ecology Using Molecular Approaches to Determine the Plant–Microbiome Interactions

Different plant diseases occur due to pathogenic microbes, which cause a severe risk to plant health, productivity, and food security. A useful tool is needed for rapid response to mitigate the biological invasions of plant pathogens. Various molecular techniques are used to identify the plant pathogen's genes, defense-related genes, protein functions, and expression profiles. Multiomics approaches screen the processes to solve the challenges of understanding plant host and pathogen and the interactions between two species at the genomics, transcriptomics, proteomics, and metabolomic level (Neik et al. 2020). Metabolomics is used to identify and detect pathogenic compounds synthesized during infection, which will provide more information on the microbial side of the relationship between pathogens and their hosts.

3.2.1 Metagenomics

Metagenomics is the molecular technique used to investigate the DNA taken directly from the environment. This field involves the extraction and amplification of microbial DNA from different samples like water, soil, air, plants, and animals (Handelsman et al. 2007). This method grew out of the need to understand the functional role and diversity of uncultivable microbes. Plant pathologists and microbiologists first discovered this method in the late 1990s (Chen and Pachter 2005). In plant health, this study has revolved around two main categories: to study the endophytic microbes within the shoots and roots in model plants such as maize to expand other crops (Fadiji and Babalola 2020). Again, the few studies conducted here are in crop pathosystems, and there is a need to study the phenomenon of suppressive soils in other ecosystems, for example, managed and unmanaged forests and grasslands.

3.2.2 Metabolomics

It refers to the study of the host plant metabolism changes during the pathogen attack. After the black rot infection in *B. oleracea*, the alkaloids, photosynthesis, sphingolipids, and coumarins were involved, and it is detected based on metabolite profiling by liquid chromatography-quadrupole within 48 h after the interaction with the host (Tortosa et al. 2018). In the *Brassica–Alternaria* pathophysiology, the essential elements were identified to pinpoint the regulation mechanism, and molecular targets for the construction of systems biology were constructed to enhance *Brassica* crops (Pathak et al. 2017). For example, systems biology was constructed for *A. thaliana–S. sclerotiorum* pathosystem, where gene expression was to be assessed to determine the hyphal cell's metabolic activity, which supports the hypothesis that hyphal cells of *S. sclerotiorum* are essential for pathogenesis and colonization of the host (Peyraud et al. 2019). Gluconasturtiin's role was discovered with the help of metabolomics and quantitative genetics in the *B. napus* that showed resistance against cabbage clubroot.

3.2.3 Transcriptomics

This is helpful to understand the molecular mechanism of host–pathogen interaction and which gene is responsible for invasion and pathogenesis (Kashyap et al. 2022). RNA sequencing and transcriptomics analysis allow the gene expression studies for host–pathogen interaction and detect the genomic loci responsible for susceptibility and resistance in the host and pathogenic virulence activity (Fu et al. 2019). The new techniques including RNA sequencing and transcriptomics analysis give the fast track method for identification and expression of a gene that is responsible for pathogenesis after the pathogen attack, for example, *P. carotovorum* and *X. campestris* that break the host resistance and release the extracellular enzymes like amylase, protease, cellulase, pectinase, mannanase, pectate lyase, and polygalacturonases as well as the biofilm production (Chen et al. 2019b). Genome editing and resistant cultivars can undermine the virulence mechanism of the pathogens.

3.2.4 Proteomics

During different conditions, the proteomics study provides actual contributions, while the genomic study provides a potential contribution toward cellular function. The early stages of *P. brassicae* infection in *B. oleracea* showed the thioredoxin protein expression associated with oxidative stress as a defense response against a pathogen (Moon et al. 2020). Proteomics analysis of *P. brassicae* infected Chinese cabbage revealed 487 proteins that were involved in defense response, as these proteins are involved in tryptophan and glutathione biosynthesis (Lan et al. 2019).

3.2.5 CRISPR

Clustered regularly interspaced, palindromic repeats system can be used to study the functional study of genes in plants and genome editing techniques used to engineer disease resistance traits. This technique is expected to resolve the significant challenges to crop improvement and engineering resistance toward viruses, fungi, bacteria, and pests (Zaidi et al. 2020). One of the most important bacterial diseases for which CRISPR provides a resistance solution is the citrus canker caused by *Xanthomonas citri*. CRISPR-mediated editing of CsLOB1 in grapefruit and Wanjincheng orange significantly reduced citrus cancer (Peng et al. 2017; Jia et al. 2017). This technology has been used against oomycete infection as well. *Phytophthora palmivora* is a destructive oomycete pathogen of papaya, and CRISPR-Cas-9 was used to develop papaya plant mutant for cysteine protease inhibitors, which increased resistance toward *P. palmivora* (Gumtow et al. 2018).

3.3 Application of Plant–Microbe Interactions for Sustainable Agriculture in CRISPR Era

With the population explosion, the increasing demand for food worldwide is the primary task for governments. To ensure food security, higher crop production in a sustainable way is the major challenge. With the antagonistic impacts brought about by traditional farming practices on the climate alongside food security, a manageable and sound agrarian yield should be worked on utilizing valuable microorganisms. Throughout life, all living organisms are surrounded by various microbes (Bulgarelli et al. 2013). For many years, the process of evolution has been taking place, which involves interaction among microbes and plants (Fig. 3.1). The endosymbiotic theory explains the origin of plastids of the plant kingdom (Keeling 2010) that happened through these interactions to survive in nature, called natural selection. These plant–microbe interactions are bidirectional, which may be beneficial/pathogenic or neutral (Thrall et al. 2007; Rodriguez et al. 2019). The metagenomic study of the interaction between plants and microorganisms is available in several kinds of literature (Shelake et al. 2019). In modern-day agriculture, cutting-edge technology could play an essential role in enhancing the beneficial plant–microbe interaction for sustainable agriculture. The use of microbiota for agricultural yield enhancement requires a better understanding of the interaction at the molecular level (Bulgarelli et al. 2015; Cavicchioli et al. 2019). The technique of the era, i.e., CRISPR (clustered regularly interspaced short palindromic repeats) technology, is the potential platform to make desirable changes to get higher yield and stress resistance in plants (Zaidi et al. 2018). Therefore, further understanding of plant–microbe interaction sustainably improves the agricultural produce using CRISPR technology.

The microbial interaction with plants may be in the rhizosphere, rhizoplane, endosphere, and phyllosphere region. In these different regions of the plant–microbe interface, the composition, role, and effects are determined by the compatibility and

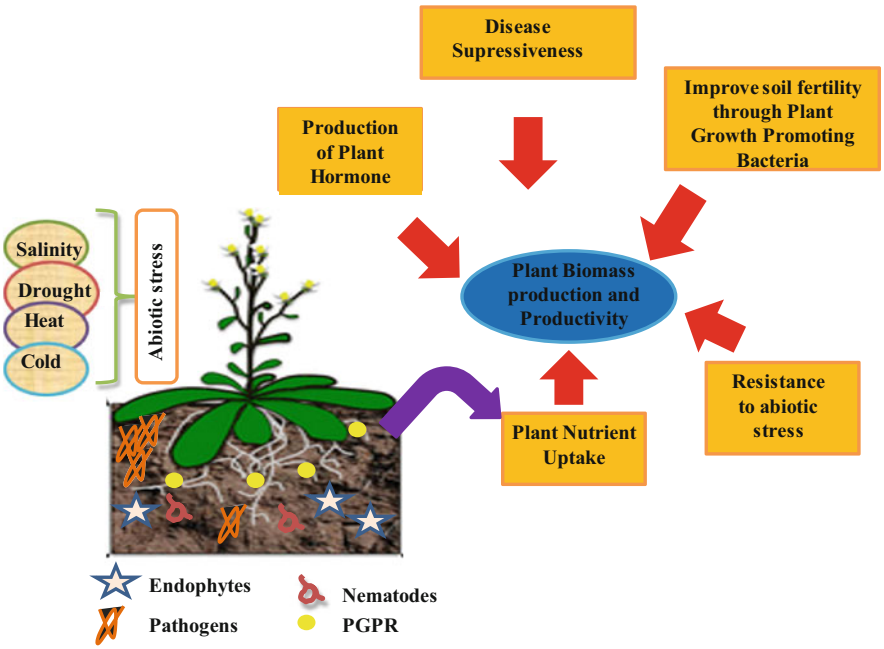

Fig. 3.1 Understanding the role of microbes in alleviating biotic and abiotic stresses in plants

requirement of both participants that can be studied by next-generation sequencing of microbiota (Hassani et al. 2018; Vorholt 2012; Muller et al. 2016; Walters et al. 2018). Apart from this, genetic modification of the plants or microbes is also very useful for a better understanding of the role and mechanism of beneficial microbes but on the other hand unacceptance of the technology is a major drawback. At present, genome editing is the perfect tool to establish the precise function of the gene involved in plant–microbe interaction (Knott and Doudna 2018). The complete dysfunction and function regain of the targeted genes by genome editing technology is CRISPR advantage to get precise genetic information about plant–microbe interactions. CRISPR could be used to know the plant–microbe interaction to avoid pathogens on the crops well elaborated and documented in various reports (Yin et al. 2017; Langner et al. 2018; Chen et al. 2019a). Apart from model organisms, the genetic interaction among plants and microbes could be studied using nonmodel microorganisms using CRISPR/Cas technology, which is not possible by conventional methods (Eoh and Gu 2019). Due to the increasing population of the world, using CRISPR technology to boost the plant growth-promoting activity, leading to more yields sustainably, is the most potential technique and is the need of the hour.

3.3.1 Case Studies

Several case studies are available in the public domain indicating CRISPR/Cas technology application for sustainable agriculture.

Bacterial blight is the major pathogen in rice, causing severe yield and quality loss. Jiang et al. (2013) tried to target *OsSWEET14* and *OsSWEET11* genes in the model and nonmodel plant systems by CRISPR/Cas9/sgRNA. They found that the disease symptom of blight was positively controlled in rice in terms of better yield and quality of the grains. Wang et al. (2016) found that InDel mutant were resistant to rice blast. In polyploidy crops such as wheat, desirable changes in the genome by targeting the homo-alleles were not reported. Wang et al. (2014) and Zhang et al. (2017) targeted *TaMLO-A1* and Taedr1 alleles, respectively, in hexaploid bread wheat by genome editing. Alteration of these genes in wheat resulted in a wide range of resistance against powdery mildew in wheat. In the Solanaceae family, tomato is among the important vegetable crops. *Phytophthora syringae*, *P. capsici*, and *Xanthomonas* sp. infestation are too severe. The tomato-caused disease was regulated in tomatoes by altering the *SlDMR6–1* gene through CRISPR/Cas-9 by de Toledo et al. (2016). The genome-edited tomato plants were showing increased resistance against various fungal pathogens. They also revealed that the effect of this mutation on the plant's other agronomic traits is negative. In tomatoes, *Pseudomonas syringae* (Pto) DC3000 produces a compound known as coronatine that induces stomata opening. Through these stomatal openings, *Pseudomonas* enters into plant system. In *Arabidopsis*, the synthesis of coronatine requires a coreceptor AtJAZ2. They edited the tomato gene SIJAZ2, which led to the uncoupling of SA-JA hormonal antagonism in the stomata. The study will set a reference in agricultural biotechnology to develop resistant varieties in other crops as well.

The canker disease caused by *Xanthomonas* is yield loss and deteriorating quality agent of citrus fruits. The study conducted by different researchers used CRISPR tools and targeted the CsLOB1 canker susceptibility gene. The citrus canker frequency and efficacy on citrus crops were reduced in engineered plants (Jia et al. 2017; Peng et al. 2017). Powdery mildew of grapevine and apple are devastating in nature. Some susceptible genes are potential targets for editing. The known gene MLO-7 in grapevine and *DIPM-1*, *DIPM-2*, and *DIPM-4* genes in apple were knockdowned by genome editing, and resistant phenotypes were observed (Malnoy et al. 2016).

Theobroma cacao suffers severe yield losses due to pathogens. The resistance gene against *Phytophthora tropicalis* is not present in the available germplasms of cocoa. Therefore, to make cocoa plants resistant to *Phytophthora*, Fister et al. (2018) targeted the suppressor of the defense response gene *TcNPR3*. The deletion mutation generated by genome editing resulted in increased resistance in cocoa against *Phytophthora tropicalis*. Severe crop yield losses by *Phytophthora sojae* in soybean is of great attention. Fang and Tyler (2016) used CRISPR/cas technology to target RXLR effector gene *Avr4/6* and found that the effector gene is replaced by NPTII

through nonhomologous end joining (NHEJ), resulting in resistant soybean against *P. sojae*.

The functional genomics study by mutagenesis through CRISPR/Cas is of great importance to know the function of the gene. Oxathiapiprolin is an agrochemical that is being used to control *Phytophtora capsici*. Regular use of the chemical selected the mutant population with a point mutation in G839W and G770V ORP1 protein-making *Phytophtora capsici* resistant to oxathiapiprolin. Miao et al. (2018) validated the point mutation by CRISPR cas and targeted G839W and G770V genes. The study validated that the G839W and G770V genes are responsible for developing resistance against Oxathiapiprolin in *Phytophtora capsici.* The Blackleg disease of canola is caused by *Leptosphaeria maculans*. Among available fungicides against *Leptosphaeria maculansis*, iprodione is the most common. The natural mutation in hos one gene makes *Leptosphaeria* resistant to agrochemicals. Ali et al. (2016) targeted the noncoding intergenic sequence of geminivirus using CRISPR/Cas9 and observed reduced capacity of the generation of viral variants. In this way, other viruses could also be targeted to reduce their multiplication and durability. Apart from developing resistance to various diseases, CRISPR can also be used for a breeding program.

3.4 Composition and Driving Factors of the Plant–Microbiome (PM) Interactions

In this environment, a complex food web is developed in which macroorganisms are connected to microorganisms and vice versa. This interaction mainly occurs above the ground surface and below the ground surface. Here we focused on the interaction that occurred below and above the ground surface in which different complex microbiota are interlinked with higher systems, i.e., with plants either positively or negatively. These parameters are responsible for the behavior and function of both plants and microorganisms with each other.

3.4.1 Plant-Driven Forces

Plants are versatile hosts that are associated with different microorganisms (bacteria, fungi, and archaea) in their underground (rhizosphere) and above the ground surface (phyllosphere). These partnerships control the health and efficiency of their host plant. Ecologist Dr. Peter Chesson postulated that stabilizing mechanisms are important for preserving species diversity and coexistence (Chesson 2003). Some of the host factors that affect this plant–microbial partnership are listed below.

3.4.1.1 Plant Species

The host plant's identity has a strong effect on its microbiome. Separate microbiomes can be harbored by different plant species growing adjacent to each other. A report by Samad et al. (2017) explored the soil microbiome of grapevine and some weeds of the rhizospheric region using 16S rRNA gene sequencing method and investigated that different hosts harbor different types of microbial communities whether growing under similar field conditions. Phylogenetically, distant-related plants display more significant variation in associated microbiome compositions, indicating that plant phylogeny functions in structuring root microbiomes (Bouffaud et al. 2014). Lundberg et al. collected more than 600 *A. thaliana* plants and analyzed their core root microbiota through 16S rRNA gene sequencing. They noted that plant root microbial communities are appropriately dependent on the genotype of the host. In addition to the rhizospheric microbiota, the phyllosphere is also a complex landing surface that bears different microbial communities, which depend on the host genotype (Vorholt 2012). Kembel et al. (2014) have shown that the leaf microbiome population is closely associated with plants' evolutionary relationship. Plant characteristics can determine their hosting microbiota, which include leaf permeability, leaf topography, cuticle structure, and root exudates, as well as plant immunity, which may also play a role in microbial type.

3.4.1.2 Plant Age

Various symbiosis researchers have reported that the plant age is an essential factor affecting their microbial communities for governing the plant health status (Sugiyama et al. 2014). *Arabidopsis* bacterial rhizosphere population research showed that at developmental time points, the seedling stage selects different microbiomes. Plants produce a diverse group of compounds in the root exudates and unique phytochemicals. Marques et al. (2014) studied DGGE fingerprints of three sweet potato cultivars, and the analysis revealed that the plant age significantly influences bacterial community composition. Similarly, the rhizospheric bacterial community of potato, maize, and soybean has been demonstrated and the outcome of the analysis showed different bacterial communities in different host systems (Lerner et al. 2006).

3.4.1.3 Plant Health Status

The state of plant health plays an influencing role in the composition of the microbiome. Plants have defense mechanisms against plant pathogens: damage-associated molecular patterns and pattern-triggered immunity (PTI) (Monaghan and Zipfel 2012). During the period of any pathogen or herbivorous attack, plants released exogenous volatile compounds and hormones, altering the composition of

root exudates and the microbial community (Erb et al. 2009). Lee et al. (2012) investigated the increased population of rhizospheric *Bacillus subtilis* around sweet pepper rhizosphere in response to pathogenic microbial and aphid infestation.

3.4.1.4 Microbial-Driven Forces

Soil microorganisms play a crucial role in the plant community, growth, plant exudates, and their performance. These microbiotas can influence root exudation by affecting the permeability of root cells and metabolism. Soil microorganisms receive the secretion of root exudates in the rhizosphere zone, and in response to exudation, microbes also secrete other volatile compounds that affect the plant–microbial interaction (Valladares et al. 2007). Some soil microbes can also induce phenolic compound exudation to boost the iron absorption from soil to plant under low-Fe availability conditions (Whitaker et al. 2018). In nature, plants are constantly encountered by different microbial communities, including pathogens, symbionts, and commensals affecting the root metabolite composition. The main reason behind this alteration is not precisely understood, but it was hypothesized that the rhizospheric microbiome exerts host systemic signals, which trigger an alteration in root exudation. This host systemic signal modulates the local microbial colonization on roots that alter the root exudate composition (Manzar et al. 2021). The most renowned group of bacteria called plant growth-promoting rhizobacteria plays a pivotal role in shaping the rhizospheric microbiome. These organisms interact with plants through a chemical communication route established in the rhizosphere, causing a positive effect on plant growth. In an experiment, *Arabidopsis thaliana* was co-cultivated with two PGPRs (*Bacillus subtilis* GBo3 and *B. amyloliquefaciens* IN937a) under in vitro conditions and resulted in enhanced growth of the plant. The study suggested that the promoter effect occurred due to the diffusion of some bacterial volatiles that triggers a positive effect on the *A. thaliana* plants (Ryu et al. 2003). A variety of microorganisms often harbor the above-ground surfaces of plants (the phyllosphere), and this phyllosphere microbiome communicates with the host plant, influencing its health and work. Microorganisms of the phyllosphere, mainly bacteria and fungi, can function as mutualists promoting plant growth and environmental stressor tolerance by using the leaf surface for habitat. Alphaproteobacteria and gammaproteobacteria are particularly well-dominated groups of commensal microbe in the phyllosphere and play many ecological roles (Ruinen 1965; Redford et al. 2010; Vorholt 2012). Molds belonging to the Ascomycota are often the dominant fungi on the leaf surface before senescence (Abdelfattah et al. 2015). The phyllosphere microbiome has traditionally been regarded as being relatively inert, with little function in supplying the host plant with nutrients. In surveys of phyllosphere population composition, nitrogen-fixing bacteria were reported and helped fix atmospheric nitrogen for plant growth (Ruinen 1965; Holland 2011). Interestingly, by oxidizing ammonia to nitrate by nitrification, phyllosphere microorganisms may play a protective role in areas prone to contamination from high nitrogen levels (Guerrieri et al. 2015).

3.4.2 Soil Effect

Soil performs different functions, and one of the key functions is to serve as a supportive resource for human and animal communities to feed plants or food crops. The soil microbiome composition close to the roots is highly affected by the root microbiome populations. Likewise, the fungal community's composition is affected more primarily by the kind of soil than by the host plant. The plant–microbial interaction is a mirror effect of sustainable agriculture, in which both the partners are affected by various biotic and abiotic factors.

3.4.3 Salicylic Acid (SA)

Salicylic acid is a beta hydroxyl phenolic acid that regulates various biological processes in plants at low concentrations as chemical messengers. Exogenous SA treatment enhances the host defense system. This defense-related hormone is also involved in many physiological processes, such as storage, seed germination, stomatal movement, flowering development, and fruit ripening. In addition, SA controls its signaling pathways metabolites besides getting engaged in cross-talk with another pathway mediating resistance. SA affects plant development under stressful conditions through water relations, stomatal regulation, nutrient uptake, and photosynthesis. It is also involved in the activities of various enzyme regulations such as peroxidase, polyphenol oxidase, superoxide dismutase, phenylalanine ammonia-lyase, etc., which are the components of inducing plant defense against biotic and abiotic stresses. Both in PAL and IC pathways, the biosynthesis of SA mainly takes place in the cytosol. For different plant species, the biosynthesis of SA pathways differs. In rice, the PAL pathway are the most important pathway for the accumulation of SA, whereas the ICS pathway is essential for SA accumulation in *Arabidopsis*. ICS and PAL pathways are responsible for SA accumulation in Soyabean. However, biosynthesis and accumulation of SA are different in the plant systems; for example, SA accumulation is more in shoots than in roots in the case of rice (Duan et al. 2014).

3.4.3.1 Phenylalanine Ammonia-Lyase (PAL) Pathway

This pathway converts phenylalanine (Phe) into trans-cinnamic acid (t-CA), then to benzoic acid (BA), and finally to salicylic acid. Basal SA levels differ widely between different plant species, with up to 100-fold differences recorded among numerous species (Dean et al. 2005). The enzyme phenylalanine ammonia-lyase converted Phe deaminase to ammonia and t-CA, and this enzyme was isolated from barley in 1961 and considered the key enzyme in the pathway of PAL. However, in the development of the seed process in *Arabidopsis*, the PAL enzymes that belong to

the cytochrome P450 protein family-like aldehyde oxidase 4 were observed, which converted benzaldehyde to BA, and in oxygenase activity BA 2-hydroxylase (BA2H), which converted BA to SA.

3.4.3.2 Isochorismate (IC) Pathway

In the late 1990s, three major genes, i.e., ICS1, EDS5, and PBS3 were cloned and soon after found to encode three enzymes that describe the IC pathway of SA biosynthesis. ICS1 and its nearest homolog ICS2 are homologs of PchA, an isochorismate synthase (ICS) controlling the first committed step of SA biosynthesis. Both of the homologs are requisite for the biosynthesis of SA, present in chloroplasts, and transform chorismate to IC. In this biosynthesis, the PmsCEAB gene cluster represents a vital role. Two investigations have indicated that in *Arabidopsis*, SA synthesis by ICS varies from that in bacteria. The synthesis of SA results from the amino acid conjugation of IC, accompanied by enzymatic conversion. In *Arabidopsis*, the responsible gene, PBS3, was described (Rekhter et al. 2019; Torrens-Spence et al. 2019). Ding and Ding (2020) reported that *eds5* is a mutant located in the membrane of chloroplast and responsible for transportation of IC and increased susceptibility of bacterial disease in the plants, considered a multidrug and toxin transporter protein family, and it is responsible for transportation of SA from plastids to cytosol. Nevertheless, soybean (*Glycine max*), which has five PAL and two ICS and homologs, demonstrates similar essential functions for both PAL and ICS pathways in the accumulation of SA. A threefold rise in SA is observed when treated with *P. glycinea*, *P. syringae*, or *Phytophthora sojae*. During the pathogen infection, downregulation of either the ICS or PAL pathway succeeded in substantially lowering SA accumulation levels. In addition, such silent plants were more vulnerable to any of these pathogens (Shine et al. 2016). Salicylic acid can go through numerous modifications in the plant system. Most of them cause SA to become inactive. SA glucoside (SAG) and salicyloyl glucose ester (SGE) are formed during SA glucosylation. These compounds can be amassed in the vacuole in large amounts (Dean et al. 2003).

3.5 Rhizosphere Engineering

The rhizosphere represents the plant-influenced habitat found by soil microorganisms in the limited region of interaction between roots and soil particles (Dessaux et al. 2016). The rhizosphere is an area of complex and tightly condensed inhabited soil zone within this region of plant–soil interactions, with a complex collection of food web interactions and inter–intra-species communication that have a major effect on transition and carbon flux. The CO_2 decreased due to reduced crop production, natural vegetation, and loss through abiotic stresses like high temperatures, drought, and salinity. Drought stress severely affects root growth and

photosynthesis (Verslues 2017). Excessive Na+ and Cl^- causes have detrimental effects on plant production and growth (Negrão et al. 2017). Drought and salinity also contribute to increased ethylene levels, which are suppressive of root growth and affect many plant physiological pathways (Sun et al. 2007). Plant hormones are involved in root formation and development when abiotic stress happens. The auxin is the main regulator for about all parts of plant growth. Other plant hormone types, such as gibberellic acids and cytokinins (GAs), serve as restrictive in root growth development and supportive in root elongation. By controlling auxin transportation inside the root tip, ethylene regulates root formation (Swarup et al. 2007). Therefore, plant phytohormones should be considered as one of the key factors in all efforts dealing with rhizosphere engineering. The pathogen-triggered immunity and plant defense machinery are stimulated by applying biocontrol agents or biopesticides (production of antifungal, antibacterial, or nematicide compounds) (Saraf et al. 2014). As demonstrated by experiments on the bacterial pathogen *Pectobacterium* described earlier, engineering the interactions among microbes and plants is also an exciting strategy that is more than a simple opportunity. Likewise, the plant engineering techniques presented in the related part of this document (see above) associated with the plant immune system are also illustrations of what can be known as the engineering of plant–microbe interactions. The *Burkholderia cepacia* strain transformed with toluene degrading gene incorporated in yellow lupine plants. The transformed strain incorporated in the plants showed no response of phytotoxicity at a high toluene level even up to 1000 mg/mL. A 50–70% reduction in evapotranspiration of toluene through the leaf also led to the association of engineered plant microbes. In phytoremediation and plant growth promotion situations, the possible function of plant–endophyte interaction engineering and the related problems have been thoroughly researched (Barac et al. 2004; Gaiero et al. 2013; Sessitsch et al. 2013). Many PGPR is being used as microbial inoculants in different situations and, unlike the abundance of PGPR choice and use data, there are fewer PGPR engineering reports to make them more successful. There are a few disadvantages and potential negative effects of engineering in the rhizosphere. For example, it is not clear that the plant defense system's exploitation is entirely benign for plant fitness. In comparison, the incorporation into the rhizosphere of bacteria decreases QS signals and may have deleterious effects on PGPR bacteria generating antifungal proteins under the influence of QS regulation (Molina et al. 2003). Likewise, since these can also trigger pathogenic agrobacteria, establishing a trophic connection between microbial populations or communities and the plant cannot be focused on opine-producing plants in the field. Therefore, as a global law, precautions should be taken about exploiting the ecological characteristics of components of the rhizosphere.

3.6 Frontiers in Multiomics for Plant Disease Ecology

Like every other organism, plants, as well as plant pathogens, inherit their genetic constituents from their predecessors, indicating that closely related species are more likely to share characteristics in common than are far related ones; i.e., traits are phylogenetically conserved, which can be of great significance in understanding plant disease ecology (Gilbert and Parker 2016). Plant disease is not a rule rather it's an exception. Plants remain healthy until they get sick, and the consequences can be quite alarming if leading to economic losses. Apart from managing the diseases, generally fewer efforts are made to keep plants healthy and understand what's going on within the plant while they are disease-free. In present times, the entire DNA, proteins, and metabolites of the plants can now be sequenced to understand the genetic basis of disease at a level that was never been possible before.

Omics technology is one of the advances in molecular biology that has helped understand plant–pathogen interactions by facilitating all-encompassing approaches underlying the pathogenesis mechanisms (Crandall et al. 2020). Integration of different types of molecular information through multiple omics approaches including genomics, transcriptomics, proteomics, epigenomics, metabolomics, etc., makes it a revolutionary field of study (O'Donnell et al. 2020).

Concerning emerging plant diseases, climate change, and invasive species introduction make epidemics management more challenging. Under such circumstances, multiomics come as a rescue by providing us with complete detail of plant–pathogen interactions and can be significant in designing disease forecasting models under the present changing scenario. It is better to integrate findings of genetics studies with metabolomics and spectrasonics to complete the omics cascade, which can provide insight into plant disease ecology.

One significant frontier of multiomics understands the association of endophytes with plant disease management (Sahu et al. 2020). Endophytes are the beneficial microbes harbored by the plant itself, which play an important role in well-being of the plant via several mechanisms regulating plant immune responses, oxidative burst protection (Kusajima et al. 2018), reduced gene expression, metabolomic cascade, etc. (Khare et al. 2018). A promising frontier in multiomics is the metagenomic studies that have been extensively performed to explore plant growth-promoting factors through a detailed study of endophytic microbes' interactions within the plants (Lindeberg 2012; Fadiji and Babalola 2020). In the case of endophytes, there are a handful of microbes that have been explored. For instance, a study conducted on *Burkholderia phytofirmans* PsJN (an endophyte belonging to potatoes) showed how different extra-cytoplasmic functional group elements (sigma factors, group IV) were crucial in assisting other bacteria to sense temperature or moisture alterations in their surroundings and switch their metabolism as a part of their survival strategy (Meireles et al. 2020).

Under multiomics, studies regarding spectranomic characterization of leaves are another frontier that can be utilized for plant disease management under different circumstances. Multi-dimensional phylogenies thus obtained can be employed in an

understanding of evolutionary dynamics of leaf structures, their chemical constituents, etc. Recent advances in multiomics have shown that spectroscopy is proficient in sensing phylogenetic signals to identify a large number of plant orders and families through the reflectance spectra technique (Meireles et al. 2020). Results from such studies have revealed that contrasting spectral regions have evolved at distinct rates at varying constraints, thus resembling the corresponding evolutionary trait. This innovative frontier demonstrates that both spectranomics, as well as spectroscopy, can bring forth novel cognizance into leaf evolution and phyto-phylogenetic diversity altogether.

In many ways, the multiomics approach can be utilized in plant disease ecology to best understand the various aspects like evolution, positive plant–microbe dynamics, and antagonistic microbe interactions. The application and scope of such approaches in plant–microbe interactions are beyond magnificent, and if explored, they can bring breakthroughs in our understanding of plant disease ecology. Using multiple omics approaches integrated in the best possible ways can provide us a plentiful knowledge with respect to minute details of the basic mechanisms in plant disease ecology, such as strengthening plant defense, thereby giving novel insight into very fascinating plant disease ecology.

3.7 Multiomics in Suppressive Soils for Plant Disease Management

Many options are now available to scientists, researchers, and farmers to manage plant diseases caused by different plant pathogens. Research on suppressive soils is also useful for solving yield losses caused by diseases because no or few diseases occur in certain soil types. Baker and Cook (1974) defined disease suppressive soils as "soils in which the pathogen does not establish or persist, 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 soils." Despite its well-known benefits, ecologists were eluded for many decades about the exact composition of soil microbiota of specific suppressive soils. Recently, with the availability of many -omics approaches like metagenomics, metabolomics, metatranscriptomics, and volatilomics, comparison between the microbiota of suppressive and conducive soil became much easier. With advancements of these approaches in an integrated manner, it expands the understanding of the composition of these soils and mechanisms for particular disease suppression. Several researchers studied different host–pathogen models through these approaches and found that certain fungal and bacterial genera are present in suppressive soils and not present in conducive soil. Several new, cultivation-independent technologies, including population profiling by denaturing gradient gel electrophoresis (DGGE), PhyloC, restriction fragment length polymorphism (RFLP) or quantitative PCR (qPCR), DNA stable isotope probing (DNA-SIP), are now able to determine a more detailed

image of the microbial consortia and basic activities for functioning in disease suppressive soils. By comparative microbiome study of a *Fusarium* wilt conducive soil and *Fusarium* wilt suppressive soil, Siegel-Hertz et al. (2018) found that many antagonistic compounds in suppressive soil, genera of bacteria, and fungi are exclusively or more present predominantly. Li et al. (2017) reported that *Proteobacteria*, *Acidobacteria*, *Chloroflexi*, *Verrucomicrobia*, and several *Archaea* were more abundant in soils without bacterial wilt of tomato, while another eight phyla were more abundant in soils with disease symptoms. Trivedi et al. (2017) developed a prediction model for *Fusarium* wilt suppressiveness known as "Know before you sow" to support the choice of crops or cultivars. In this, they included three keystone genera in combination with several edaphic factors. European Union-sponsored METACONTROL Project revealed several interesting facts about novel biological functions through soil metagenomics. Several biocontrol agents are present in different suppressive soils. Isolation, identification, characterization, and reintroduction of these biocontrol agents into nonsuppressive soils lead to inconsistent performance.

3.8 Conclusion

Omics techniques have highly developed our perception of plant–pathogen interactions in numerous ways and will become the conventional approach to quickly identifying pathogenicity genes for breeding crops resistant to the main pathogens. This will foster success in the development of new varieties of different crops for sustainable agriculture.

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Chapter 4
Detection and Diagnosis of Important Soil-Borne Diseases: An Overview

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Abstract Soil borne pathogens are major group of phytopathogen causing numerous soil-borne diseases. Due to their persistent behaviour, huge losses in yield have been reported. Thus, to build an effective and precise management approach, these soil-borne diseases must be detected early, quickly, and accurately. The most common methods for identifying plant diseases in the past were basically based on morphological approaches and such approaches are highly time-consuming and lab or intensive. Molecular detection techniques could address these issues with greater precision and dependability. Collection of information regarding pathogen presence through molecular approach assist in taking timely decisions for early-stage treatments and pre-plant evaluation of the fields. Nowadays, polymerase chain reaction along with high-throughput sequencing methods provides a best window to check the soil health status, in which specific conserved region present in the microbes (16s and ITS) are amplified and sequenced. However, the effect of environmental condition on dynamics of phytopathogens could be exploited to develop prediction model, which allow anticipating the attack of soil borne pathogen prior to disease establishment.

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4.1 Introduction

The plant kingdom is recorded to have been infected by about 80,000 diseases, of which the soil-borne diseases occupy a majority of stake. It is more challenging to control soil-borne diseases as their diagnosis is difficult during the early stage of infection because the host is symptomless and has a long latent period (DeShields et al. 2018; Tarafdar et al. 2019). The diseases from soil-borne phytopathogens are key constraints in limiting the production and productivity of crops. Many of the soil-borne phytopathogens like *Fusarium*, *Rhizoctonia*, *Pythium*, *Phytopthora*, *Verticillium*, *Sclerotinia*, etc., cause yield loss in cereal, pulse, vegetable, fruit, and ornamental crops to the tune of 50–75% (Mihajlovic et al. 2017; Baysal-Gurel and Kabir 2018). The phytopathogens inhabiting soil are a diverse group of microbes from lower fungi to higher fungi to bacteria to viruses to even nematodes. However, all the members of this group share some basic features, which enable them to survive and live their part of life in the soil (Ghosh et al. 2019). Some of these phytopathogens form specialized survival structures like resting spore or melanized hyphae that are capable of surviving in the soil for a long period of time (Baysal-Gurel and Kabir 2018). Major diseases caused by soil-borne phytopathogens include rots (root rot, collar rot, stem rot, and head rot), wilts, blights, and damping-offs.

4.2 Important Soil-Borne Phytopathogens

In a list of top ten fungal phytopathogens of economic and scientific importance published by Dean et al. (2012), two genera namely *Botrytis* and *Fusarium* are soil-borne fungi. The species of these two genera cause massive losses in many agricultural and horticultural crops. In addition to these, *Verticillium*, *Phytopthora*, *Plasomodiaophora*, and *Sclerotinia* are also soil-borne fungal phytopathogens that are of economic importance. Among the soil-borne diseases of crop plants, rots are majorly caused by species of *Phytopthora*, *Pythium*, *Rhizoctonia*, *Aphanomyces*, etc., which have lowered the production of many crops very significantly (Clarkson et al. 2015). Additionally, wilt, yellowing, dieback, stunting, damping-off, root blackening, and cracking are other common diseases caused by soil-borne phytopathogens (Ghosh et al. 2019; Panth et al. 2020). Apart from the below-ground infections, some of these phytopathogens, namely species of *Sclerotinia,* cause infections at and aboveground levels in the form of collar and stem rots. The prevalence of soil-borne fungal phytopathogens spread from the Southern to Northwest pacific.

Even with the abundance of fungal genera in the group of soil-borne phytopathogens, many bacterial phytopathogens are also an eminent part. These soil-borne

phytopathogenic bacterial genera include *Erwinia*, *Streptomyces*, *Rhizomonas*, etc., and are responsible for causing scabs and soft rots. The viral diseases of soil-borne nature are rare as they have a necessity of living host, but many of them are carried by fungi and nematodes dwelling in soil or flow with the thin film of water around soil particles (Ghosh et al. 2019). Nematodes also constitute the soil-borne phytopathogen group and are responsible for about 10% annual global loss of agricultural production, amounting to over $125 billion each year (Chitwood 2003). With the increasing temperature of the world, it is now suggested that the infection by soil-borne phytopathogens might increase as the reservoir of inoculum increases (Egidi et al. 2019).

4.3 Detection and Diagnosis

For a very long time period, the identification of plant diseases has been carried out by the experts on the basis of their knowledge and experience after viewing the affected plants through their naked eyes. Since the process of infection is influenced by various parameters consisting of inoculum type, growth stage of plants, and weather, it has become a tedious and exhaustive process for the experts to identify the disease. This process of disease identification has now become time consuming and expensive, so modern techniques are being utilized for the same (Mishra et al. 2020). The different methods utilized in the identification of soil-borne phytopathogens are mentioned in Fig. 4.1.

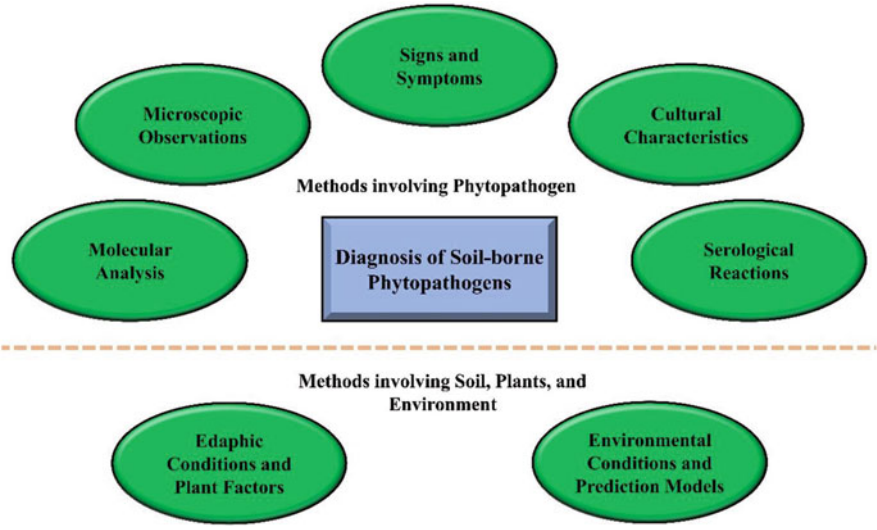

Fig. 4.1 Different methods utilized in the identification of soil-borne phytopathogens

4.3.1 Identification Through Visual Symptoms

The initial step of plant disease diagnostics is the identification of the phytopathogen (Riley et al. 2002). Whenever there is an infection in crop plants from soil-borne pathogens, there is a deviation from the normal functioning of the plants, which helps in predicting, confirming, and managing plant diseases prior to loss. The diagnosis of soil-borne diseases can be challenging, and it usually depends on a combination of observable symptoms and prior knowledge of common diseases that may be prevalent in your region. By comparing infected and healthy plant samples in the lab, a particular phytopathogen can be identified. The parts of phytopathogens that become visible on plants after infection are called signs and are more reliable than symptoms in the identification process. A hand lens and a knife are sufficient to diagnose disease in the field initially. Study of symptoms serves as additional support in the process of phytopathogen identification. Wilting of foliage, tissue discoloration, root decay, loss of vigor, stunted growth, distortion of tissues, and sudden death are some of the major symptoms in crops when they get infected by soil-borne diseases. These symptoms can be differentiated from the abiotic stress symptoms if the chlorosis, stunting, and deformation spread to the whole plant. As discussed earlier, the symptoms due to the soil-borne pathogen can be identified by the presence of physical signs of the phytopathogens, i.e., mycelial growth in the case of fungi, ooze in case of bacteria and cysts in case of nematodes. The signs and symptoms can help in distinguishing between the phytopathogenic causes and even predicting them up to the genus level (Table 4.1).

4.3.2 Identification Through Cultural Characteristics

When a particular phytopathogen of soil-borne origin is isolated and grown under lab conditions on different media, it shows certain characteristic features, which are utilized as one of the tools for identification. Different soil-borne phytopathogens grow and multiply on different media depending on their nutritional requirements. Some of the selective, semi-selective, and nonselective media for the growth of soil-borne fungi are PARP medium (selective for *Pythium* spp.), Mathur's medium (semi-selective medium having iprodione for *Colletotrichum* spp.), Czapek dox agar medium (semi-selective medium for *Verticillium* spp.), and potato dextrose agar medium (non-selective medium for *Rhizoctonia* spp., *Macrophomina* spp., *Fusarium* spp., etc.). Type of spore(s), its morphology, and mycelial structure are helpful in identification. Pigmentation is yet another cultural characteristic that is utilized for the identification of a particular phytopathogen. Development of orange-colored pigmentation with single-celled conidia confirms the suspected pathogen as *Colletotrichum* spp. Furthermore, if the conidia are acute on both ends, the species is *Colletotrichum acutatum* and if they are round, the species is *Colletotrichum gloeosporioides* (Freeman and Katan 1997). *Phytophthora* spp. have coenocytic

Table 4.1 Signs and symptoms of major soil-borne diseases

Signs and symptoms	Disease	phytopathogen (s)
Small elliptical, water-soaked lesion on root, poor stands, wilting, death of emerged seedlings, and whitish mycelial growth on the stem at soil level	Damping off	*Pythium* spp. *Rhizoctonia* spp.
Dark-brown sunken lesion near the crown and on leaves near the ground	Crown rot, crater spot, bottom rot	*Rhizoctonia* spp.
Soft, sunken dull orange lesion, accompanied with ooze and bad odor	Bacterial soft rot	*Erwinia* spp.
Soft and watery rotting of lower stem with profuse mycelial growth and formation of black sclerotia	Cottony rot, pink rot, white mold	*Sclerotinia* spp.
Thinning of crown, dieback, chlorosis of leaves, and decaying of roots and inner tissues at the base of stem	Root rot	*Armillaria* spp.
Stunting, wilting, darkened roots, and collapsing of plants	Phytophthora root rot	*Phytophthora* spp.
Stunted growth, leaf yellowing, and brown discoloration of vascular tissue	Fusarium wilt	*Fusarium* spp.
Stunted growth, leaf yellowing, and black discoloration of vascular tissue	Verticillium wilt	*Verticillium* spp.
Profuse gumming on stem from small and black lesions	Charcoal rot	*Macrophomina* spp.
Stunting and yellowing of plants, formation of galls, and distortion of roots	Root knot nematode	*Meloidgyne* spp.
Uneven root growth with tiny white to brown cysts	Cyst nematode	*Heterodera* spp.
Tan discoloration and rot of stem, bulbs, and basal plates	Stem and bulb nematode	*Ditylenchus* spp.

Source: Harrington et al. (1992), Koike et al. (2003)

mycelium with double-celled oospore and lemon-shaped conidia (Meszka and Michalecka 2016). The presence of white septate mycelium with branching at 45° and 90° angles and formation of brown to black sclerotia confirms the phytopathogen to be *Rhizoctonia* spp. Formation of melanized dark-brown microsclerotia at 10–14 days after inoculation confirms the phytopathogen to be *Verticillium* spp. (Zveibil and Freeman 2005).

Soil-borne bacterial phytopathogens grow in general on nutrient agar medium, King's B medium, and yeast extract mannitol agar medium. Colonies of bacteria are white or clear, transparent or opaque, smooth or rough, shiny, and mucoid-type. Some of the bacterial phytopathogens also produce pigmentation from cream to pale yellow to light pink (Khedr et al. 2014). *Rhizomonas* spp. produce opaque, sticky, and yellowish circular colonies on nutrient agar medium, which confirms their presence. *Streptomyces* spp. can be identified through their mycelial-type colonial growth on yeast extract malt extract medium (Shepherd et al. 2010).

4.3.3 Identification Through Microscopic Observations

Soil-borne phytopathogens can be very easily identified to genus level through microscopic observations. The differentiation between fungi and bacteria can be very well achieved through cultural characteristics. Thereafter, fungi can be identified through microscopic observations of their mycelium and spores, as knowledge about the spore shape, size, color, and arrangement is sufficient for predicting their taxonomic position. On the other hand, most of the bacterial phytopathogens are Gram-negative rods; hence, they can be identified by observing their shape and color after Gram's staining. The only Gram-positive bacterial group that are phytopathogenic are *Actinomycetes* spp. Furthermore, the Gram-negative ones can be identified by observing the presence/absence, number, and position of flagella. For example, *Erwinia* spp., which are responsible for soft rot, have a peritrichous flagellar arrangement.

4.3.4 Identification Through Serological Reactions

In serological detection techniques, unique antibodies react to phytopathogen-specific protein(s), giving a positive or negative result. Different soil-borne phytopathogens have varied reactions to different antibodies, and hence, a combination of antibody reactions is devised to form a serological study for identification. Most of the bacterial phytopathogens produce antibodies or related compounds, which are exploited in the serological assay test for their diagnosis. Most common serological methods used in the diagnosis of soil-borne phytopathogens are enzyme-linked immunosorbent assay (ELISA), tissue-blot immunoassay (TIBA), and quartz crystal immunoassay. For example, the resting spores of *Plasmodiophora brassicae* are detected through the use of highly specific monoclonal antibodies in indirect enzyme-linked immunosorbent assay and indirect immunofluorescence assay (Wallenhammar et al. 2012). *Rhizoctonia solani* are identified by using IgM monoclonal antibody in an LFD-based assay (Thornton 2008). One advantage of using the serological methods of phytopathogen identification is that through these methods we can diagnose them even at a very low detection limit (Lopez et al. 2003).

4.3.5 Identification Through Molecular Methods

Use of molecular techniques in the diagnosis of soil-borne phytopathogens has increased over the years. These techniques offer us an option of determining the phytopathogen at the species level with the help of a specific primer with a high level of sensitivity and precision (Zveibil and Freeman 2009). Polymerase chain reaction (PCR) is an economical and powerful tool that amplifies small segments of DNA or

RNA for identification. The working principle behind this technique is the hybridization of nucleic acid with complementary bases in recurrent cycles. The identification of phytopathogens through conventional approaches is a time-consuming process and requires proficient knowledge of physiology and taxonomy of the phytopathogen (Leslie et al. 2006; Thokala et al. 2015). In this method of identification, the conserved genomic regions (generally ITS in the case of fungi and 16S rRNA gene in the case of bacteria) of phytopathogens are amplified and sequenced. After sequencing, the obtained nucleotide sequence is aligned to the database sequences (Extaxon or Blast), thereby giving the name of identical organisms (Chittem et al. 2015). The process of identification through molecular methods is described in Fig. 4.2. The advent of real-time PCR has now provided the option of detection and quantification of soil-borne phytopathogens on a real-time basis. Various soil-borne phytopathogenic fungi such as *Colletotrichum michiganensis*, *Rhizoctonia solanacearum*, *Fusarium oxysporum*, *Alternaria* spp., *Phytopthora* spp., etc., are now commonly diagnosed by the scientific community using the PCR method. It can also be used additionally to study the genetic diversity within the species of soil-borne phytopathogenic fungi (Steimel et al. 2004; Reznikov et al. 2018). Rapid development in PCR has now enabled on-site detection of soil-borne phytopathogens (Ghosh et al. 2019). High-throughput sequencing methods are now additionally exploited with PCR for the diagnosis of soil health (Yuan et al. 2020).

4.3.6 Identification Through Analysis of Edaphic and Plant Factors

Different edaphic factors consisting of soil pH, temperature, nutrition, moisture, etc., are universally recognized as crucial factors in the development and spread of soil-borne diseases. These edaphic factors often affect soil micro-flora and fauna by regulating their production and diversity (Rajakaruna et al. 2008). When it comes to soil-borne phytopathogens, physical, chemical, and biological soil properties play a far more important role in defining their population and diversity (Nielsen et al. 2010). Thus, sampling of soil followed by phytopathogen-specific testing gives us an initial idea for moving forward with the diagnostics (Clarkson et al. 2015). There are different proposed soil sampling methods for the detection of different phytopathogens (Wallenhammar et al. 2012; Clarkson et al. 2015). Determination of soil pH also gives us an idea of the putative phytopathogens that can be present (Ghosh et al. 2019) as alkalinity and acidity of soil significantly influence diseases like clubroot of crucifers caused by *Plasmodiophora brassicaea* and common scab of potato caused by *Streptomyces scabies*. Similarly, the level of nutrients also gives an idea of potential disease as, for example, higher levels of potassium in soils lessen the chances of occurrence of *Fusarium* spp. (Panth et al. 2020). Soil temperature is a key regulator in disease development; thus, by calculating it, the disease can be predicted (Onwuka and Mang 2018). Genetic background of the cultivar infected

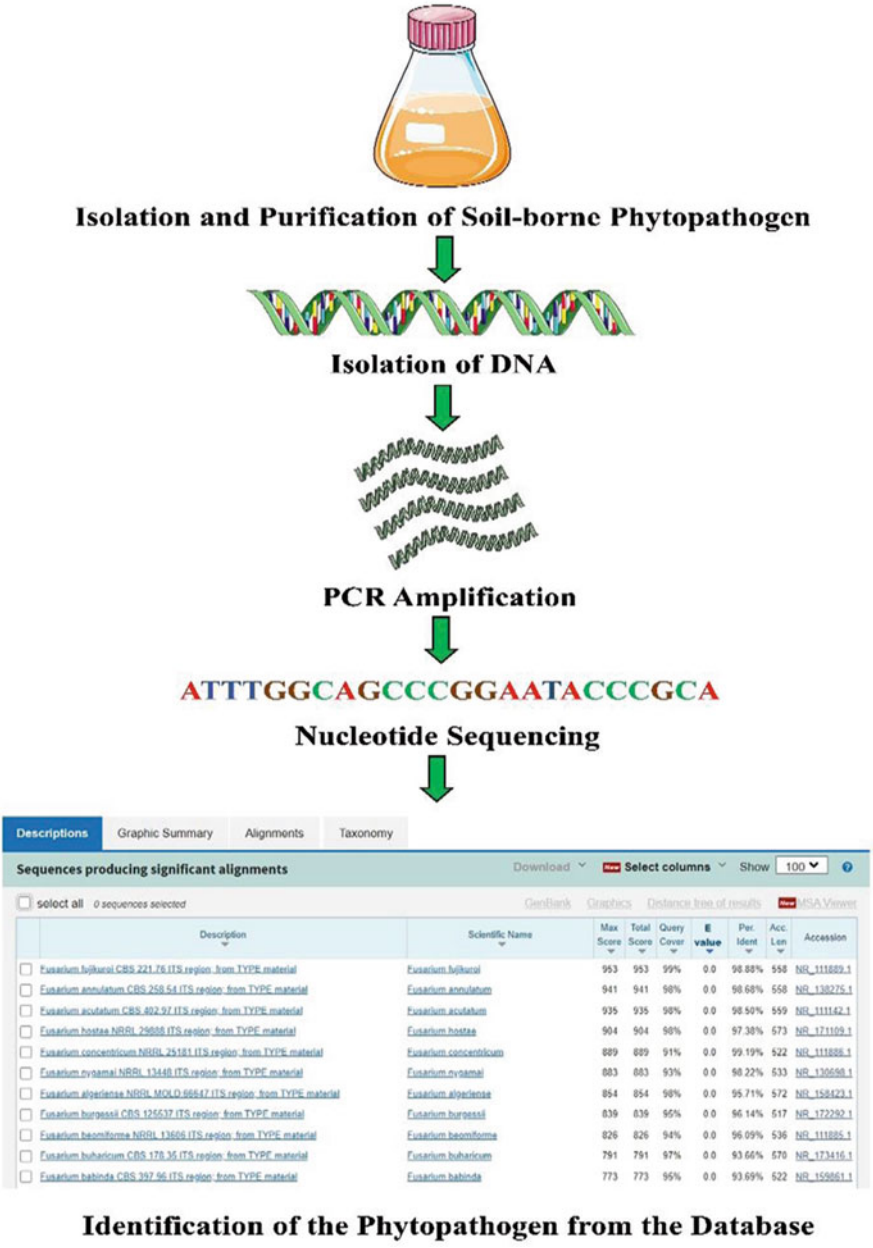

Fig. 4.2 Process of identification through molecular methods

also provides us with an initial idea of probable soil-borne phytopathogens that can incite disease in them (Riley et al. 2002). Analysis for the presence and absence of resting and reproductive structures of phytopathogens in the debris of previous crops also serves as a tool for disease diagnosis (Panth et al. 2020). Even the presence or absence of volunteer plants or weeds or alternate/collateral hosts serves as a general way for the detection of soil-borne phytopathogens.

4.3.7 Identification Through Environmental Factors and Prediction Model

For proper detection of disease, it is vital to know the activities that have been performed in and around the infected plant and field. This information contributes to the microenvironment regulation, which is a very important piece in solving the puzzle of disease diagnostics. Each phytopathogen is favored by different sets of environmental conditions, and thus, knowledge about the same completes the disease triangle concept, ultimately providing a way to proceed further for diagnostics of soil-borne diseases. For example, the probability of infection by *Aphanomyces euteiches* in pea growing in moist soil conditions is very high; thus, we can proceed with the idea that the phytopathogen can potentially be *Aphanomyces euteiches* after analyzing the initial symptoms (Clarkson et al. 2015). Prediction models are also a new upcoming tool that is used for the diagnosis of diseases. Environmental factors are fed into machine learning methods and are employed in the detection of soil-borne diseases prior to their onset in prediction models. In this method, various parameters comprising symptoms, morphological parameters, physiological parameters, etc., from the previously diagnosed soil-borne disease are computationally analyzed and stored in the machine database. When a similar set of parameters are observed in plants, the model can successfully predict the disease incidence. Different prediction models have been developed and are already in use for the detection and diagnosis of soybean charcoal root rot (Khalili et al. 2020).

4.4 Future Aspects and Conclusion

For proper management of soil-borne diseases, it is inevitable to properly detect and diagnose the disease-causing phytopathogen. At present, the techniques utilized for the same are faster, sensitive, and reproducible than the conventional ones. However, all the current techniques have their lacunas and are not ready for implementation in field conditions. These techniques rely on heavy and sophisticated instruments, which are unaffordable at the individual level. Moreover, all the current techniques have a much more complicated process than the conventional ones making them

difficult for a person to use without prior scientific know-how. Thus, in the future, the researcher and technology developers have to work together to modify the current techniques to improve their applicability in field conditions and should be simple for the use of ordinary personals without having scientific knowledge. A rapid, mobile, and accurate method or device or tool for diagnosis of soil-borne diseases is essential for monitoring their development and progression to apply management practices timely. This would reduce the chances of heavy crop losses due to those diseases and also reduce the probability of the development of resistance in soil-borne phytopathogens through the judicious application of pesticides.

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Chapter 5
Omics Approaches to Revisit Rhizobacterial Biome

Mala Trivedi and Parul Johri

Abstract The new mantra "omics" of molecular research describes something big and refers to a field of study in life sciences that focuses on large-scale data (information) to understand life summed up in "omes" and "omics." The integrated omics approach is a combination of genomics, transcriptomics, proteomics, metabonomic, phenomics, and ionomics. These technologies are high-throughput technologies that aim for the nontargeted identification of all gene products present in a specific biological sample. In the present chapter, we will focus on the workflow of metagenomics and various computational approaches to study them. This chapter also gives an insight into pangenome analysis and their level and formats with a special focus on plant growth-promoting rhizobacteria (PGPRs). Additionally, genome-wide association studies (GWAS) of both plants and microbes have made the selection of looked-for traits in plants and management of the genomes of individual plants and microbes easy and less time-consuming.

Keywords Plant–microbe interaction · GWAS · PGPR · Omics · RhizoBase

5.1 Introduction

In the last ten years, the world has invested well over a trillion dollars in pursuit of deeper insights into disease and better therapies. Scientists all over the world believe that many answers they were seeking can be found in DNA and the human data that

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have now been collected around the world. The challenge is working effectively with the oceans of data needed to paint a clear picture of how disease happens and how we can stop it. Life is encoded in the genetic code, and how the genetic code is translated into the living and breathing molecules and the body and overall, in the lifetime, how these molecules interact with each other will determine a health and disease spectrum.

We are healthy most of the time, but we get sick and sometimes we get really very sick and the consequences can be quite serious. There are several problems with our health system. First, we actually treat people after they get sick. We actually don't do too much to keep them healthy and understand what's going on while they are healthy. And the other is that we are in a one size fits all; we basically give people the same treatment even though we are all individuals and even though the way we respond will be very different (Baltimore 2001). So, what we need to do is to switch from this one size fits all to something that's individualized, where every person is treated according to their genetics, all their life history, and their environmental exposures. There is an amazing revolution going on right now. Omics is really seeing the entire collection of molecules that make up an individual. Basically, it's the DNA, the protein, and metabolites. The sequence of the human genome was a major step in making all this happen. For the first time in 2001, people were able to decode the DNA of a human being and basically set up a reference onto which we could actually do a lot of mapping. We could now sequence the DNA and understand the genetic basis of any disease at a level that's never been possible. Mass spectroscopy lets us follow tens of thousands of molecules, and because of this, we are getting an IMAX experience, like watching a movie of what's going on when people are healthy and when they are falling sick.

5.2 Computational Strategies behind "Omics" Approaches

Microbes were the first life form on our Earth. About 4600 million years ago, the Earth declared its independence, and about 3400 million years ago, the first photosynthetic bacteria (that can convert light energy to create biomass) emerged (Table 5.1).

5.2.1 Genomics, Proteomics, and Metabolomics

All the three branches—genomics, proteomics, and metabolomics—are the study disciplines of biology and, more specifically, genetics. Genotype is the genetic material or the inner side, and the phenotype is visible, which we can observe from the outside. So, whatever is there on our gene (inside) is controlling what would be reflected outside. If we traverse from genotype to phenotype, there are various molecules that we encounter like DNA, RNA, protein, and metabolite (Brown et al. 2009). The various studies on these molecules constitute the world of "omics" science (Table 5.2).

Table 5.1 Timeline of the events on the planet Earth

S. No.	MYA scale (million years ago)	Event happened
1.	4600 MYA	Earth declaring its independence
2.	3400 MYA	First photosynthetic bacteria
3.	2700 MYA	First oxygen producers
4.	2300 MYA	The atmosphere has O_2
5.	500 MYA	First terrestrial plant
6.	200 MYA	First mammal emerged
7.	13 MYA	Bats able to fly
8.	10 MYA	The branch of life that is currently represented by humans emerged
9.	0.0004 MYA	Humans made the first observation of microbes through simple microscopes

Table 5.2 Various branches of "omics" and their equipment and techniques

S. No.	X—ome	Equipment	High-throughput data
1.	Genome	DNA sequencer	DNA sequence data
2.	Transcriptome	Microarray	mRNA profile
3.	Proteome	2D Gel, MS-MS	Protein profile
4.	Metabolome	GC-MS, NMR	Metabolite profile
5.	Fluxome	Flux and isotopomer balance	Flux profile

1. **Genomics**: Genomics is the study of genes and the genetic makeup, the chromosomes, and the DNA on it. So, the study of the DNA complement of a cell forms the branch of genomics. In simple words, genomics is the discipline in genetics that applies recombinant DNA technology or DNA sequencing methods as well as bioinformatics techniques to sequence and analyze the structure and function of the genome, that is, the complete set of DNA within a single cell or an organism.

   Rhizobium species have been studied widely in the field of genomics. One of the examples shows the evaluation of the sequences of the complete genome for nine species of *Rhizobium* using in silico methods at the molecular level. In this study, the techniques used were restriction digest, AFLP PCR (amplified fragment length polymorphism polymerase chain reaction), and AMPylating enzymes. Also, alignment and visualization of the gene sequences were done with progressive Mauve and construction of the phylogenetic tree, respectively.
2. **Transcriptomics**: The study of RNA in a cell in a specific condition falls under the header of transcriptomics, that is, the study of the transcript that is the complete set of RNA produced by the genome of a cell under a specific circumstance. We use high-throughput methods like microarray technology to study the function and expression of these transcripts. Transcriptomics field has explored the *Rhizobium* species in detail to study various functions of proteins involved in different biological pathways. One of the studies through the in silico transcriptome profiling technique reveals that the PssZ gene present in *Rhizobium*

leguminosarum bv. *Trifolii* species affects the expression of a set of genes and has established its significant role in the regulatory network of *Rhizobium*.

3. **Proteomics**: The study of protein structure, functions, interactions, and expression in a system (cell) is coined proteomics, that is, the study of protein complements of a cell at a specific time (protein content changes from time to time as per the requirement of the cell). In the field of proteomics, a lot of in silico scientific research has been done on different *Rhizobium* species. One of them is in silico designing of the three-dimensional (3D) structure of an important protein—DehrP present in *Rhizobium* species—RC1 through Homology Modelling. This was accomplished with the use of various in silico tools such as Phyre, 3D refine, RAMPAGE, etc.
4. **Metabonomics**: The study of the various cycles running in our body like glycolysis, Krebs cycle, electron transport chain, and their interaction with each other in terms of requirement and generation of primary and secondary metabolites comes under the canopy of metabonomics. It is the scientific discipline of studying the chemical process involved in metabolites inside a cell. Metabolomics means the systemic study of unique chemical fingerprints that specific cellular process leaves behind during different metabolic reactions. Each cell comprises various metabolites (chemical compounds), which are generated during the different cellular processes of metabolism (both anabolism and catabolism) (Debnath et al. 2010). All the metabolites (primary or secondary) coming from a metabolic pathway, which are found inside a particular cell at a particular time, are called metabolome, and the study of all the metabolome inside a cell is known as metabolomics. As we come from DNA to protein, the information content as well as the level of variation is increasing.

   Sinorhizobium meliloti, a *Rhizobium* species, is very well known for its ability of establishing symbiotic nitrogen fixation (SNF) with various leguminous plants. With the help of an in silico reconstruction process, a metabolic network has been created, which gives the overall information about the key metabolic properties of SNF process in the *Rhizobium* species.
5. **Flux Analysis**

   It involves a steady-state mass balance. A steady state means that there is no accumulation, substitute is coming out, there is some conversion, and in a steady state some substances are going out. Flux analysis can be used to determine the ability of the metabolic networks to produce significant outcomes such as chemicals or responding to any environmental change and many more. One of the in silico studies involving a *Rhizobium* species created a modeling system/framework through which the interlinking of the metabolic processes of plants and nodules can be analyzed in depth. Flux balance analytics was used to exhibit that the pathway/network has the ability to produce biomass constituents in controlled sizes during different times of the day.

5.2.2 *Metagenomics*

Metagenomics, also known as environmental and community genomics, is the genomic analysis of microbes by direct extraction and cloning of DNA from a microbial community. Metagenomics provides a culture-independent insight into microbial communities. It has emerged as a powerful centerpiece since most microorganisms cannot grow in a pure culture and that culturing cannot capture the full spectrum of the microbial community. The entire community of taxonomic profiles can provide a massive amount of information on genome assembly, gene prediction, and species diversity. Comparative metagenomics can provide additional insight into the function of complex microbial community and their role in host health; therefore, metagenomics has the potential role in attaining knowledge and serves practical knowledge in a wide spectrum of fields such as infectious medicine, engineering, agriculture, sustainability, and ecology. Metagenomics approaches are roughly classified into two groups:

- Whole metagenomics.
- Targeted metagenomics.

Whole metagenomic analysis reveals that the microbial communities are well adapted to the different ecosystems. They can provide evidence for a positive selection of enzymes for key ecological processes under ecological pressures due to insufficient sequence data for the targeted enzyme group. Targeted metagenomics is a suitable tool for constructing gene collection of a specific group of enzymes that are used to study adaptive evolution. Whole Genome Shotgun Sequencing (WGS) has been recognized as the most powerful, comprehensive, and robust approach for metagenomics. It offers the advantage of identification of specific level taxonomy and the estimation of metabolic pathway activities from human and environment samples. Several large-scale metagenomics projects have been recently conducted or are under way utilizing WFS (Kell 2007). With the generation of a vast amount of data, bioinformatics and computational analysis of the WGS results become vital for the success of metagenomics study. However, WGS has several limitations:

1. Each step in the metagenomics data analysis, genome assembly, gene prediction, taxonomy annotation, functional annotation, and WGS data analysis is complicated by the sheer amount of data. But algorithms and tools have been developed specifically to handle WGS-generated metagenomics data with the hope of reducing the requirement of computational storage and time.
2. One of the biggest considerations for library preparation of environmental samples for shotgun metagenomic sequencing has to do with amplifications. Certain types of samples, such as water and swats, yield a small amount of DNA, necessitating sample amplification during the library preparation step. Amplification by PCR can overamplify certain fragments over others, cofounding abundance and microbial diversity measurements. If you are able to extract a good amount of DNA (more than 250–500 nanograms), an amplification-free base library preparation method is recommended. Targeted metagenomics studies

involve the extraction of DNA samples from environmental samples, cloning the DNA into a suitable vector, transforming the clones into the host bacterium, and screening the resulting transformants. The clones can be screened for phylogenetic markers such as 16 S rRNA or for other conserved genes by multiplex PCR or for expression of specific traits such as enzyme activity or antibiotic production. A high number of biocatalysts have been identified by function-based or sequence-based screening of metagenomic libraries derived from various environments. The sequence-based approach has been used extensively to retrieve a specific gene from a pool of DNA by PCR or hybridization. Instead of cloning all the extracted DNA, primers are designed specifically to identify the target gene (Westerhoff and Palsson 2004). The advantage of using sequence-driven screening is that it uses well-established and high-throughput techniques such as PCR and hybridization for different targets; for example, the 16s ribosomal RNA gene is a taxonomic genomic marker that is almost common to all bacteria and archaea. The microbial world was revolutionized by the analysis of 16S rRNA genes. Nevertheless, when it comes to fungi, it's an altogether different scenario. Instead of 16S, its 18S rRNA is commonly used for phylogenetics since it has more hypervariable domains than 16S. In addition to this, the internal transcribe spacer regions (ITS) removed in the posttranscriptional process have been widely regarded as the fungi biomolecular marker for the successful identification of the wide range of fungi, and compared to 18S, ITS is more variable and hence more suitable as a genetic marker for measuring intergenetic diversity (Evans and Relling 2004).

Functional metagenomics studies the function of encoded proteins. Functional metagenomics involves isolating DNA from the microbial communities, cloning the DNA fragments, expressing genes in the host, screening for enzymatic activities, sequencing, and functional analysis. When it comes to a construction of a metagenomic library, cosmids- or phagemids-based libraries are always preferred due to their large and consistent insert size and high cloning efficiency.

DNA is first extracted from the environment sample, then size selected and repaired, and ligated to a cos-based vector, allowing packing by lambda phage for subsequent transduction. The resulting library contains a relatively large size of the insert DNA (25–40 kB). Functional metagenomics has identified novel antibiotic resistance genes.

5.2.2.1 Metagenomics Analysis Methods

The reads could be analyzed in multiple ways. They can be used for classification, and they could be used for assembly into contigs and scaffolds where they can go to the binning or annotation analysis, where annotation could be functional annotation or taxonomic annotation. Classification methods can be generally grouped into four groups:

(a) Sequence similarity-based methods.
(b) Sequence composition-based methods.
(c) Marker-based methods.
(d) Hybrid methods (combination of sequence similarity-based and sequence composition-based methods).

5.2.2.1.1 Sequence Similarity-Based Methods

Sequence similarity-based methods use homology-based search against the database of reference organism. It's a good method and widely used; however, it has its own disadvantages, such as one cannot identify organisms that are not present in the reference database. So, one has to be really careful about what one has in the reference database. The reference database should be bigger and should have more variety so as to have better rates to assign weights more correctly (Dunn et al. 2005).

5.2.2.1.2 Sequence Composition-Based Methods

Sequence composition-based methods are based on the nucleotide compositions (for example, the GC percentage, the codon usage, and many more). They find the best fitting model for each sequence read. However, these methods have a very major drawback that they can't be used for short reads (<1000 bps).

5.2.2.1.3 Hybrid Methods

Sequence similarity and sequence composition methods can be combined to make the hybrid methods. It contains the advantages of both the methods and is widely used for all the reads.

5.2.2.1.4 Marker-Based Methods

Marker-based methods compare each metagenomic read to the curated collection of marker genes to identify high-confidence matches. The disadvantage of this method is that it achieves a low level of sensitivity if the reads don't come from genomes represented by the marker gene database. Additionally, marker genes could be used for functional analysis or aligning reads against different databases based on their, for example, antibiotic-resistant genes or virulence factors or transposons or enzymes, which are involved in various metabolic pathways.

5.2.2.1.5 Commonly Used Sequence Search Algorithms

There are five commonly used sequence search algorithms:

1. BLAST and variations of BLAST such as nucleotide BLAST, protein BLAST, MEGA BLAST, etc. BLAST finds sequence similarities between biological sequences.
2. Hidden Markov Models (HMMER) use protein sequences or amino acid sequences and search sequence profile (model) databases for sequence homologs.
3. Bowtie and Bowtie 2 read alignment to long reference sequences.
4. Burrows–Wheeler Aligner (BWA) aligns nucleotide sequences or maps of low divergent sequences against a large reference genome.
5. K-mers method searches against a database of substrings of length k that are contained in a string (Table 5.3).

5.2.2.2 Bioinformatics Analysis of Metagenomics Data

Metagenomics involves a collection of samples from environments such as clinical samples from humans, pigs, beef, and other animals and fresh water and sewage samples. Collected samples undergo their purification and extraction of DNA, and the latter are being sent for sequencing. Sequence reads that come out of the sequencing machines undergo quality assessment steps. Fastqc is a commonly used quality control tool. It helps to find out if the sequencing machine has left some adapters; one can also look at the GC content, the k mers composition of your reads. We can also trim the low-quality bases from the end and can also fix the cutoff score based on the strictness. Additionally, as mentioned, one can also look at the left-out adapters, primers in the sequence. Some of the read trimmer and adapter

Table 5.3 Some Commonly used tools and their functional classifications

S. No.	Name of the methods	Class of the method	Sequence search method	Composition	Functional classification
1.	RITA	Hybrid	Pipeline of BLAST	NB	N/A
2.	MEGAN4	Similarity	BLAST program	NA	KEGG, SEED
3.	CARMA3	Similarity	BLAST program	NA	Pfam, COG, GO
4.	TACOA	Compositions	NA	k-NN	NA
5.	MetaPhlAn	Marker	Mega BLAST, Bowtie2	NA	NA
6.	phymmBL	Hybrid	Mega BLAST	IMM	NA
7.	MGRAST	Similarity	BLASTN, BLAT	NA	SEED, NOG, KEGG, COG

removal tools are Cutadapt, Trimmomatic, PRINSEQ, and BBDuk. Using these tools, we are left with the sequence having high-scoring reads.

5.2.2.3 Metagenomics Workflow

After the quality assessment steps, reads can be used for taxonomic identification to see who is out there for quantitative analysis and how many are there for quantitative analysis and for functional analysis (Fig. 5.1).

5.3 Plant Growth-Promoting Rhizobacteria (PGPR)

The rhizosphere is the region of soil found around the roots of the plants. This region has a very high microbial load and is influenced by the exudates of the roots. The organisms that are present in the rhizosphere may include bacteria, fungi, and some viruses, the root exudates have an effect on the rhizosphere flora, and the rhizosphere flora in turn affects the plant with which they are associated. The plant growth-promoting rhizobacteria (PGPRs) are bacteria that colonize the rhizosphere and improve the growth of plants directly or they act as a biocontrol agent against minor pathogens like DRMO (deleterious rhizosphere microorganisms) like DRB (deleterious rhizosphere bacteria) and DRF (deleterious rhizosphere fungi) (Theodorescu and Mischak 2007). These DRMOs are minor pathogens that can be controlled and can be inhibited by these PGPRs by either secretion of some

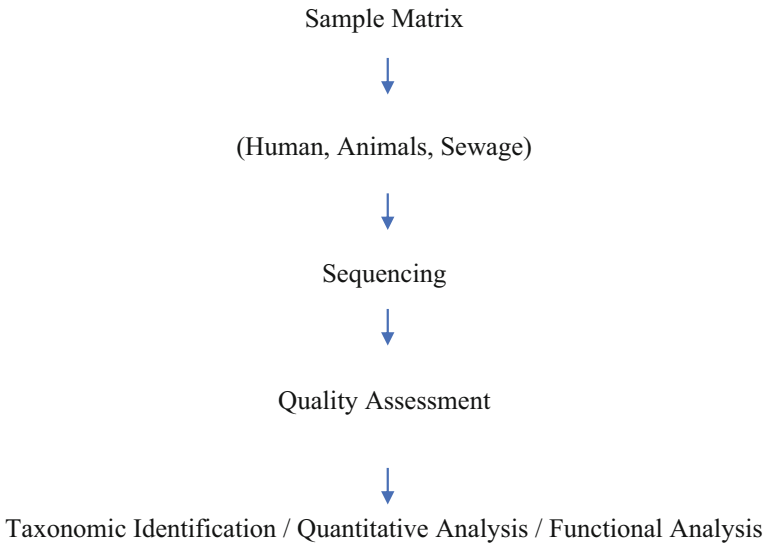

Fig. 5.1 Flow chart for metagenomics workflow

antibiotics or certain chemicals or by aggressive colonization of the roots, which prevents these deleterious organisms from coming near the roots. The primary examples of PGPRs are *Pseudomonas fluorescens* and *P. putida*, *Acinoplanes*, *Agrobacterium* species, *Cellulomonas*, many species of *Bacillus*, *Azotobacter*, *Erwinia*, *Serratia*, etc. (Vlahou and Fountoulakis 2005).

The survival of PGPRs is influenced by certain factors like the soil conditions—soil temperature, soil pH, amount of humidity in the soil, composition of soil particles like loamy soil, and clay soil. There are various mechanisms by which these PGPRs are improving the growth of plants. The first is that they provide a competition for substrate and niche exclusion (for example, growth of lateral roots, increased nutrient absorption, and space). PGPRs are competing with the other deleterious organisms and inhibiting the other organisms from growing. Many of these PGPRs produce plant growth-stimulating hormones like gibberellic acid and indole acetic acid (IAA), so they produce hormones that directly increase the growth of the plants. Third, they show increased nutrient utilization in the soil, which means that the PGPRs are able to show nitrogen fixation and solubilization of the inorganic phosphates in the soil. This way, whatever they are fixing or solubilizing is being provided to the plant, the plant is able to uptake better nutrients or effective utilization of the nutrients and it grows better. The fourth mechanism by which the PGPRs can work is by the production of antibiotics; for example, *Agrobacterium radiobacter* produces Agrocin 84, which is a compound that inhibits the crown gall caused by *A. tumefaciens*. Some of the rhizobacteria produce antifungal metabolites like phenazine, phloroglucinols, or pyoluteorin, which help in protecting the plants from these fungi. Lastly, PGPRs can also produce siderophores. Siderophores are low-molecular-weight, high-affinity iron chelators (absorb all the iron in the vicinity and ensures that it is being released slowly to the plant).

It acts as a sequester for the limited supply of iron in the rhizosphere, thereby reducing the availability of trace elements to the pathogens. Some examples of siderophores are yellow green florescent Pseudobactin by *P. fluorescens* against *Erwinia carotovora*. *Pseudomonas* has been known to increase crop yields by the production of siderophores. Hydrocyanic acid (HCN) produced by harmful microorganisms or deleterious rhizosphere microorganisms reduce the yield of the plants. If PGPRs are present in that particular soil, they reduce the HCN production by producing siderophores, which will in turn compete for the ions and keep it with themselves. So, ions are not available in the soil, which is very important for the HCN production; hence, there is no HCN production and hence the plant is not affected. This is how PGPRs work. So, every plant growth-promoting rhizobacteria could have one or more mechanisms by which it is promoting plant growth (Khandagale 2020).

5.4 Pangenome

A pangenome represents the full complement of the diversity within a clade, or the union of all the genes or SNPs across a representative selection of genomes. The reason why people want to make a pangenome is that a single reference genome cannot represent the diversity within a single species. Also, advances in sequencing technology and lowered costs have made pangenome a feasible goal for many genome research groups (Kenny et al. 2008). The first pangenome of microbe made for *Streptococcus agalactia* was released in 2005 and was composed of eight genomes (about 1.5 Mb in size). The whole genome alignment was done using the MUMmer alignment program, and the gene sequence similarity was determined by translated protein sequence similarity (Fig. 5.2) (Malla et al. 2018).

For creating a pangenome usually a whole genome alignment is used (MUMmer, Blast), or alignment of short reads to a reference sequence (Fig. 5.3). There are three main features of a pangenome:

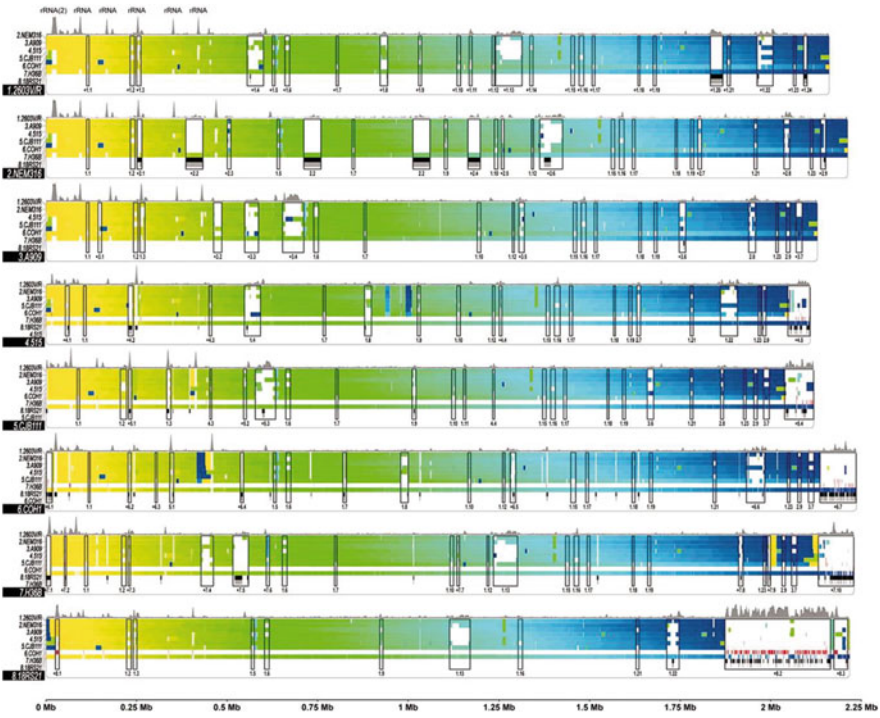

Fig. 5.2 Pangenome of *Streptococcus agalactia* (eight panels represent one of the eight genomes used as a reference for the comparison to the others, and the colors represent the coordinates from the beginning to the end of the chromosomes—yellow is the beginning and blue is the end of chromosomes)

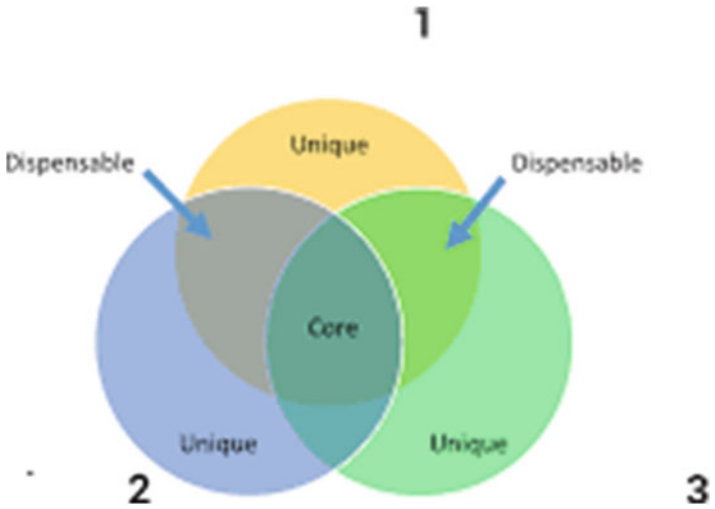

Fig. 5.3 Construction of a pangenome

(a) Core Genome: It is made up of genes present in all accessions in pangenome.
(b) Dispensable Genome: It refers to the genes that are present in some but not all accessions in the pangenome.
(c) Orphan Gene: These are linkage-specific genes, found only within a particular accession.

5.4.1 Types of Pangenome

There are basically two types of pangenome:

1. Reference-Based Pangenome: These pangenomes have all accessions mapped to a single reference. These types of pangenomes are really fast and easy and can be represented as a fasta file (consensus sequence). Their main drawback is that they do not represent genes that are present in other accessions, but are present in the reference sequence.
2. All Against All Pangenome: The pangenome includes the total diversity of all accession studied. So, it contains the total diversity of all the accessions, but on the other hand, they can't be represented as a fasta file, are very slow to compute, and need a lot of space in CPU.

5.4.2 *Pangenome Formats*

There are various types of pangenome formats:

1. Consensus Fasta File: In this type of pangenome format, all the variations across the accessions are collapsed into a consensus sequence. This could be used for SNP diversity or whole genome alignment against a reference sequence. The best thing about this is that it is bastable and alienable, so one can create this pangenome and reuse it for new and incoming pangenomes. The drawback of this is that it is hard to represent large regions of variations and difficult to represent all against all pangenome.
2. Graphical Format: Another way of representing a pangenome is the graphical format. This is where the variations are represented as a graph with nodes and edges; shared sequence similarity is collapsed into a single node. This is a very useful format; it can be used for all types of pangenomes. It can represent large regions of diversity better than a fasta file. But the drawback is that it is not bastable and alienable.

5.4.3 *Levels of Pangenomes*

Among the levels of pangenomes, there are species-specific pangenomes, which are cultivars within the species. These are the most common types of pangenomes that are usually generated. There are genus-specific pangenomes, and these are closely related species within a genus. There can also be family-specific pangenomes, the species within a family. Higher order clade pangenomes are possible, but complexity increases as you go up in a clade since the number of shared genes declines (Romero et al. 2006). There are some challenges in creating a whole genome pangenome for complex genomes such as maize and wheat. Maize and wheat have undergone recent polyploidy events and have large inversions and translocations relative to outgroups and contain a great number of repeat elements. The genomes also contain a large number of duplicated genes in cis and trans and have simultaneously undergone a great deal of gene deletion relative to the outgroup.

5.5 RhizoDB (http://xbase.warwick.ac.uk/rhizodb/)

RhizoDB is a genome resource for the rhizobia research community. Currently, there are about 107 genomes in the database, out of which 61 are complete and 46 are incomplete. This database is maintained by the xbase, which in turn is maintained by the EnteroBase team (http://enterobase.warwick.ac.uk/) (Fig. 5.4). The xbase receives funding from BBSRC (Biotechnology and Biological Science Research

Fig. 5.4 Homepage of xBASE database

Council). It uses a large number of externally developed databases, tools, and services.

Enterobase is a user-friendly, powerful online resource for visualizing and analyzing various genomic variations within enteric bacteria. xBASE is a group of multiple individual databases to store genomic and other biological details of numerous existing bacterial taxa. It has been created along the pattern of coliBASE, which stores data for *Escherichia coli*.

The URL for xBASE is http://xbase.warwick.ac.uk/ (Fig. 5.4).

The databases that have been included under xBASE are as follows:

(a) Campy DB for *Campylobacter*, *Helicobacter*, and *Wolinella.*
(b) PseudoDB for pseudomonads.
(c) ClostriDB for clostridia.
(d) RhizoDB for *Rhizobium* and *Sinorhizobium.*
(e) MycoDB for *Mycobacterium* and *Streptomyces* and its related organisms.

xBASE is a user-friendly web-based graphical user interface, which provides easy access for the comparison of annotations and genomes stored. The features that have been newly added when compared to coliBASE are the following:

(a) Displaying the whole genome.
(b) Painting of genes based on various properties like the content of GC.
(c) An improvised system of pattern search for identification of conserved motifs.
(d) Batch BLAST search of proteins region-wise.

Below are the snapshots of how the RhizoDB works and what information it contains.

(a) Fig. 5.5 is the homepage of RhizoDB. The specific species can be searched through the search box.
(b) Figure 5.5 depicts the search, which gives the overall information on the Rhizobium species in the subject.

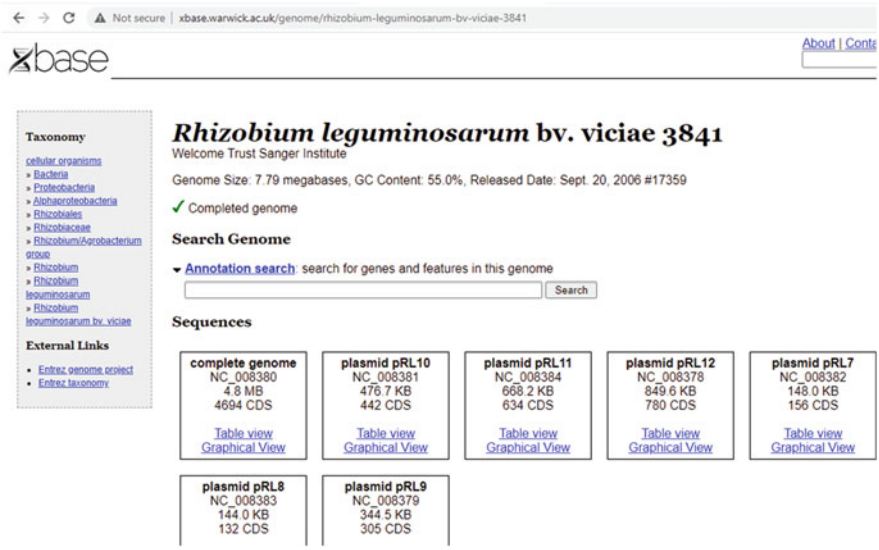

Fig. 5.5 Information about *Rhizobium* species

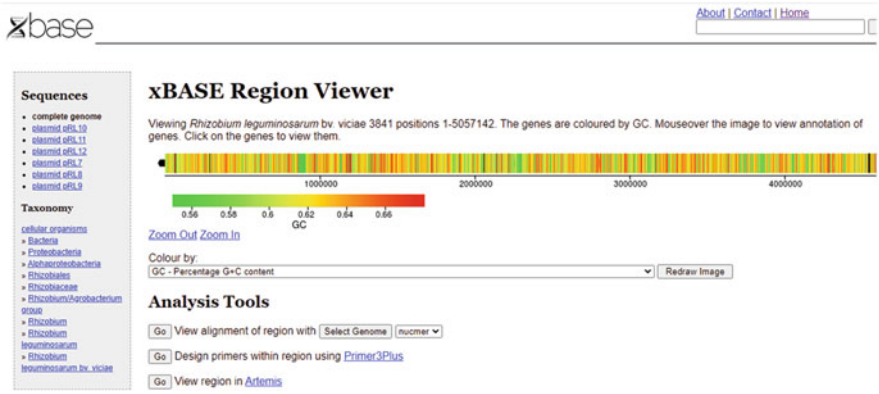

Fig. 5.6 Graphical representation of genome information

(c) Each group of data available, as shown in Fig. 5.5, for example, complete genome, plasmid pRL10, etc., can be viewed in a tabular as well as in a graphical view. Figures 5.6 and 5.7 show the graphical and tabular views, respectively.

The database results upon search can be drilled down in different ways as per requirement in order to view/extract more information available in the database.


Not secure | xbase.warwick.ac.uk/genome/rhizobium-leguminosarum-bv-viciae-3841/NC_008380/features

xbase

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Search

Rhizobium leguminosarum bv. viciae 3841, complete genome. features

Type	Gene	Protein id	Locus	Product	Start pos	End pos	Strand
CDS	hemE	YP_765604.1	RL4742	uroporphyrinogen decarboxylase	1	5057142	-1
CDS	-	YP_765605.1	RL0001	hypothetical protein	474	1295	1
CDS	-	YP_765606.1	RL0002	Maf-like protein	1329	1928	1
CDS	aroE	YP_765607.1	RL0003	shikimate 5-dehydrogenase	1921	2778	1
CDS	coaE	YP_765608.1	RL0004	dephospho-CoA kinase	2778	3389	1
CDS	-	YP_765609.1	RL0005	DNA polymerase III subunit epsilon	3382	4104	1
CDS	secB	YP_765610.1	RL0006	preprotein translocase subunit SecB	4172	4654	-1
CDS	fixA	YP_765611.1	RL0007	FixA	4756	5271	-1
CDS	-	YP_765612.1	RL0008	hypothetical protein	5394	6098	1
CDS	mltA	YP_765613.1	RL0009	putative membrane-bound lytic murein transglycosylase a precursor	6091	7209	1

Fig. 5.7 Tabular representation of the genome information

5.6 Genome-Wide Association Studies (GWAS) for Understanding Plant–Microbe Interactions

Genome-wide association studies (GWAS) is an efficient in silico tool used for the detection of genomic regions present in wild and cultivated plants. These genomic regions associated with variations occur naturally in disease resistance. This tool uses the preexisting data of recombination events occurring in populations naturally and permits the study of relationships between traits in a population that are genetically diverse. The GWAS is used in detecting the presence of single nucleotide polymorphisms (SNPs), which are further studied for establishing relationships with their significant phenotypes. It has also transformed the process of identification of genes that cause a particular disease and result in characterizing the traits, which are quite complex in nature.

The SNPs are assessed statistically for the identification of relationships to a well-stated phenotype. The GWAS follow a case–control design pattern in which SNPs are compared in terms of their frequency and distribution between subjects/individuals with and without a particular trait/disease.

Various GWAS outcomes in combination with multiple other techniques can be utilized for the improvisation of the ability for the detection of the presence of SNPs having limited effect on the phenotypes. One of the most positive outcomes of GWAS is the identification of numerous associations between the changes of DNA base combinations and the characteristics they impact, but on the contrary GWAS still needs to improve the process of predicting disease traits that are common in individuals.

The following are a few examples that demonstrate that GWAS has proved beneficial in the identification and management of diseases.

(a) Diabetes Mellitus

With the help of GWAS, a considerable number of genes have been identified for Diabetes Mellitus Type 1 disease. A few genes to mention are Arg620Trp, Ala946Thr, Trp262Arg, etc. In this study, numerous loci with associations have also been found through the help of GWAS meta-analysis in huge population data.

(b) Ovarian Function Disorders

The GWAS approach can significantly prove beneficial in one of the most complicated diseases, which is Polycystic Ovary Syndrome (PCOS), and also in premature ovarian failure (POF/I), which has very complex phenotypes. It contributes to the total heritability of characteristics of PCOS and POI/F. The occurrences of SNPs and other genetic variations can be compared in control and diseased individuals from a common set of populations, and as a result unique genetic factors and the biological pathways involved, which lose their functionality during the disease, can be identified. Furthermore, based on the outcomes, GWAS can suggest new therapies or treatment options for the prevention and management of the disease. Though, analyzing the humongous amount of GWAS data for an advance study about ovarian disease remains a challenge for researchers.

Even though with a huge number of advantages, GWAS still has some challenges/limitations, which further have to be addressed. One of the primary issues is that at a genetic level, not all the subjects are equally related distantly, and hence disregarding the correct consideration of population in the scope of study results in the false establishment of an association between the genotypes and characteristics. Also, unaccounted genotypes, heterogeneity at genetic levels, complicated gene design, low frequency of alleles, etc., contribute to the challenges with GWAS.

5.7 Conclusion, Future Prospects, and Challenges

Environmentally friendly sustainable farming benefits immensely from useful microbe–plant interactions. This beneficial interaction has been proved to be significant in the development of bio-remediation, biofertilizers, and biocontrol agents in the field of sustainable agriculture. The main challenge that researchers of this area are facing is the unavailability of information about the mechanisms of underlying gene functions and signal transduction when the plant and microbes interact.

The unavailability of data will majorly contribute to challenges in the near future:

(a) Identification of crucial elements which take part in immune responses of plants.
(b) Detecting and managing newly emerging and re-emerging plant-related pathogens.
(c) Development of pathogen-resistant plants/crops.

These hurdles give rise to the necessity of studies in order to understand the genetics of the microbe–plant interaction, which can be performed through next-generation sequencing in combination with the available “omics” technologies in silico. The detailed study through the combination of various in silico technologies will provide vast, in-depth information about the biological processes, which would thereby enhance the health of the crops/plants and the quality of food, and also help in improvising the management process of plant stress—both biotic and abiotic

types of plant stress. As a solution, the enhancement of sustainable agricultural techniques such as GWAS will help in overcoming the challenges and thus contribute to crop advancement.

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Chapter 6
"The Key Influencers" of Rhizosphere Microbial Population Dynamics

Surinder Paul, Poonam Kumari, Rahul, and Mushineni Ashajyothi

Abstract The plant rhizosphere is the center of immense microbial activity. It harbors a diverse microbial population that plays diverse important functions ranging from nutrient cycling to the protection of the host from different abiotic and biotic stresses. Any change in the composition of the microbial population may severely impact these vital processes and thus impact plant growth and survival and productivity. The factors or "the key influencers" that may have a great impact on the normal rhizosphere microbial population dynamics and thus play important role in shaping the microbial community structure around the rhizosphere need to be studied to understand the complex host–microbe and microbe–microbe interactions taking place in the rhizosphere of the plant, which play an important role in shaping the microbial community structure around the rhizosphere and thus have a strong influence on the microbial population dynamics around rhizosphere. It is very important to explore the various crucial factors, including environment, edaphic, host-specific factors (root secretions or root exudates), etc., and their impact on the population structure of rhizosphere microorganisms. Furthermore, this knowledge may be utilized in designing the "Rhizosphere Engineering" strategies for harnessing the untapped potential of rhizosphere microorganisms in sustainable agriculture.

Keywords Rhizosphere · Abiotic and biotic stresses · Microbial community structure · Rhizosphere engineering · Sustainable agriculture

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6.1 Introduction

The rhizosphere harbors a diverse set of microbial populations estimated to contain up to 10^{11} microbial cells per gram root (Egamberdieva et al. 2008). The collective genome of these plant-associated microorganisms is very important for plant survival, thus described as the plant's second genome (Berendsen et al. 2012). The plant rhizosphere -associated microbial communities also play a crucial role in carbon sequestration, proper functioning of the ecosystem, and regulation of nutrient cycling in natural as well as agricultural and forest ecosystems (Somers et al. 2004). Diversity of microbes inhabiting plant rhizosphere and their complex interactions with the host plant significantly affect plant morphology, physiology, growth, development, and health (Philippot et al. 2013). Each plant has various biochemical processes in the rhizosphere leading to specific microenvironmental conditions in the rhizosphere, which provide dwelling ground for the specific microbial population subset with distinct functional capabilities (Bulgarelli et al. 2012). Any factor causing a change in the microbial community structure, composition, or activities has a strong effect on the normal growth and development of the plant in a particular environment.

Many factors viz. soil physiochemical profile, environmental conditions, type of plant and its growth stages, and secretions by the plant through roots, i.e., root exudates and its composition, play an important role in shaping and determining the community structure and composition in the rhizosphere of the plant (Marschner et al. 2001; Buyer et al. 2002). Thus, in order to understand the composition of microbial community structure in the rhizosphere of a particular plant and complex plant–microbe interactions, it is very important to explore the various environmental and physiological factors that play a crucial role in this complex and dynamic process (Fig. 6.1).

6.2 Role of Edaphic Factors

Soil is the ultimate source of all the nutrients needed for the development of the plant; thus, soil type, its chemical and physical composition, and nutrient profile have huge effect on the plant's physiological processes (Garbeva et al. 2004, 2006). Physical factors viz. soil moisture, temperature, pH, salinity, and organic and inorganic nutrient profile have a strong impact on rhizosphere microflora.

Role of Soil Moisture, Temperature, and pH The moisture content and the temperature of the soil considerably affect the microbial cenosis of the soil and the rhizosphere, since these factors can essentially change the amount and the pattern of nutrients secreted by plant roots (Melent'ev et al. 2000). Availability of water or moisture is a very crucial factor for determining the microbial growth around the rhizosphere. Very low water content in the soil generally reduces the microbial population around the rhizosphere. These conditions favor the dwelling of the

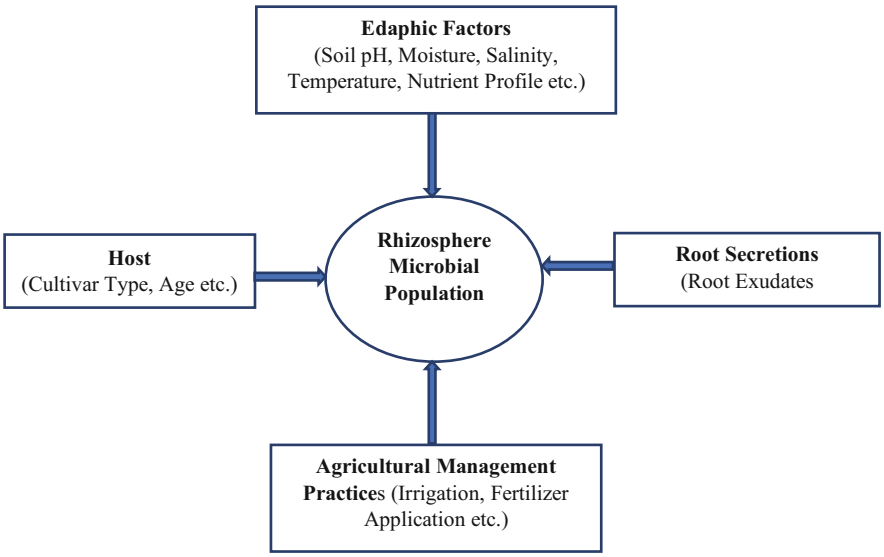

Fig. 6.1 Various factors affecting rhizosphere microbial population structure

microorganisms, which require less water to carry out their important physiological processes. Similarly, excess of water (above optimal level) also has a detrimental impact on the type, growth, and survival of rhizosphere microbes. Excess of water may cause water logging, which may prevent proper aeration and thus may reduce the population of aerobic microorganisms and increase the number of micro-aerobes and anaerobic microbiota.

Soil temperature is also a very important factor, which influences the rhizosphere microbial population dynamics. Rhizosphere microorganisms require a particular range of temperature to thrive. Most of the microorganisms termed mesophiles thrive in moderate temperature conditions (25–37). Some bacteria called thermophiles can tolerate a high-temperature range (45–65). Some rhizosphere microorganisms can thrive at temperature below 20. Thus, soil temperature can be considered an important factor that may influence the microbial population in terms of number and type. Similar to moisture and temperature, pH (the hydrogen ion concentration) is a crucial determinant of microbial population structure and composition. Most of the microorganisms prefer pH ranging from 6.5 to 7.5. pH above or lower than the optimal range may result in drastic changes in the rhizosphere microflora. Salinity is a major factor in controlling microbial abundance, diversity, composition, and functions (Borneman et al. 1996). Soil salinity is also a very important factor that imparts significant effects on the soil microbial structure, diversity, and function (Borneman et al. 1996). Ibekwe et al. (2010) also studied the impact of rhizosphere bacterial diversity in cucumber (*Cucumis sativus*) rhizosphere in response to salinity, soil pH, and boron. Neal et al. (2012) reported that increased boron and salt concentration led

to change in rhizosphere microbial population structure indirectly by altering the composition and quantity of plant exudate secretion.

6.3 Effects of Agricultural Management Practices

Agricultural management practices change the many soil physicochemical parameters and thus has a strong impact on soil microbial composition as well as structure and function (Schmidt et al. 2019). The organic and inorganic fertilizers are important drivers of soil microbial population structure and function (Ling et al. 2016). Cropping intensity or continuous cropping is also known to influence the rhizosphere soil bacterial community structure. It was found that bacterial community structure was altered as a result of monocropping of Tibetan barley. They reported that relative abundances of families *Pseudomonadaceae*, *Cytophagaceae*, and *Nocardioidaceae* significantly increased, whereas *Chitinophagaceae* and *Sphingomonadaceae* decreased significantly. They also reported an increased abundance of bacteria associated with chemoheterotrophy, aromatic compound degradation, and nitrate reduction.

İnceoğlu et al. (2012) proposed and confirmed that soil type has the most significant role in deciding the structural and functional community structure of potato rhizosphere-associated bacteria. They also confirmed that the same potato cultivars grown in two different soils had different rhizosphere inhabiting microbes with different functional capabilities. Breidenbach et al. (2016) also studied the dynamics of rhizosphere microbiota of rice plants and further confirmed that community structure is greatly affected by the soil environment type (i.e., rhizosphere versus bulk soil) than did time (e.g., plant growth stage). Root exudates secreted by plants' roots provide nutrition and attractant for the specific microbial community and thus may be playing a deciding role in the change of microbial population dynamics in the rhizosphere of a plant (Lynch and Whipps 1990).

6.4 Role of the Host Plant in Rhizosphere Microbial Population Structure Composition and Function

Role of Host Plant Researchers have shown that different plant species growing on the same soil type can have different rhizosphere-associated microbial population structures (Berg et al. 2006; Garbeva et al. 2008; Viebahn et al. 2005). Moreover, some plant species can recruit similar microbiota even in different soils (Miethling et al. 2000). Reports also demonstrated that even within species, different genotypes could have distinct rhizosphere microbial communities (Micallef et al. 2009). All these reports suggest that in order to shape the microbial community structure associated with the rhizosphere, the host plant also plays a very crucial role.

Researchers have proved that the rhizosphere-associated microbial population composition also depends on the host plant genotype (cultivar) (Lundberg et al. 2012). This is termed as the "rhizosphere effect," describing that the root-associated microbiota community structure often remarkably varies across host plant species and among different genotypes within a single species (Hentzer and Givskov 2003; Berendsen et al. 2012; Bokulich et al. 2014). Jiang et al. (2017) revealed that blueberry host cultivars exerted substantial effects on the root-associated bacterial diversity along with complex co-occurrence networks and host genotype and, thereby, directly influenced the microbiota profiles.

6.5 Role of Root Exudates

The active root secretions or root exudates comprise a diverse range of low-molecular-weight compounds that enable the host plant to modulate (stimulate or suppress) the growth and colonization of selective members of rhizosphere-associated microbes (Doornbos et al. 2012). The root exudates composed of ions, enzymes, free oxygen and water, mucilage, and a diverse set of primary and secondary metabolites are utilized by the microbes as a carbon source (Bertin et al. 2003; Bais et al. 2006). Furthermore, root exudates can be broadly divided into two classes of compounds. The low-molecular-weight fraction of root exudates is highly diverse and composed of amino acids, organic acids, sugars, phenolics, and other secondary metabolites, whereas mucilage (polysaccharides) and proteins are the main components present in less diverse high-molecular-weight exudate fraction (Bais et al. 2006). Some root exudates also contain chelating agents, which form complexes with metallic micronutrients, including iron, zinc, manganese, and copper, and thus affect the nutrients' availability in rhizosphere soil (Dakora and Phillips 2002).

The amount and compositions of the root exudates are also known to be affected by nutrient availability, soil type, physiology, growth, and developmental stage of the plant (Brimecombe et al. 2001).

The root exudates from the plant in particular conditions can favor the establishment of a distinct rhizosphere microbial community by providing a wide variety of carbon sources (Philippot et al. 2013). Root exudate components such as carbohydrates and amino acids act as a stimulant and help plant growth promoting bacteria (PGPB) colonization through chemotaxis (Somers et al. 2004). Soil microflora establishes various interactions with the host plant through rhizosphere via a well-known mechanism of chemotaxis (Bais et al. 2004). de Weert et al. (2002) reported the chemotactic effect of root exudate components on the flagella-driven motility of bacterium *Pseudomonas fluorescens* and elucidated its role in tomato root colonization. Flagella-driven motility in microbes is considered an important trait, which can significantly affect the population structure of competitive pathogens and beneficial microbes in the plant rhizosphere and thus enables various microbe–microbe and plant–microbe interactions (Lugtenberg et al. 2002). Early host recognition by the

bacteria is also mediated by the bacterial Major Outer Membrane Protein (MOMP). *Azospirillum brasilense* MOMP exhibited stronger adhesion to membrane-immobilized root extracts of cereal as compared to legumes and tomato extracts. This indicated that MOMP may act as an adhesin and plays a role in root adsorption and cell aggregation of the bacterium to further colonize its specific host plant rhizosphere (Burdman et al. 2001).

Root exudates are known to influence and maintain rhizosphere-associated core and cultivar-specific microbiota (Jiang et al. 2017). Secondary metabolites representing a specific subclass of flavonoids are known to play an important role in the very specific plant–microbe interactions between legumes and nitrogen-fixing rhizobacteria. These interactions further enable a specific strain of rhizobacteria to form nodules in its specific leguminous plant host (Peters et al. 1986; Perret et al. 2000). Peters et al. (1986) established that isoflavonoids are specifically produced only by leguminous plants and they are known to regulate the expression of nod genes in specific nitrogen-fixing microbes. Apparently, flavonoids are perceived as aglycones by the rhizobacteria and further they interact with nod D protein (a LysR-type regulator) and alter its conformation to facilitate its binding to nod box elements in the promoters of the nod genes and induce the expression of nod genes to synthesize Nod factor molecules (Perret et al. 2000). Chemically, Nod factors are lipochitooligosaccharides, usually consisting of four or five β-1,4-N-acetylglucosamines, with the terminal nonreducing sugar N-acylated by a 16–18 carbon fatty acid. Nod factors may also contain acetate, sulfate, or carbamoyl groups or different sugars such as arabinose, fructose, and substituted fructose. They also vary in terms of the degree of saturation of the acyl tail (Perret et al. 2000). All these chemical modifications form the basis of host-specific recognition of a specific nod factor in legume. For instance, daidzein and genistein isoflavonoids produced by soybean (*Glycine max*) positively regulate nod gene expressions in *Bradyrhizobium japonicum* but negatively regulate nod gene expression in *Sinorhizobium meliloti*. The nod gene expression in *S. meliloti* has been instead found to be specifically induced by luteolin (Peters et al. 1986).

In plant–mycorrhiza interactions, signaling molecules known as "branch-inducing factor" present in the root exudates of plants critically help the mycorrhizal fungi in hyphal branching, root colonization, and in establishing a symbiotic relationship with the host (Giovannetti et al. 1996; Buee et al. 2000; De Carvalho-Niebel et al. 2002). Akiyama et al. (2005) have isolated a "branch-inducing factor" chemically identified as 5-deoxy-strigol, a strigolactone from the root exudates of *Lotus japonicus*, which at very low concentration induced extensive hyphal branching in germinating spores of the AM fungus *Gigaspora margarita*. Nutrient availability for the plant host is also reported to affect the activity of production and/or exudation of "branch-inducing factor" present in the root exudates of host plant. Nagahashi and Douds (1999) reported that root exudates from the plants growing in phosphate (P)-limited conditions had a high activity of branching factor than the plants growing in phosphate (P)-sufficient conditions.

Secondary metabolites in the plant root secretions also inhibit the growth of particular microbes (Zhang et al. 2011) and thus influence the microbial population

dynamics in the rhizosphere. Bais et al. (2002) reported the secretion of rosmarinic acid in the hairy root cultures of *Ocimum basilicum* and its role in exhibiting specific antimicrobial activities. 2,4-Dihydroxy-7-methoxy-2H-1,4-benzoxazin-3(4H)-one (DIMBOA), a benzoxazinoid present in large quantities in *Zea mays* root exudates, has been reported to exhibit potential antimicrobial activity as well as selective chemotactical attractants for the plant beneficial rhizobacterium *Pseudomonas putida* KT2440 (Neal et al. 2012). Plants' secondary metabolites are also reported to interfere with "quorum sensing" (QS)-regulated responses either positively or negatively by altering the expression of several QS-related genes in bacteria. As QS is very important for cell-to-cell communication and colonization in bacteria, it may influence rhizospheric microflora population structure. Several compounds interfering with bacterial activity have been reported in many important crops, including pea (*Pisum sativum*), rice (*Oryza sativum*), and *Medicago truncatula* (Teplitski et al. 2000; Gao et al. 2003; Ferluga and Venturi 2009).

6.6 Conclusion

It is very clear that the plant–microbe interactions in the rhizosphere are influenced by several factors and our present knowledge is not sufficient to fully understand these complex interactions. As several studies have established that rhizosphere microbiome structure greatly affects plant health, the plant employs several mechanisms to recruit its specific microflora. Recent omics -based studies based on next-generation sequencing techniques are able to unravel the complex mechanisms employed by the plant to recruit its specific microflora and to establish microbial communities in the rhizosphere and its impact on plant health. This knowledge can further be utilized to increase crop quality and productivity in the present changing climate scenario.

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Chapter 7
Engineering the Plant Microbiome for Biotic Stress Tolerance: Biotechnological Advances

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Abstract The transformation of rhizosphere microbiota is essentially the result of a series of events that can enhance the formation of constant and different microbial associations in the plant microbiome/holobiome based on supportive information/communications. Beneficial microbial communities act as influential identities for the elevation of ecological stresses in plants and ultimately decrease the usage of fertilizer and pesticides in order to increase the crop yield. Microbiome has the capability to stimulate the growth of plants, develop resistance to stress, and enhance the health of plants. To accomplish these objectives, it is essential to learn more about the relationship between plant, microbiome, microbial community present in soil, and their resilience to environmental changes. The information acquired will help in understanding the effect of these microorganisms on the biotic resistance, biogeochemical cycles, and productivity of the crops. A comprehensive understanding of the biological mechanisms underlying stress-induced microbiome modifications would also allow for the development of personalized DefenseBiomes and chemicals in order to combat with crop stresses.

Keywords Rhizosphere · Designer microbes · Rhizosphere engineering · DefenseBiomes · Holobiome · Biotic and abiotic stresses

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7.1 Introduction

Rhizosphere can be defined as the fine region of soil that is in uninterrupted accessibility to the roots of plants and is the hotspot of numerous microorganisms. The plant stimulates the neighboring soil microorganisms by releasing or secreting diverse compounds consisting of amino acids, carbohydrates, and secondary metabolite organic acids, commonly known as "rhizodeposits" (Ahkami et al. 2017). Consequently, the rhizosphere soils that encourage the progression of microbial communities are referred to as mesotrophic. The rhizosphere has been distinguished into three zones: endo-rhizosphere (the section of root cortex, endodermis, and extracellular space between cells), rhizoplane (the superficial surface of the root), and ecto-rhizosphere (the section ranging from the surface of the root to loose soil) (McNear 2013). The rhizosphere holds on to the various microbial communities that accomplish different purposes and employ several effects on the growth of the plant. They actively engage in nutrient cycling, amelioration of biotic and abiotic stresses, and defense against pathogens (Fig. 7.1). As a result of the microbial venture in the rhizosphere, the changes in the quantity, composition, and quality of root exudates are generated, affecting the microbial constituents/composition (Philippot et al. 2013). According to the phenomenon of "rhizosphere feedback," the plants alter the configuration of microbial community in the rhizosphere with rhizodeposition,

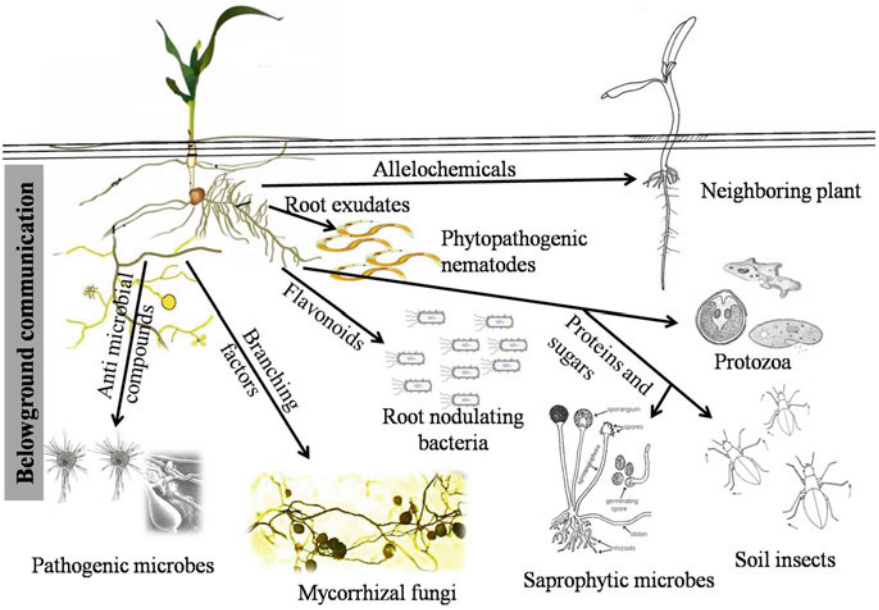

Fig. 7.1 Belowground communication in the rhizosphere of plants

which in turn affects the growth and productivity of plants (Dessaux et al. 2016). This connection proposes that the rhizosphere could be used or modified for the promotion of growth, nutritional requirement, and development of plants. A study was conducted on the bacterial species present in the rhizosphere of progeny from eight plant species that had been grown for the previous 11 years in the outdoor environments under monoculture and mixed cultivation. The results of the study demonstrated that the history of soil plantation and the identity of plant species define the structure of the soil community (Schmid et al. 2018). There is generous evidence to suggest that the interaction between plants and microbes affects the plants' overall health, efficiency, and development (Masood et al. 2020). As a result of this complex association, it is possible to engineer the rhizosphere that signifies an environment-friendly method for more ecological agronomic production, in order to enhance the growth, development, and yield of the plants as well as to protect them from biotic and abiotic stresses.

7.2 Rhizosphere Engineering for Stress Management

The improvement in tolerance to the biotic stresses in crops and plants is an important issue that could be resolved with the help of molecular biology. There are different mechanisms that are developed by the plant itself to deal with environmental stresses. Plants react to the stresses by assembling the molecules, osmolytes, and metabolites (Singh et al. 2013; Singh et al. 2019a, b, c). This article demonstrates the use of molecular biology and its implementations for plant and microbes engineering in order to escalate the tolerance against the biotic and abiotic stresses and increase efficiency through genetic analysis of gene-specific and stress-responsive overproduction of metabolites and proteins (Singh et al. 2020a, b, c). Genetic engineering provides the foundation for transferring single or multiple genes in regulating and signaling pathways that encrypt the compounds responsible for the structural and functional defense. Various approaches, such as DNA microarray, differential display PCR, cDNA-amplified fragment length polymorphism, and serial analysis of gene expression, have been characterized to detect the expression of genes during various environmental stresses in the rhizosphere. The main objective of agricultural research is the development of genetically modified, stress-tolerant crops through the bioengineering of stress signaling pathways. Designing of the rhizosphere microorganism or plant mainly concerns the study of the molecular part (genes, protein, and metabolites) and attempt to insert them into a functional model or signaling pathway to change the dynamics/activities of the organism under different abiotic and biotic stress conditions.

7.2.1 Natural Processes

7.2.1.1 Soil Suppressiveness

This approach represents soils that are suppressive in nature to major soil-borne plant pathogens such as *Rhizoctonia*, *Fusarium*, *Pythium*, *Phytophthora*, and *Gaeumannomyces*. This type of nature helps prevent pathogen establishment in the soil (Yadav et al. 2017). Suppressiveness of the soil is due to the diversity of organisms present in the soil, level of fertility, and soil texture. The soil microorganisms are the primary factor contributing to the suppression of disease through various mechanisms such as induced resistance, nutrient competition, and direct inhibition through antibiotics secreted (Yadav et al. 2011; Yadav and Verma 2014).

7.2.1.1.1 Allelopathy

Beneficial soil microorganisms like plant growth promoting rhizobacteria (PGPR) produce an extensive range of secondary compounds including antibiotics, metabolites, volatiles, siderophores, enzymes, etc. These compounds act as signal molecules for plant interaction, which is known as allelopathy, and compounds are called allelochemicals (Sturz and Christie 2003). For example, siderophores chelate iron available in the soil and deliver it to plants, antibiotics inhibit bacterial growth and their colonization, lytic enzymes rupture fungal cell wall, detoxification enzymes help in preventing the damage from pathogenic toxins, and volatiles such as hydrogen cyanide, tridecane, quinoline, etc., suppress the development of fungal pathogens (Saraf et al. 2014). Nowadays, allelochemicals producing microbes are being used in rhizosphere engineering for integrated disease management. *Alcaligenes* spp. have been studied for siderophore production, which inhibited the growth of *Aspergillus niger*, *A. flavus*, *Fusarium oxysporum* and *Alternaria alternate*, *Metarhizium anisophilia*, and *Pseudomonas solanacerum* (Sayyed and Chincholkar 2009; Sayyed and Patel 2011). *Pseudomonas fluorescens* and *P. chlororaphis* are good examples of antibiotic and volatiles producers that are responsible for the suppression of fungal diseases in plants (Fernando et al. 2005; Asadhi et al. 2013; Kumari et al. 2015; Vaishnav et al. 2014, 2016). Jain et al. (2017) reported lytic enzymes like chitinase and 1,3-glucanase from *Bacillus* sp. SJ-5 for enhanced defense against *Rhizoctonia solani* and *Fusarium oxysporum* in soybean plants (Jain et al. 2014, 2018).

7.2.1.1.2 Niche Competition and Microbiostasis

In the soil environment, microorganisms of the same population are always in competition for the acquisition of essential nutrients and better colonization. Soil nutrients become depleted by the growing population. In such conditions, PGPRs have the ability to survive, grow, and strive with other microbes existing in the

rhizosphere. These microorganisms colonize the target host plant effectively and promote plant growth. PGPRs also provide enhancement to other microorganisms of plant growth-promoting group (Garbeva et al. 2014). For example, *Pseudomonas fluorescens* produce extracellular polysaccharides (EPS) in the rhizosphere, enabling them to better colonize as compared to other microorganisms. The mats formed due to the EPS around the root surface restrict the progression of plant pathogens (Ahkami et al. 2017). The siderophores-producing bacteria vary in their abilities to confiscate iron from other organisms; they deprive pathogenic fungi of this essential element since the fungal siderophores have a lower affinity (Meziane et al. 2005). Similarly, rhizobacteria can decrease the quantity of carbon and nitrogen existing for the germination of fungal spore and growth of phytopathogen in the region of roots (Hibbing et al. 2010).

7.2.1.1.3 Antibiosis

Antibiosis is the mechanism of inhibition of pathogen by the metabolic products released by antagonist microbes. These products consist of volatile compounds, lytic enzymes, antibiotics, and antimicrobial peptides that perform an important role in the suppression of disease present in the soil (Jayaprakashvel and Mathivanan 2011; Singh et al. 2016a, b). These compounds have been reported from a diverse variety of bacterial genera, but particularly from *Pseudomonas* and *Bacillus* genera. Different strains of *Pseudomonas* bacteria produce 2, 4-diacetylphloroglucinol (DAPG), phenazines, and pyrrolnitrin-like antibiotics that are well known to suppress different soil-borne pathogens (Yadav et al. 2017). *Bacillus* strains are reported to produce lipopeptides (bacillomycin D, surfactin, zwittermicin), lytic enzymes (cellulases, glucanases, proteases, and chitinases), and volatile compounds (HCN, butendiol, acetoin), which have a broad spectrum of action against various plant pathogenic fungus (Jha and Saraf 2015).

7.2.1.1.4 Induced Systemic Resistance

Beneficial microbes present in the rhizosphere improve plants' health by induced systemic resistance (ISR) mechanisms in which plant defense is enhanced against an extensive series of pathogens and insect herbivores (Bakker et al. 2013; Pieterse et al. 2014). A varied range of microorganisms associated with roots including *Pseudomonas*, *Bacillus*, *Trichoderma*, and Mycorrhizae species are reported to alert the plant immune system for improved defense. ISR is mediated by different antioxidant enzymes, phytoalexins, jasmonic acid (JA), and ethylene (ET)-dependent signaling pathway (Choudhary et al. 2015; Singh et al. 2016a, b, 2019a, b). Inoculation of chitinolytic microorganisms such as *Chitiniphilus* sp. MTN22 and *Streptomyces* sp. MTN14 has been observed to modulate the Bacoside A pathway of biosynthesis and defense mechanism against *Meloidogyne incognita* in *Bacopa monnieri*. These microorganisms support the host resistance by

boosting the chlorophyll a, defense enzymes, and phenolic compounds and also elevated lignification and callose deposition (Gupta et al. 2017a, b). A nonpathogenic rhizobacterial strain *Pseudomonas fluorescens* WCS374r has been found to initiate ISR in rice against the leaf blast pathogen *Magnaporthe oryzae*. It was observed that WCS374r-induced resistance is regulated by jasmonic acid/ethylene-modulated signal transduction pathway (De Vleesschauwer and Hofte 2009).

7.2.1.2 Crop Rotation

Crop rotation is the method of growing multiple crops sequentially on the same field to avoid the build-up of soil-borne pathogens and to maintain soil fertility. Soil-borne pathogens have restricted the host range that's why they will decline in 2–3 years without a suitable host (Krupinsky et al. 2002). Cultivation of nonhost crops for a particular pathogen will inhibit the build-up of large populations of pathogens. For example, continuous potato cultivation resulted in the formation of stem lesions caused by *Rhizoctonia solani*. However, oat–potato, ryegrass–potato, and clover–potato cultivation have been shown to reduce *R. solani* inoculum in soil and successive disease suppression in potato crops. Transpositions of dicotyledonous with monocotyledonous crops are efficacious in restricting the majority of soil-borne plant pathogens (Kheyrodin and Antoun 2011). Likewise, green manure cultivation in wintertime is part of crop rotation. The green manure protects soil from erosion, helps in preventing the leaching of mineralized nitrogen, and suppresses plant pathogens (Hiddink et al. 2010). For example, Marigold (*Tagetes* spp.) is specifically cultivated to suppress *Pratylenchus penetrans* (Kimpinski et al. 2000). Cultivation of different *Brassica* species has been shown to reduce disease occurrence caused by *Rhizoctonia solani*, *Phytophthora erythroseptica*, *Pythium ultimum*, and *Fusarium sambucinum* in potatoes (Larkin and Griffin 2007).

7.2.2 Genetic Modification

Rhizosphere engineering can also be achieved by direct genetic modification of plant and microbial genes determining rhizosphere functions. Genetically modified plants and microbes have been designed and evaluated in laboratory and field environments. Root exudates are targeted to modify the rhizosphere through a plant genetic engineering approach. These exudates provide a favorable environment to beneficial microorganisms in the rhizosphere (Ryan et al. 2009). The synthesis of exudates in plant cells relies on the uptake of essential nutrients, and the transport process of nutrients across the plasma membrane depends upon the efflux of hydrogen ions to generate a membrane potential difference. The H^+ efflux acidifies the rhizosphere, which increases the availability of Fe^{3+} and phosphorus. The H^+ efflux is controlled by H^+-ATPase proteins (Palmgren 1991). The genes encoding these proteins are

used in plant genetic modification for manipulating rhizosphere pH (Ryan et al. 2009).

Some research groups have suggested developing genetically modified microorganisms rather than transgenic plants to accomplish enhanced performance of plants. It is easier to combine several PGP traits into a single bacterium, which can be used as an alternative to engineered crops (Rodriguez et al. 2008). The *chiA* gene, encoding chitinase, was first to be reassigned to heterologous bacteria with the aim of increasing protection activity against fungal plant pathogens. *E. coli* strain overexpressing *chiA* rapidly bursts the hyphal tips of *Sclerotium rolfsii* and decreases its capability to cause disease on bean (Shapira et al. 1989). An engineered strain of *Pseudomonas* expressing *chiA* gene showed more antagonism against *Fusarium oxysporum* f. sp. *redolens* and *Gaeumannomyces graminis* var. *tritici* (Sundheim et al. 1988).

7.2.2.1 Designer Microorganism Approach

Advancement in tools for genetic manipulation like genome sequencing, metabolic engineering, genetic engineering, and synthetic biology aids in designing microorganisms as per the necessity of desired qualities (Lovley 2012). Microorganisms can be used as a base for the biosynthesis of desired products. Microorganisms grow rapidly on relatively cheaper nutrient sources, mass production of desirable culture is easy on a large scale, and the metabolic fundamentals of microorganisms can make use for the production of significant quantities of beneficial compounds (Buchholz and Collins 2013). The selection of microbial hosts for secondary metabolite production depends on the malleability of the organism. It is better to select microorganisms that can easily grow in vitro and have a known genome sequence that is open to genetic manipulation possessing clearly understood metabolic pathways. The model organisms *E. coli* and *S. cerevisiae* are well-known examples of reprogramming strategies to improve our desired product quality and yield (Koopman et al. 2012; Umenhoffer et al. 2010).

7.2.2.1.1 Designing a Microorganism for Biosynthesis of Desirable Molecules

There are plenty of design tools available for genetic manipulation such as DNA assembler (Shao et al. 2009) and Gibson assembly that provides rapid integration of large segments of DNA in a heterologous host. Genome of the heterologous host can also be edited or manipulated by using high-throughput techniques like multiplex genome engineering and accelerated evolution (MAGE) (Wang et al. 2009). Site-specific engineering of cellular genomic DNA, zinc finger nucleases (ZFNs) (Doyon et al. 2010), TALE nucleases (TALENs) (Reyon et al. 2012), and recently, the more efficient RNA-linked CRISPR-Cas9 nuclease system (Cong et al. 2013; Ran et al. 2013) are successful cloning technologies that are an essential link between a

designed system and a functional programmed biosynthetic pathway in a designer microorganism. These technologies hold abundant potential for advance biology and for the speedy association of new biosynthetic pathways in a designer microorganism (Bonnet et al. 2012; Colloms 2013; Quin and Schmidt-Dannert 2014; Ghosh 2016).

7.2.2.1.2 Regulation of Gene Expression

Gene expression and its regulation is the main factor for controlling any designer microbial host for its biosynthetic pathway, which is required to ensure the necessary expression of enzymes to optimize pathway flux. Promoters can either act as "on/off" switches or permit varying degrees of gene expression. The in silico approach can help in predicting and modeling promoters and their regulatory elements (Rhodius et al. 2012; Salis et al. 2009) to specifically alter a new metabolite. Construction of promoter libraries brings together a diversity level and choice in choosing multiple promoters with different strengths that are appropriate for enhanced expression of a biosynthetic pathway (Blount et al. 2012).

Several genes are involved in the control and regulation of complex cellular phenotypes including tolerance to biotic and abiotic stimuli; therefore, reprogramming of gene and metabolic networks is necessary. The methodology to achieve this is the global transcription machinery engineering (gTME), which has recently been applied in both prokaryotes (e.g., *Escherichia coli* (Alper and Stephanopoulos 2007), *Lactobacillus plantarum* (Klein-Marcuschamer and Stephanopoulos 2008) and eukaryotes (*Saccharomyces cerevisiae)* (Alper et al. 2006; Liu et al. 2010). For example, a strain of *Escherichia coli* has been designed in such a way that could degrade toxin and atrazine and is tested on a wide scale and responding specifically to the presence of the herbicide under field conditions. Recent advancement in genome sequencing and the capability to influence DNA fragments have contributed to the successful application of design principles in creating designer microorganisms. However, there is limited progress by the fact that we do not have a completely standardized method to design and engineer a microorganism for biological control. Improving the design process would make designer microorganisms much more competent and agriculturally related. Research leads to more metabolic pathway design (Lux et al. 2012), and this emerging field of designer microorganism concept has much potential to enhance the production of designer microorganisms for biological control.

7.2.2.1.3 Designer Microorganisms: A Success Story

In the latest case, a microbial host has been used for increasing the yield by controlling the carbon storage regulator system (Csr) of *E. coli*. Expression levels of Csr and translation protein are enhanced in such a way to accumulate TCA cycle intermediates and use carbon in a more efficient manner. Furthermore, using

Csr-designed *E. coli* as a host for the production of biofuels led to the enhanced yields of n-butanol (88%) and amorphadiene (55%) in comparison to control cells (McKee et al. 2012). Another example of enhancing yields, the complete biosynthetic pathway for more production of artemisinic acid, an anti-malarial drug precursor, was demonstrated in *S. cerevisiae* (Cong et al. 2013). The effectiveness and performance of biocontrol agents can be made more intense by using gene splicing, gene cloning, and transformations. There are certain genes that encode enzymes involved in the synthesis of phytotoxins, growth hormones, or enzymes that are capable of degrading plant cell walls. Since these genes are involved positively and required for pathogenesis, their inactivation would destroy the organisms and their pathogenic potential or reduce virulence. In India, some of the genes of Baculoviruses are as such designed to self-destruct after they have inoculated, infected, and killed the insect pests. Polyhedrin is the protein coat that covers the genetic material of Baculoviruses. Suicide group of Baculoviruses was designed by eliminating the polyhedrin gene. Applications of such genetically engineered Baculoviruses infect and kill the insect larvae and millions of viruses after replication, and they are released out into the environment. Such viruses die within a few hours after release as they lack polyhedrin; therefore, they fail to survive in the environment (Ghosh et al. 1998).

7.2.3 Designer Plant Approach

The functional approach helps to know the genes encoding functional stress protein modified according to the environmental stresses. This approach is a needful tool for genetic engineering for stress tolerance. Some stress-regulating pathways in plants are analogous to that in bacteria. Transgenic plants of *Arabidopsis thaliana* were developed as an osmotolerant by the administration of *proBA* genes derived from *Bacillus subtilis* that produced a high level of free proline and increased the tolerance against osmotic stress (Chen et al. 2007). *Vigna aconitifolia* (P5CS) cDNA transformed wheat plants developed accumulated more proline than nontransformed plants in the water stress condition by higher production of main enzymes responsible for proline biosynthesis (Vendruscolo et al. 2007). Functional genomics plays a key role in the development of varieties tolerant to biotic and abiotic stresses with the genes mostly expressed at transcriptional and translational levels. Tolerance to different stresses is a multifunctional syndrome instead of a function of single gene or reaction (Mittler 2006). Modification in the genetic make-up of the plants to enhance the expression of the genes involved in the signaling and regulatory pathways results in the synthesis of functional and structural metabolites. Genetically modified plants have been developed to acclimatize via the accumulation of compatible solutes (proline, betaine, and alcohol sugars) and the expression of enzymes that catalyze the synthesis of these solutes to protect plants from abiotic stress like salinity and drought (Fatemeh et al. 2012). Designer plants developed by metabolic profiling of species under stress are an important tool for studying stress-

induced changes in metabolites and analysis of transgenic plants. Engineering metabolic pathways in plants has emerged as a better approach for abiotic tolerance in plants. This targets the multiple genes in the pathways and results in an increase in the number of metabolites. Genetic modification in the root modifies uptake of the essential elements like silicon that enhance the resistance of plants to multiple stresses (Ma et al. 2004). Similarly, SNAC1 and LEA genes of rice were identified from microarray experiments under stress drought conditions (Hu et al. 2006; Xiao et al. 2007).

7.2.4 Designer Rhizomicrobiome Approach

Transgenic plants were enhanced to access the soil nutrients by insertion of desired/respective gene(s) from soil fungus *Aspergillus* sp. Engineering of microbial community structure in the rhizosphere of tobacco plants to produce phytase has been done and assessed using a TRFLP-based approach on amplified 16S rDNA. Transgenic plants have been designed with a single gene change to modify the biochemistry of the rhizosphere that helps in the releases of fungal physate for the utilization of inositol phosphates, a form of organic P in soil (George et al. 2009). Response of plants toward multiple stresses together (biotic and abiotic stress) is different as compared to a single stress. Interaction between molecular signaling pathways controlling these multiple stress has been analyzed by microarray, resulting in the expression of a unique set of multiple stress-regulated genes. AtRALFL8 (Rapid alkalinization factor like8) was induced by the root as a signal peptide for cell wall remodeling. *AtMGL* gene was upregulated in *A. thaliana* leaves. The interaction was positive as well as antagonistic to each other. AZI1 was downregulated in leaves by the effect of multiple stresses (Atkinson et al. 2013).

7.2.4.1 Rhizosphere *Vis-à-Vis* Designer Rhizosphere

The rhizosphere is an integral belowground component of a plant essential for interactions with arrays of soil microorganisms that facilitate the acquisition of water and nutrients to both the plant and microorganisms. Signals from the plant through the production of biomolecules by root systems help colonization with specific kinds of microorganisms. A designer rhizosphere, a highly precise environment, is the man-made induced environment in the rhizosphere of the plant for achieving a particular target for human benefits by modulating plant systems. Manipulating the rhizosphere to harness or enhance its potential will most probably play a key role in the future development of sustainable agricultural practices. Designer rhizosphere can be developed through rhizosphere engineering approaches by modifying agricultural soils for a short period of time to modulate plant growth and development while minimizing environmental impacts. Rhizosphere can be manipulated by manipulation in the plants, microorganisms, and soil. Rhizosphere

engineering decreases the dependency on agrochemicals by replacing their functions with beneficial microbes, biodegradable, biostimulants, and transgenic plants. Modern efforts specifically deal with the defense machinery of plants that is involved in engineering plant resistance to stresses. Plants have naturally developed certain approaches for the modification of their rhizosphere in order to reduce the impact of biotic stresses (Ryan et al. 2009). In addition, plants also release compounds that protect against pathogens or encourage the proliferation of beneficial microorganisms. In general, designer rhizosphere can be created by rhizosphere engineering approaches. Rhizosphere engineering can be done in three different ways, by (1) harnessing the potential of natural processes, (2) genetic modification, or (3) exogenous amendments.

7.2.5 Exogenous Amendments

7.2.5.1 Beneficial Microorganisms

Over the past decades, the application of beneficial microbes has increased as a valuable part of rhizosphere engineering. Based on the mechanism, beneficial microbes can be categorized as biofertilizers, biopesticides, and phytostimulators that promote plant growth and yield in different ways. These microorganisms are more effective when added to compost and other organic amendments in the soil environment. In order to reduce plant disease, biopesticides have recently attracted a lot of interest. They are based on pathogenic microorganisms specific to a target pest. The most commonly used biopesticides are biofungicides (*Trichoderma*), bioherbicides (*Phytopthora*), and bioinsecticides (*Bacillus thuringiensis*) (Raaijmakers and Mazzola 2012). *Bacillus thuringiensis* (Bt), an insect pathogenic gram-positive facultative-aerobic soil bacterium, is the most commonly used biopesticide in the world. During sporulation, this bacterium creates an insecticidal protein known as endotoxins or cry proteins. When ingested by certain insects, this protein leads to the lysis of gut cells. More than 60% of the market for bioinsecticides is accounted for by Bt, which is well-liked globally and has superior efficacy for most lepidopteran and coleopteran larvae (Sanchis and Bourguet 2008). The bases of Bt formulations are generally from their strains of the subspecies *kurstaki*, *galeriae*, and *dendrolimus*. Bt sprays are used for the control of caterpillar pests, larvae, corn borers, etc. Bt formulations have majorly been used on large and diverse crops such as maize, soybean, and cotton, where resistance caused by synthetic chemical insecticides is a major problem. The toxins present in Bt are also genetically engineered in numerous crop plants in order to make resistance to insect attack (Chandler et al. 2011).

Entomopathogenic baculoviruses and fungi are also used in the development of additional bioinsecticides. Baculoviruses are the specific type of infection that primarily affects lepidopterous pests. For instance, in the USA, the biopesticide Cydia pomonella granulovirus (CpGV) is primarily employed to combat codling

moths on apple crops (Chandler et al. 2011). Additionally, the use of nucleopolyhedro virus has been done on around 35% of crops of soybean against the caterpillar *Anticarsia gemmatalis* found in Brazil (Moscardi 1999). The major constituents in the entomopathogenic fungal products are *Beauveria bassiana* and *Metarhizium anisopliae*. These fungi penetrate their hosts through the cuticle, feed on the nutrients found in the intercellular spaces, and then produce poisons. *Cydia pomonella* L. has been found to be restricted by the insecticide "Boverin," which is based on the *Beauveria bassiana* inoculated plants (Ferron 1971). Spraying of *B. bassiana* has been shown to produce the most favorable and rapid outcomes even though only a small amount of imidacloprid was being used (Ambethgar et al. 2007).

Trichoderma spp., *Pseudomonas* spp., and Mycorrhizae are some other biopesticides that are used against plant pathogens. *Trichoderma* is important for dry land crops, i.e., groundnut, black gram, mung, and chickpea that are susceptible to *Rhizoctonia*, *Pythium*, *Fusarium*, and other soil-borne pathogens. It secretes a variety of secondary metabolites (volatile, nonvolatile, and diffusible) for the suppression of pathogen attack in the plant environment (Waghunde et al. 2016). Because of their broad-spectrum antagonistic activity and efficiency in colonizing the rhizosphere, *Pseudomonas* spp. are by far the most widely studied bacteria. They produce a wide range of bioactive molecules for plant pathogen control, including siderophores, gluconic acid, rhamnolipids, 2,4-diacetylphloroglucinol (2,4-DAPG), 2,5-dialkylresorcinol pyrrolnitrin, pyoluteorin, phenazines, hydrogen cyanide (HCN), quinolones, and various lipopeptid (Raaijmakers and Mazzola 2012). *P. fluorescens* has been found to be effective against multiple plant species from various infections due to its ability to adapt effectively to soil and colonize plant roots (Couillerot et al. 2009). By producing a fungal mat, mycorrhizae can surround plant roots. This fungal mat creates a physical barrier against soil pathogens such as insects, nematodes, bacteria, and fungi (Harrier and Watson 2004). Inadvertently, mycorrhizae improve the ability of plant roots to absorb nutrients, making plants stronger to withstand or resist pathogens (Ortaş et al. 2017).

Nonpathogenic organisms are also being used to control soil-borne pathogens in the context of rhizosphere engineering. Nonpathogenic organisms compete with pathogens for nutrients and infection sites for colonization. Furthermore, plants induced "memory" defenses against nonpathogenic determinants, resulting in quick and durable initiation of basal tolerance mechanisms upon pathogen exposure (Vaishnav et al. 2017).

Alabouvette (1999) described the ability of nonpathogenic *Fusarium oxysporum* and fluorescent *Pseudomonas* spp. to suppress fusarium wilt disease for the first time. These organisms are in charge of carbon and iron competition. It has been demonstrated that the presence of nonpathogenic Fusarium strains such as Fo47 is responsible for the control of *Fusarium* wilt in suppressive soils (Alabouvette et al. 1979). The nonpathogenic *Fusarium oxysporum* strain Fo47 has been found to protect cucumber from *Pythium ultimum* infection via a combination of antibiosis and mycoparasitism (Benhamou et al. 2002).

7.2.5.2 Chemical Nutrients

The nutrient status of the soil and the amendments of fertilizers can play major roles in the management of diseases. Soil pH, calcium level, and the use of nitrogen form have significant impacts on the pathogen's environment. Different types of nitrogen sources change the pH of soil, which reduce the incidence and severity of soil pathogens. These fertilizers shift the pH that alters the chemistry of soil around roots and stimulate the growth and composition of microbial communities (Yadav et al. 2017). For example, ammonium-based fertilizers acidify the rhizosphere, whereas nitrate-based fertilizers make the rhizosphere more alkaline (Kheyrodin and Antoun 2011). The alkaline soils are resistant against *Fusarium* diseases. In a study, the use of nitrate form of nitrogen suppresses *Fusarium* wilt disease in tomatoes by making the root zone less acidic (Woltz and Jones 1973). Likewise, calcium has also been used to control soil-borne diseases such as damping off caused by *Pythium* and club root in crucifer crops (Ko and Kao 1989). These diseases are inhibited in neutral to slightly alkaline soils (pH 6.7 to 7.2). On the other hand, potato scab disease is more severe in alkaline soils, and below pH 5.2, the disease is generally suppressed. Studies on potato scabs showed reduced disease levels by using sulfur and ammonium sources of nitrogen (Kheyrodin and Antoun 2011).

Salicylic acid (SA) is involved in systemic acquired resistance (SAR) signaling in which uninfected systemic plant parts become more resistant in response to a localized infection in the plant. Salicylic acid is also described in modulating the colonization of roots by specific bacterial families. In a study, *Arabidopsis thaliana* mutants with reformed immune systems in wild soil were demonstrated for their colonization pattern. It was observed that foliar application of salicylic acid is required to assemble a normal root microbiome (Lebeis et al. 2015).

7.2.5.3 Compost

Compost is being extensively used in field areas such as nursery and pot soil for the control of root rot diseases. In the compost, organic matter is already digested through the aerobic process, which is an advantage over other amendment techniques. Compost acts as a food source for the antagonists that compete with plant pathogens and the organisms that prey on pathogens and produce antibiotics (Singh et al. 2018). It fosters a high diversity of beneficial microflora in the soil, which suppresses the root rot causing pathogens like *Pythium* and *Phytophthora* (Harrison and Frank 1999). Depending on the feedstock, inoculum, and composting process, composts have a different characteristic that affects the disease management potential. The high carbon to nitrogen ratio (C:N) in compost suppresses *Fusarium* wilts, while lower C:N ratio favors *Fusarium*. Compost is also amended with specific biocontrol agents. Two biocontrol strains of *Trichoderma* and *Flavobacterium* have been added into the soil with compost, resulting in the suppression of *Rhizoctonia solani* (Hoitink et al. 1991).

7.3 Conclusion and Future Prospects

Several microbial inoculants have been created in order to accomplish the achievement in the field by either designing the smart microbial community or by engineering the microbes with advantageous traits. Since individual microbes are the primary controllers, hence, a thorough research study of these bacteria as well as the soil's microbial community can contribute to escalating the possibilities of this field. Additionally, it will promote discovering the new and innovative approaches for microbiome engineering in order to advance toward sustainable agricultural production. Presently, the high-throughput methodologies containing proteomics, genomics, metabolomics, and transcriptomics are available for a better understanding of metabolic networks and signaling pathways in the plant–microbe connections. The use of synthetic microbial communities (SynComs), metabolomics, and metagenomics methodologies are also in progress for understanding the biological significance of the changes caused in microbiome under various stresses. CRISPR-Cas is anticipated to be an important tool for engineering plants and microbes in order to overcome the limitations such as low cultivation, less nutritive assessment, and exposure to infection. Another system-based method is needed that incorporates plant physiology and genetics, and the DefenseBiome is required for understanding the plant defense mechanism against biotic and abiotic stresses.

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Chapter 8
Potential of Bacterial Endophytes in Biological Control of Soil-Borne Phytopathogens

Vellaichamy Mageshwaran, Rishabh Gupta, Pramod Kumar Sahu, Pratyush Tripathi, and Ritu Vishwakarma

Abstract Soil-borne pathogens pose a serious threat in crop production worldwide due to the occurrence of wide pathogenic variability, loss of resistance in cultivars, and survivability of these pathogens in soil for longer periods. The important soil-borne pathogens having economic importance that cause root and stem rot and wilt in a wide range of host plants including cereals, pulses, vegetables, fruits, etc., are *Rhizoctonia* spp., *Sclerotium* spp., *Fusarium* spp., *Sclerotinia* spp., *Verticillium* spp., *Pythium* spp., and *Phytophthora* spp. Among the different strategies of soil-borne disease management, biological control receives much attention due to its inherent nature of eco-friendliness and sustainability. Bacterial endophytes are the group of rhizobacteria that has the capability to colonize the host plant and resides inside the plant parts such as root, stem, leaves etc. Bacterial endophytes are considered potential candidates for biocontrol as they reside in the immediate vicinity of the invasive pathogens and compete with the pathogens for nutrients and space. Besides, endophytes are capable of inducing systemic resistance and host defense properties through the synthesis of pathogenesis-related proteins and enzymes, secrete antimicrobial substances, and produce siderophore and hydrogen cyanide (HCN), thereby suppressing the invading soil-borne pathogens. In this chapter, we discuss the bacterial endophytes of different crop plants and their ability to suppress the multi-host soil-borne pathogens in crop plants.

Keywords Bacterial endophytes · Biological control · Soil-borne phytopathogens · Induce systemic resistance · Antimicrobial metabolites

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8.1 Introduction

By 2050, the world's population is expected to grow to almost 10 billion, warranting the agricultural demand to 50% in the scenario of modest economic growth (FAO 2017). Agriculture remains to stand as the primary occupation, and more than 50% of the population directly or indirectly depends on agriculture. In India, about 17 to 18% of gross domestic product (GDP) depends on agriculture. Every year more than 40% of crop losses occur due to insect, pest, and plant diseases. Farmers use chemical pesticides to overcome this problem, but these chemicals rigorously pollute the environment and deteriorate soil fertility. Though the development of resistant cultivars against the emerging pathogens is a superior strategy as a part of Integrated Disease Management, the occurrence of wide pathogenic variability and development of resistance in different populations of soil-borne pathogens presents a serious threat to the development of resistant cultivars. The foremost soil-borne diseases that occur globally in wide crop plants and cause crop losses to the tune of 50–60% are wilt caused by *Fusarium oxysporum*, collar rot (*Sclerotium rolfsii*), wet root rot (*Rhizoctonia solani*) and stem rot (*Sclerotinia sclerotiorum*), dry root rot (*Rhizoctonia bataticola*), *Verticillium* wilt (*Verticillium* spp.), *Phythium* (Damping off), and stem and root rot (*Phytophthora*). These pathogens often form resting structures such as microsclerotia, sclerotia, chlamydospore, or oospores and survive in plant debris, soil organic matter, etc., for long periods.

Biocontrol is an eco-friendly way to reduce the use of chemical pesticides in agriculture. Rhizobacteria function as biological control, which is the primary indirect mechanism for promoting plant growth (Glick 2012). Bacterial endophytes are a new tool to control pathogenic microbes and enhance plant growth. Bacterial endophytes are present in almost all plant species. They present inside the root, stem, leaves, and fruits. They produce plant growth-promoting substances and antimicrobial compounds. Some of them are also responsible for phytoremediation and xenobiotic degradation (Ryan et al. 2008). Bacterial endophytes show plant growth-promoting traits, including phosphate solubilization, N_2 fixation, and phytoremediation, and produce antimicrobial compounds that are used in agriculture and medicine.

Santos et al. (2018) reviewed the metabolic effect of endophytic bacteria on the plant during its colonization in plant tissue. Bacterial endophytes showed antagonistic properties against plant pathogens and increased plant growth traits such as biomass, grain production, root length, etc. Similarly, Rosenblueth and Matrinez-Romero (2006) reviewed the effect of bacterial endophytes on the growth of the plant. Bacterial endophytes eliminate plant pathogens and solubilize minerals like phosphate, zinc, and potassium. Most of the endophytes have metabolic mechanisms to colonize in plant tissue, but some of them are seed-borne. Firdous et al. (2019) and Malea and Serepa-Diamini (2019) highlighted the importance of bacterial endophytes in plant growth promotion through phosphate solubilization, siderophore production, phytohormone production, etc. Endophytes induce systemic resistance

in plants, increase plant stress tolerance, and are able to degrade xenobiotic pollutants in their proximal environment.

Bacillus and *Paenibacillus* are gram-positive endospore-forming bacteria most abundant in rhizospheric soils and frequently occur in isolation and screening of plant growth-promoting rhizobacteria. The major functions of these bacteria in agricultural soil are nitrogen fixation, nutrient supplementation (major and micronutrients), synthesis of antimicrobials, phytohormone production, and supporting plant growth and defense against plant pathogens through induction of systemic resistance through the synthesis of related metabolites and pathogenesis-related proteins. More importantly, they have the ability to colonize the plant tissues as endophytes and do all the functions within the plants as described here (Govindasamy et al. 2010; Hyung et al. 2016). The bioagents employ multiple mechanisms for the biocontrol of plant pathogens. The antagonistic properties of *Bacillus* isolates included β-1,4 glucanase, chitinase, siderophore, hydrogen cyanide, ammonia, and other biocidal and thermo-stable nonvolatile antifungal metabolites (Kumari and Khanna 2016). Among the bioagents, *Trichoderma* spp. is considered a popular bioagent for the control of soil-borne pathogens. Rudresh et al. (2005) reported potential *Trichoderma viridie* and *T. harzianum* strains for the biocontrol of wilt complex disease in chickpea. Though *Trichoderma* sp. are reported as a potential biocontrol agent, *Bacillus* and *Pseudomonas* are frequently encountered as rhizobacteria and endophytes and have multiple traits of plant growth and biocontrol of plant diseases.

8.2 Isolation and Characterization of Bacterial Endophytes from Different Crops

The bacterial endophytes were isolated from different crop plants such as maize, peanut, banana, *Artemisia* sp., ginger, soybean, *Scutellaria baicalensis* Georgi, *Lonicera japonica*, pearl millet, holy basil, etc. *Bacillus* and *Pseudomonas* are the predominant genera obtained as endophytes from different crops. The summary of the isolation of bacterial endophytes from different crops is depicted in Table 8.1. Gond et al. (2015) isolated endophytes in maize plants to evaluate bacteria's ability to produce any antimicrobial compounds. They identified *Bacillus* spp.-producing lipopeptides and showed antifungal properties against *Fusarium moniliforme*. *Bacillus* spp. was able to induce PR-1 and PR-2 proteins against plant pathogens. Souza et al. (2014) isolated 122 bacterial endophytes from five different species of *Musa* (banana plant). They screened out four strains of bacteria that showed antifungal properties against *Colletotrichum guaranicola* and *Fusarium oxysporum* f. sp. *cubense*. Phylogenetic analysis of the 16S rRNA of these bacteria showed that they belong to three different species of *Bacillus*: *B. thuringiensis*, *B. amyloliquefaciens*, and *B. subtilis* subsp. *subtilis*. Chung et al. (2008) identified a bacterial strain YC5480 isolated from the plant *Artemisia* sp. Bacterial strain was

Table 8.1 Summary of isolation of bacterial endophytes from different crops

S. no	Crop	Bacterial endophytes	Reference
1	Maize	*Bacillus amyloliquefaciens* or *Bacillus subtilis*	Gond et al. (2015)
2	Peanut	*Bacillus subtilis* and *Pseudomonas fluorescens*	Ziedan (2006)
3	Banana	*Bacillus amyloliquefaciens*, *Bacillus subtilis* subsp. *subtilis*, and *Bacillus thuringiensis*	Souza et al. (2014)
4	Artemisia sp.	*Pseudomonas brassicacearum* YC5480	Chung et al. (2008)
5	Ginger	*Bacillus* and *Pseudomonas*	Chen et al. (2014)
6	Soybean	*Paenibacillus* sp. HKA-15 and *Bacillus* sp. HKA-121	Senthilkumar et al. (2009)
7	*Scutellariabaica lensis* Georgi	*Bacillus amyloliquefaciens* ES-2	Sun et al. (2006)
8	*Lonicera japonica*	*Paenibacillus* and *Bacillus* strains	Zhao et al. (2015)
9	Pearl millet (*Pennisetum glaucum*)	*B. amyloliquefaciens*, *Bacillus subtilis* subsp. *subtilis*, and *Bacillus cereus*	Kushwaha et al. (2020)
10.	Tomato	*Bacillus, Lysinibacillus*, and *Stenotrophomonas*	Sahu et al. (2019)
11.	Holy basil (*Ocimum tenuiflorum* L.)	*Bacillus altitudinus* (BTL-1 and GTS-16), *B. tequilensis* (BTL-4), *B. safensis* (BTL-5), *B. hayensii* (GTR-8), and *B. paralicheniformis* (GTR-11)	Sahu et al. (2020)

identified by biochemical test, physiological characteristics, and 16S rRNA gene sequencing analysis, and it was found to be *Pseudomonas brassicacearum*. This bacterium produced antifungal compounds. Kumar et al. (2016) isolated 14 bacterial endophytes from *Curcuma longa* L. and identified them by morphological, biochemical characterization, and 16S rRNA gene sequencing. Six strains, viz., *Bacillus cereus* (ECL1), *Bacillus thuringiensis* (ECL2), *Bacillus* sp. (ECL3), *Bacillus pumilus* (ECL4), *Pseudomonas putida* (ECL5), and *Clavibacter michiganensis* (ECL6). All strains produced IAA and showed phosphate solubilization activity and only two strains (ECL3 and ECL5) produced siderophore during PGP trait analysis. These strains inhibited the growth of pathogenic bacteria *Klebsiella pneumonia*, *Escherichia coli*, and some pathogenic fungi *Alterneria alternata* and *Fusarium solani.*

Chen et al. (2014) isolated 248 bacterial endophytes from the ginger plant. They screened out 107 isolates from 248 on the basis of functional properties, and 90 strains produced IAA and 17 strains produced antimicrobial products. Based on 16S rRNA gene sequencing, these 107 strains grouped into 16 genera in which *Pseudomonas* and *Bacillus* were the dominant genera. Sixteen strains showed antimicrobial activity against *Pythium myriotylum* Drechsler, while 7 strains showed antimicrobial activity against *Phyllosticta zingiberi* Hori. This study showed the

synthesis of antibacterial substances in ginger, which could change the endophytic bacterial community. Sun et al. (2006) isolated bacterial endophytes from *Scutellaria baicalensis* and identified them by morphology, biochemical characterization, and 16S rRNA gene sequencing. Results of identification indicate that the isolated strain was *Bacillus amyloliquefaciens* ES-2 and the strain produced antibacterial and antifungal compounds. Senthilkumar et al. (2009) isolated 137 bacterial endophytes from the root, leaves, and stem of soybean plant, and they screened out nine strains of bacteria that show antifungal properties against *Sclerotium rolfsii*, *Fusarium udum*, *Rhizoctonia bataticola*, and *Macrophomina phaseolina.* They identified them by morphology, biochemical characterization, and 16S rRNA gene sequencing. Out of nine strains, eight strains belonged to *Bacillus* and one strain belonged to *Paenibacillus.* The strains were screened on the basis of siderophore production, hydrogen cyanide production, IAA production, phosphate solubilization, and nitrogen fixation. They identified two most effective biocontrol strains *Bacillus* sp. HKA-121 and *Paenibacillus* sp. HKA-15 for control of *Rhizoctonia bataticola* causing charcoal rot disease in soybean. Vinodkumar et al. (2017) isolated 50 strains of *Bacillus* spp., from rhizosphere of cotton, banana, carnation, and turmeric in the Tamil Nadu state of India and chose 5 strains from the culture collection, Tamil Nadu Agricultural University, India. Out of 55 strains, 10 strains showed an antagonistic effect on plant pathogen fungus *Sclerotinia sclerotiorum*, which causes stem rot of carnation. They identified them at the strain level by molecular characterization and found the AMP gene in all the selected strains. *Bacillus amyloliquefaciens* (VB7) had a maximum number of antibiotic biosynthesis genes. They also identified that the metabolite produced by *B. amyloliquefaciens* strains VB2 and VB2 had antifungal activity and found that the metabolites were phenols and fatty acids. It was concluded that *B. amyloliquefaciens* could be used for the management of stem rot of cultivated carnations.

Ziedan (2006) isolated 25 bacteria from the root of healthy peanut in Nobaria province, Egypt. They screened out four strains of bacteria that showed antifungal properties against *Fusarium oxysporum* and *Aspergillus niger*. Morphological, physiological, and biochemical analysis showed that one belongs to *Pseudomonas fluorescens* and other three are *Bacillus subtilis.* These isolates were effective in inhibiting root and pod disease in peanut plants. *Bacillus subtilis* strain no. 1 showed the best result under field conditions for control of root rot disease and increased crop production. Zhao et al. (2015) isolated 48 bacterial endophytes in *Lonicer japonica*. They screened out 6 strains out of 48 on the basis of functional properties. Strain 122 and 124 showed high phosphate solubilization activity and a high amount of siderophore production, while a high amount of IAA was produced by strain 170. The wheat plant treated with the endophytic strain 130 showed increased root length, seedling growth, shoot length, and chlorophyll content. These six strains also showed antifungal properties against *Fusarium oxysporum*. Based on 16S rRNA gene sequencing, these six strains were identified as *Bacillus* and *Paenibacillus*. Kushwaha et al. (2020) isolated 19 bacterial endophytes in *Pennisetum glaucum.* All 19 endophytes showed antagonistic activity against *Fusarium solani, Rhizoctonia solani*, and *Sclerotium rolfsii*. These bacterial endophytes also showed PGP activity.

The 16S rRNA sequencing identified that the endophytes belong to three *Bacillus* species, viz., *B. subtilis*, *B. cereus*, and *B. amyloliquefaciens.* The bacterial endophytes belonging to three genera *Bacillus*, *Lysinibacillus*, and *Stenotrophomonas* were isolated from tomato plants and the endophytes had strong antagonism against *Sclerotium rolfsii* causing collar rot in tomatoes. The media used in general for the isolation of bacterial endophytes are nutrient agar, tryptone soybean agar, casein-starch agar, etc. Eevers et al. (2015) reported that the complex medium supplemented with plant extract (medium no. 869) resulted in increased recovery and optimal growth of bacterial endophytes.

8.3 Plant Colonization Ability of Bacterial Endophytes

The root exudates released by the host plants in the rhizosphere attract a variety of microorganisms for their colonization. The primary cause of colonization of microorganisms in the vicinity of the roots is the availability of carbon and other nutrient sources from the root exudates. The entry of bacteria into the root system is through the passive invasion at root hairs or through open root sites or wounds. The bacteria have the ability to colonize plant roots and are equipped with hyperproduction of cellulolytic enzymes to hydrolyze the exodermal cell walls of the host plant (Malea and Serepa-Diamini 2019). The analysis of peanut root tissues through scanning electron microscope (SEM) and transmission electron microscope (TEM) analysis revealed that endophytic bacteria (*Bacillus subtilis* and *Pseudomonas fluorescens*) are capable of colonizing in the cortex of peanut plant root (Ziedan 2006).

Many endophytes are reported to be present in the rhizosphere as well (Rosenblueth and Matrinez-Romero 2006). Figure 8.1 describes the distribution of bacterial endophytes in plant system and the mechanism of biocontrol of soil-borne phytopathogens. It is found that the diversity of bacterial endophytes is found abundant in roots followed by stems, leaves, etc. The rhizospheric bacteria are abundant in root tips, and the specialized bacteria make entry into the plant through the root hairs or wounds or cracks. After entering the root system, the bacteria mostly colonize cortical cells and the vascular system. Similarly, in the stem, the bacteria are abundant in the vascular system, while in the leaf, they make niches in the palisade and mesophyll tissue. Bacterial endophytes have the potential to protect the plants against the infestation of plant pathogens. The mechanism of antagonism against plant pathogens is related to the production of siderophores, antibiotics, or bacteriocins, induced systemic resistance (ISR), hydrogen cyanide (HCN) production, hydrolytic enzymes production, etc. Among the different mechanisms, the production of antibiotics is considered a very efficient mechanism because they directly act on the targeted pathogen.

Recently, the colonization pattern of endophytes in plant roots was established using the method of *gfp* (green fluorescent pigment) tagging and the use of sophisticated microscopy (confocal laser beam microscopy) to localize the endophyte association with the host plant (Timmusk et al. 2005; Annapurna et al. 2013). The

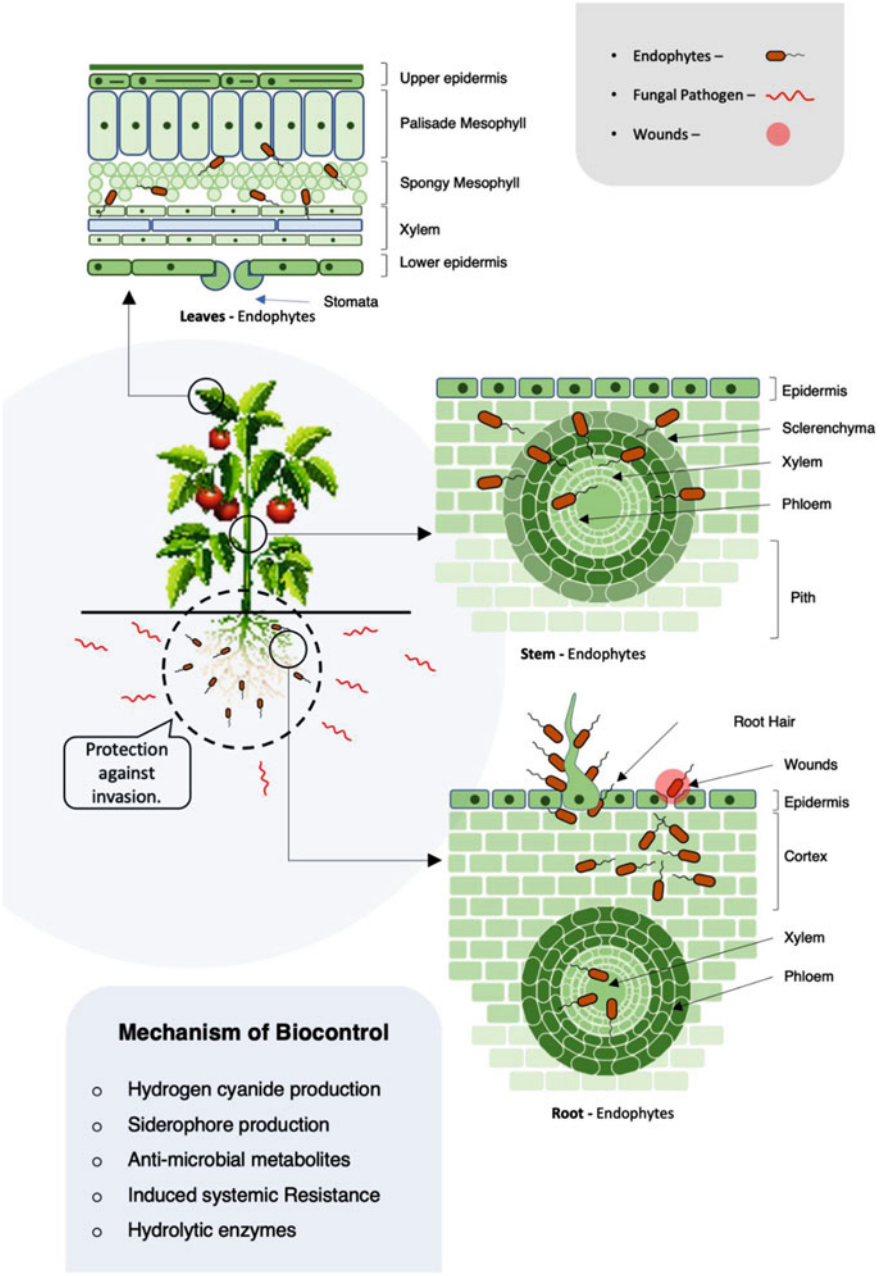

Fig. 8.1 Illustration of colonization of bacterial endophytes and their distribution in plant system

localization of bacterial cells inside the root tissue is clearly visible by green fluorescence in the given figure. Timmusk et al. (2005) showed that *P. polymyxa* invades the root tips and forms biofilm in *Arabdiopsis thaliana*. Annapurna et al. (2013) reported that *P. polymyxa* HKA-15 invade the root nodules of soybean. The co-inoculation of *P. polymyxa* and *B. japonicum* increased the plant growth than the inoculation of *B. japonicum* alone.

The inoculation of *gfp* tagged *P. polymyxa* WL Y78 in crops, wheat, maize, and cucumber seedling under the gnotobiotic system and soil indicated the colonization of *P. polymyxa* in epidermal and cortical cells, intercellular spaces, and vascular system of root, stem, and leaf tissue (Hao and Chen 2017). The comparative, genomic, and functional analysis of *P. polymyxa* strains showed that plant growth-promoting traits are conserved in these bacteria while genes relevant to nitrogen fixation and antibiotic synthesis are evolved with the diversity (Xie et al. 2016). The colonization capability of bacterial endophytes was also tested in crop plants that are different from their host plant. Confocal scanning laser microscope imaging using LIVE/DEAD™BacLight™ bacterial viability staining indicated trans-genera colonization of *O. tenuiflorum* endophytes in rice (Sahu et al. 2020) (Fig. 8.2).

8.4 Important Soil-Borne Phytopathogens

The four foremost soil-borne diseases that occur in wide crop plants and cause crop losses to the tune of 50–60% and sometimes more globally are being discussed in this section. The other pathogens are discussed in other chapters in the book. They are wilt caused by *Fusarium oxysporum*, collar rot (*Sclerotium rolfsii*), wet root rot (*Rhizoctonia solani*), and stem rot (*Sclerotinia sclerotiorum*).

*8.4.1 Wilt (*Fusarium oxysporum*)*

It causes an average yield loss of 10–12% globally. The pathogen is a facultative saprophyte and can survive in soil or crop residues as chlamydospores for up to 6 years. The pathogen is seed- and soil-borne. The symptoms are drooping of petioles, rachis, and leaves and internal discoloration (browning of xylem vessels).

*8.4.2 Collar Rot (*Sclerotium rolfsii*)*

The disease occurs 6 weeks after sowing. The pathogen produces densely floccose, white septate mycelium. The pathogen produces numerous olive brown to clove brown, lobose hard fungal bodies (sclerotia) measuring 0.8 to 2 mm on host tissues and in laboratory culture.

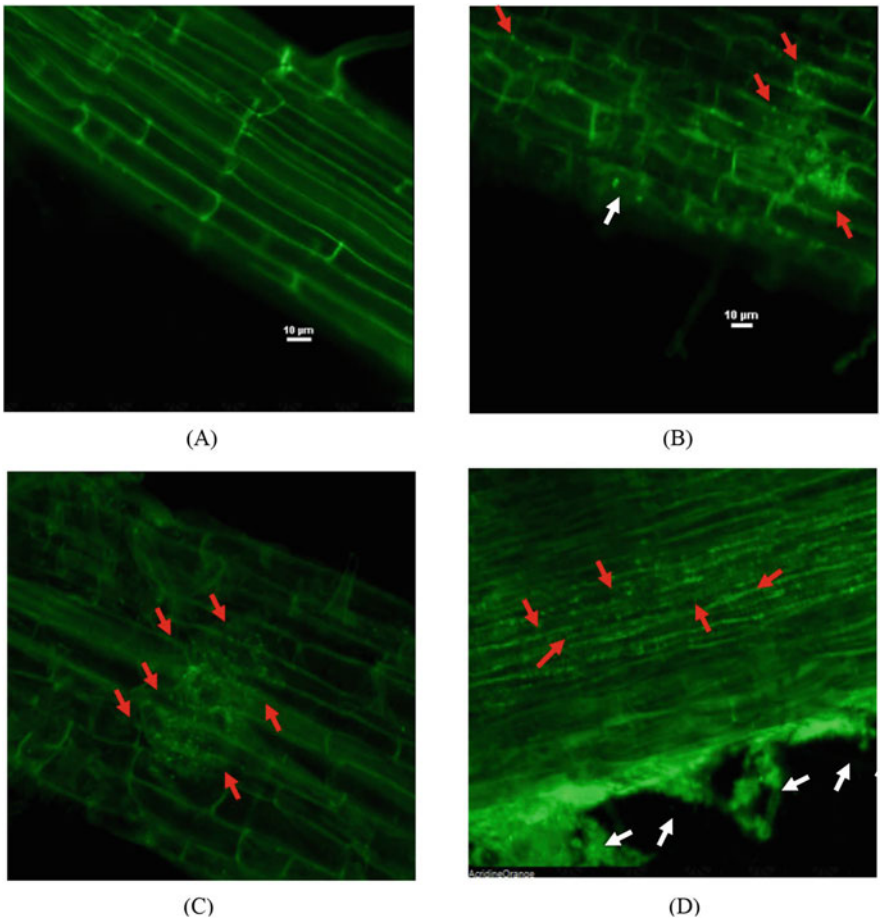

Fig. 8.2 Colonization pattern of *O. tenuiflorum* endophytes in rice roots. Red arrows indicate endophytic colonization, and white arrows indicate colonization at the external root surface. (**a**) Uninoculated control root, (**b**) *B. tequilensis* BTL-4 inoculated root, (**c**) *B. safensis* BTL-5 inoculated root, and (**d**) *B. altitudinis* GTS-16 inoculated root

*8.4.3 Root Rot (*Rhizoctonia solani*)*

Among the root rot diseases, wet root rot caused by *R. solani* is one of the important diseases. The disease occurs in the seedling stage. The pathogen is soil- and seed-borne, having a wide host range. It can infect the cop at all stages from seed germination to harvest. The symptoms are wet rotting of root initially appeared pale green and later turn into yellow color.

8.4.4 *Stem Rot (*Sclerotinia sclerotiorum*)*

It is one of the most devastating soil-inhabiting fungal plant pathogens infecting various crop plants including chickpea (Mandal and Dubey 2012). The pathogen is a homothallic ascomycetous necrotrophic fungal plant pathogen dispersed as airborne ascospores or soil-borne sclerotia. The symptoms are white or grayish white cottony fungal growth in the affected plant parts commonly known as "white mold." It is one of the emerging pathogens in vegetables, pulses, oilseed, and flower crops (Dutta et al. 2016). It is considered one of the serious pathogens of many plants because it is capable of infecting multiple organs (roots, stem, floral parts, fruits, etc.).

8.5 Biological Control of Soil-Borne Phytopathogens by Bacterial Endophytes in Different Crops

In view of the adverse effect caused by fungicides in the environment and increasing awareness about sustainable agriculture, biocontrol of soil-borne fungal pathogens is an eco-friendly alternative solution to chemical control. The different biocontrol agents commercially used are *Trichoderma viride*, *T. harzianum*, *Gliocladium catenulatum*, *Streptomyces* spp., *Pseudomonas fluorescens*, *Bacillus subtilis*, and *B. amyloliquefaciens* (Singh et al. 2018). The group of bacteria that are residing inside the plant system are called bacterial endophytes. Endophytes are involved in the synthesis of siderophores, production of plant hormones, nitrogen fixation, solubilization of immobilized phosphorus, production of volatile organic compounds, nutrients recycling, and pathogenic resistance and stress tolerance (Firdous et al. 2019).

Pseudomonas and *Bacillus* sp. produce low-molecular-weight lipopeptide antibiotics active at low concentrations against a broad spectrum of human, animal, and plant pathogens. In a commercial point of view, *Bacillus* sp. and *Paenibacillus* sp. have wider applications due to greater stability in the population in the steps of formulation and storage of inoculant products (Santos et al. 2018). *Bacillus subtilis* strain B29 produces an antifungal protein that has a broad spectrum of activity against fungal pathogens, *Fusarium oxysporum*, *Rhizoctonia solani*, *Fusarium moniliforme*, and *Sclerotinia sclerotiorum* (Li et al. 2009). Sheath blight of rice caused by *R. solani* Kuhn is an important soil-borne disease throughout the rice-producing areas in the world. Nagendran et al. (2014) reported the endophytic *Bacillus subtilis* var. *amyloliquefaciens* (FZB24) had maximum inhibition (36%) against *R. solani* over the control. The application of bioagent (FZB24) through seed treatment (4 g kg^{-1}), soil application (500 g ha^{-1}), and foliar application (500 g ha^{-1}) had 55% reduction of disease incidence over the control under glasshouse conditions. In another study, Jamali et al. (2019) reported that biocontrol agent *B. subtilis* RH5 inoculation in rice plants pre-challenged with *R. solani* resulted in a significant increase in plant growth and triggered resistance in rice

Table 8.2 Benefits associated with inoculation of bacterial endophytes in crop plants

S. no.	Crop evaluated	Bacterial endophyte tested	Associated benefits	Reference
1.	Cotton	*Paenibacillus polymyxa* ShX301	Biocontrol of *Verticillum* wilt in cotton	Zhang et al. (2018)
2.	Soybean	*P. polymyxa* HKA-15 *Bacillus subtilis* PBRS-1, AP-3	Biocontrol of *R. bataticola* (dry root rot and *X. campestris* pv. *phaseoli* (common blight) in soybean, production of bacterial lipopeptides against seed-borne pathogenic fungi in soybean	Senthilkumar et al. (2009), Mageshwaran et al. (2012), Araujo et al. (2005)
3.	Peanut	*Bacillus subtilis, Pseudomonas fluorescens*	Biocontrol of soil-borne diseases of peanut (*A. niger* and *F. oxysporum*)	Ziedan (2006)
4.	*Lonicera japonica* (medicinal plant)	*Bacillus* and *Paenibacillus* strains	Plant growth promotion in wheat	Zhao et al. (2015)
5.	Rice	*Herbaspirillum seropedicae*, *Serratia* sp., *Bacillus altitudinus* GTS-16, *Bacillus subtilis* var. *amyloliquefaciens* (FZB24)	Plant growth promotion in rice; suppression of sheath blight disease	Bao et al. (2013), Sahu et al. (2020), Nagendran et al. (2014)
6.	Canola	*P. polymyxa*	Production of fusaridicin type of antibiotics	Beaty and Jensen (2002)
7.	Banana	*Herbaspirillum seropedicae*	Plant growth promotion	Weber et al. (1999)
8.	Carrot	*Pseudomonas fluorescens*	Plant growth promotion	Surette et al. (2003)
9.	Citrus	*Bacillus pumilus*	Plant growth promotion	Araujo et al. (2002)
10.	Sugarcane	*Herbaspirillum seropedicae*	Plant growth promotion	Olivares et al. (1997)
11.	Carnation	*Bacillus amyloliquefaciens* VB7	Biocontrol of stem rot caused by *Sclerotinia sclerotiorum*	Vinodkumar et al. (2017)
12.	Tomato	*Bacillus* sp. 2P2	Induction of systemic resistance against collar rot pathogen *Sclerotium rolfsii*	Sahu et al. (2019)

plants through the production of defense-related antioxidant enzymes. Similarly, Sahu et al. (2020) reported that rice plants challenged with *R. solani* and inoculated with *B. altitudinis* GTS-16 exhibited the lowest sheath blight incidence than control.

Table 8.2 describes the benefits associated with the inoculation of bacterial endophytes in crop plants.

The bacterial endophytes have also been reported for biocontrol of horticultural and floricultural crops. The bacterial endophyte *B. amyloliquefaciens* VB7 was found to reduce significantly the disease incidence of stem rot caused by *Sclerotinia sclerotiorum* and enhance the plant growth in carnation than the control (pathogen alone) (Vinodkumar et al. 2017). Chickpea *Fusarium* wilt severity caused by *Fusarium oxysporum* f. sp. *ciceri* (FOC1) was significantly reduced from 60 to 99% in susceptible cultivar ILC 482 treated with antagonistic *Bacillus* spp. (Rb29, Rb6, Rb12, Rb4, and Rb15) under field trials (Zaim et al. 2013). Thus, *Bacillus* strains were found to be efficient in the control of chickpea wilt disease.

8.6 Mechanism of Biological Control of Soil-Borne Phytopathogens by Bacterial Endophytes

The bacterial endophytes possess different antagonistic traits for suppression of soil-borne pathogens, which include competition for colonization site or nutrients, production of volatile/diffusible antibiotics, synthesis of PR proteins in host plants, and production of enzymes and biocidal compounds. Zaim et al. (2016) described that the various mechanisms used by bacterial endophytes and plant growth-promoting rhizobacteria for the suppression of soil-borne fungal pathogens are synthesis of enzymes that hydrolyze fungal cell walls, synthesis of hydrocyanic acid (HCN) that suppresses the growth of fungal pathogens, production of antibiotics that kill the phytopathogen fungus, induction of systemic resistance (ISR), and antagonism against phytopathogenic microorganisms by production of siderophores. The illustrative description of the mechanism of biocontrol of plant pathogens by bacterial endophytes is given in Fig. 8.1. In addition, the bacterial endophytes and PGPR directly enhance the growth of host plants through solubilization of minerals like phosphates and micronutrients, secrete phytohormones like IAA, gibberellic acid, etc., and fix atmospheric nitrogen. In this chapter, we discuss antimicrobial metabolites, induction of systemic resistance, and HCN and siderophore production by antagonistic bacterial endophytes as the major mechanism of suppression of potential soil-borne fungal pathogens.

8.6.1 Antimicrobial Metabolites

The endophytes and other biocontrol agents are reported for the synthesis of antimicrobial compounds of small molecular weights, usually less than 3.5 kDa. They are generally classified as lipopeptides (LP) containing amino acid chains with a lipid moiety. They are composed of hydrophobic tail, which is usually a fatty acid,

linked to a hydrophilic head between 4 and 12 amino acids. The major group of microorganisms that produce LPs are *Bacillus*, *Pseudomonas*, yeasts, etc. The cyclic lipopeptides contain a lactone ring in the amino acid chain. The different classes of cyclic lipopeptides reported in bacterial endophytes and rhizobacteria are bacilysin, subtilin, fengycin, surfactin, iturins, lichenysins, viscosins, amphisins, etc. (Biniarz et al. 2017). The structural details of selected cyclic lipopeptides are presented in Fig. 8.3.

An endophytic bacterium, *Bacillus amyloliquefaciens* ES-2 isolated *from Scutellaria baicalensis*, produced two families of secondary metabolites with broad-spectrum antibacterial and antifungal activities. This bacterium showed an antagonistic effect on plant pathogens, food spoilage bacteria and fungi, and food-borne pathogens. The electrospray ionization/collision-induced dissociation spectrum analysis revealed that the antimicrobial metabolites belong to fengcyin and surfactin homologs, respectively. These lipopeptide antibiotics could be used against fungal plant diseases and in food preservation (Sun et al. 2006). Mageshwaran et al. (2012) isolated an antimicrobial compound from endophytic bacteria *Paenibacillus polymyxa* HKA-15. The antimicrobial compound produced by *P. polymyxa* HKA-15 was partially characterized using mass spectrophotometry and SDS-PAGE. The lipopeptide compound produced by *P. polymyxa* HKA-15 showed antagonism against plant pathogenic fungi and bacteria, viz., *Xanthomonas campestris* pv. phaseoli M-5, *Xanthomonas oryzae*, *Ralstonia solanacearum*, *Xanthomonas campestris* pv. *phaseoli* CP-1-1, *Fusarium udum*, *Rhizoctonia bataticola*, and *Macrophomina phaseolina*.

Hyun et al. (1999) isolated an antibiotic compound from *Bacillus polymyxa* strain KB-8. The isolated compound was active against *Fusarium oxysporum* f. sp. *sesame* causing Fusarium wilt of sesame. The media and pH were optimized for large-scale production of the antibiotic produced by *B. polymyxa* KB-8. The optimized parameters were yeast-malt extract medium, pH 5, and 13-day incubation. The isolated antibiotic inhibited the growth of *F. oxysporum*, *Rhizoctonia solani*, *Alternaria mali*, *Colletotrichum gloeosporioides*, and *Phytopthora* sp. Soil drenching of the lipopeptide antibiotic at the concentrations of 13.0 μg/ml and 26.0 μg/ml effectively inhibited the *Fusarium* wilt of sesame under greenhouse conditions. Kim et al. (2003) isolated and characterized the antibiotic produced by *Bacillus* strain GB-0356 and GB-017. The partial characterization of antibiotic revealed that the antibiotic belongs to lactone and polyene groups. Both strains showed antifungal properties against *Rhizoctonia solani*, *Fusarium* sp., *Pythium* sp., and *Botrytis cineria.*

Screening of antimicrobial peptide genes elucidates the potential of a biocontrol agent for the synthesis of antimicrobial peptides that are active against pathogens. The biocontrol potential of *B. subtilis* RH5 against sheath blight of rice caused by *R. solani* was evaluated through the presence of antimicrobial peptide (AMP) biosynthetic genes (bacylisin, surfactin, and fengycin) (Jamali et al. 2019). *Pseudomonas brassicacearum* strain YC5480 inhibited the growth of plant pathogenic fungi like *Phytophthora capsici*, *Colletotrichum gloeosporioides*, and *Fusarium oxysporum*, but they also produce some compounds that inhibit the growth of seed

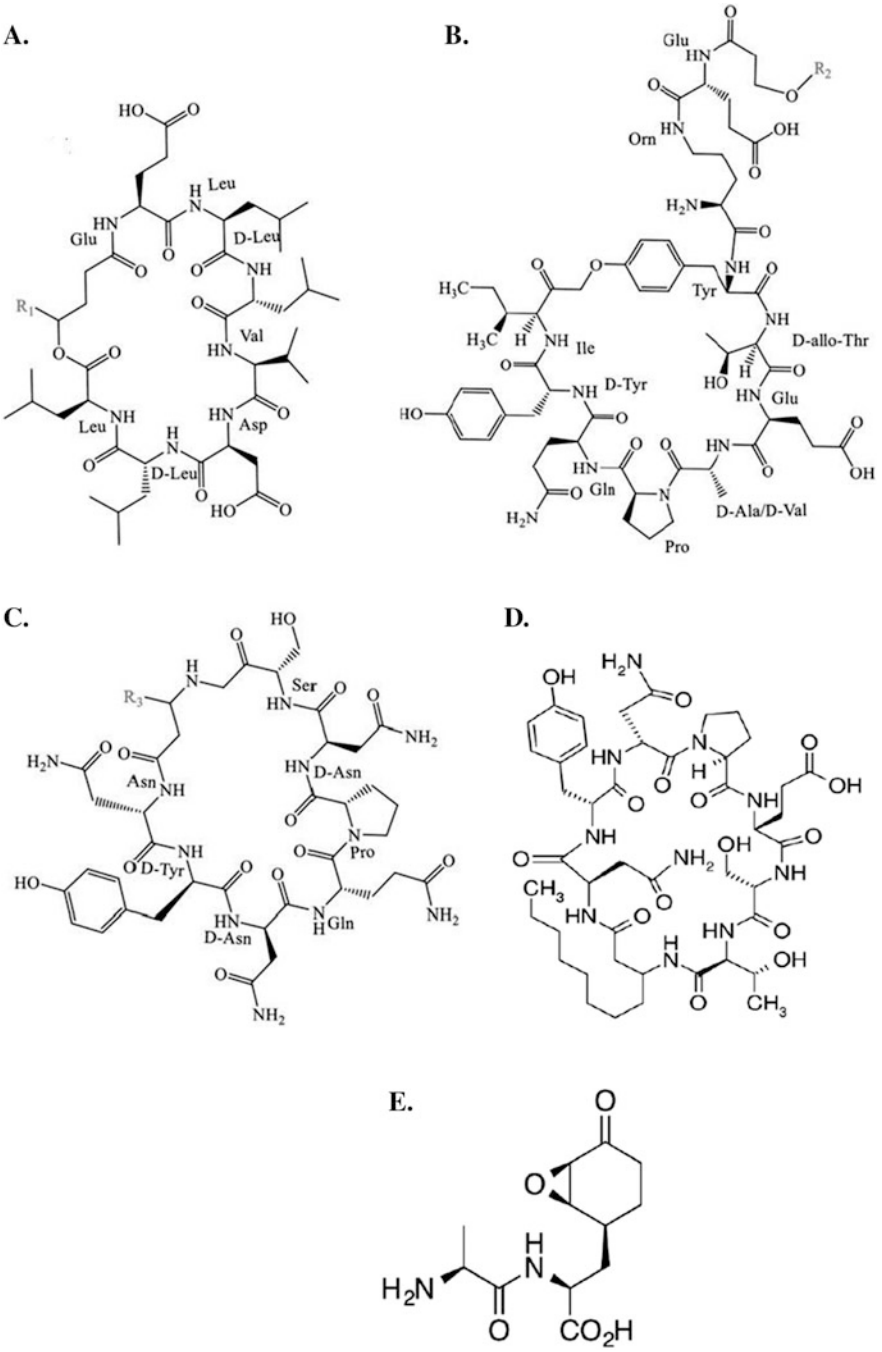

Fig. 8.3 Structural details of cyclic lipopeptides: surfactin (**a**), fengycin (**b**), iturin (**c**), bacilomycin (**d**), and bacilysin (**e**)

germination. These compounds identified KS-1 and KS-2 and chemically found to be 2,4-diacetylphloroglucinol (DAPG) and 2,4,6-trihydroxyacetophenone (THA), respectively. Tendulkar et al. (2007) reported that *Bacillus licheniformis* BC98 was able to produce surfactin with a molecular mass 1035 Da and suppress the phytopathogens *Magnaporthe grisea*, *Curvularia lunata*, and *Rhizoctonia bataticola*. The endophytic *B. amyloliquefaciens* strain VB7 was able to harbor ten diverse antibiotic biosynthesis genes, namely, *ituD*, *ipa14*, *bacA*, *bacD*, *bamC*, *sfP*, *spaC*, *spaS*, *albA*, and *albF*, which correspondingly produce the antibiotics iturin, bacilysin, bacilomycin, surfactin, subtilin, and subtilosin (Vinodkumar et al. 2017).

8.6.2 Induced Systemic Resistance

The expression of certain defense-related genes in the host plant indicates the resistivity of the plants against the invading pathogens. Gurjar et al. (2012) studied the expression of defense-related genes during wilting in chickpea caused by *Fusarium oxysporum* f. sp. *ciceri*. Defense-related genes such as Chalcone Synthase (*CHS*) gene, Isoflavone Reductase (IFR) gene, 60s ribosomal protein (60srp), etc., are upregulated in chickpea root tissues in resistant cultivar (Digvijay) compared to the susceptible cultivar (JG62). The presence of plant growth-promoting bacteria as endophytes inside the host plants stimulates the production as well as activity of pathogenesis-related (PR) proteins such as peroxidiase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), chitinases, lipoxygenases, and glucanases to suppress the invading pathogen and host plant self-defense mechanism. The inoculation of antagonistic bacteria (*Bacillus* and *Pseudomonas*) along with *Mesorhizobium* in chickpea plants pre-challenged with *Fusarium oxysporum* f. sp. *ciceri* induced PAL and malonic aldehyde concentrations in stem tissues revealed the plant defense response to reduce the disease incidence and to improve plant growth and yield (Kumari and Khanna 2019). Nagendran et al. (2014) reported that in rice plants pre-challenged with *R. solani* causing sheath blight disease, higher induction of defense-related enzymes—PO, PPO, and PAL—and higher accumulation of total phenols were observed in *B. subtilis* var. *amyloliquefaciens* (FZB24) inoculated rice plants than in untreated (pathogen alone). Similarly, Jamali et al. (2019) reported that in addition to the enhancement of plant growth-promoting traits, the inoculation of *B. subtilis* RH5 triggered defense-related enzymes PO, PPO, and PAL in rice plants pre-challenged with *R. solani*. The endophytic strain *Bacillus* sp. 2P2 showed strong inhibition against collar rot pathogen in tomatoes. The strain induced systemic resistance in the host plant, elicited PAL, PO, and PPO, and upregulated pathogenesis-related proteins PR1a, PR2a, and PR3, which are responsible for the synthesis of glucanases and chitinases. Sahu et al. (2020) reported the PAL and PO activity was higher in *R. solani* pre-challenged and endophytes inoculated rice plants, and the value recorded was 30 nM cinnamic acid h^{-1} g^{-1} fresh weight and 3.2 units min^{-1} mg^{-1} fresh weight, respectively.

8.6.3 HCN and Siderophore Production

Hydrogen cyanide (HCN) is the secondary metabolite produced at the end of the exponential phase and the start of the stationary phase. It is synthesized by *hcn*ABC, which oxidizes glycine to produce HCN and CO_2 (Laville et al. 1998), and it is volatile and controls the growth of surrounding microorganisms (Akhtar and Siddiqui 2006). HCN inhibits electron transport, which disrupts the cell's energy supply and causes cell death. In particular, cytochrome oxidase and other metallozymes are inhibited by HCN and hence are toxic molecules for all aerobic organisms. HCN production has been demonstrated in a wide range of bacterial genera and endophytes, including *Alcaligenes*, *Aeromonas*, *Bacillus*, *Pseudomonas*, and *Rhizobium* species (Bhuiyan et al. 2008). According to several studies (Gagné et al. 1991), volatile compounds may also help prevent several plant diseases. According to Ramette et al. (2003), fluorescent *Pseudomonads* associated with several plants were using HCN, a broad-spectrum antibacterial chemical, to biologically control root diseases. HCN inhibits electron transfer and through reversible mechanisms of inhibition, it interferes with the efficient functioning of the enzymes and natural receptors (Corbett 1974). For example, *Macrophomina phaseolina* can be efficiently controlled by *Pseudomonas fluorescence* production of HCN (Reetha et al. 2014). Many rhizobacteria produce hydrogen cyanide (HCN), which is known to be involved in the biological control of pathogens (Defago et al. 1990). *Phytophtora infestans* was inhibited by strains of *Pseudomonas* associated with potatoes at various developmental stages. Multiple mechanisms, including the production of hydrogen cyanide, were identified as potentially contributing to this anti-oomycete activity using a comparative genomics approach. In this study, HCN-negative mutants (*Dhcn*) were generated and their activities were compared to those of their corresponding wild types in order to quantify the contribution of HCN in biocontrol (Anand et al. 2020). Numerous species have evolved various defense mechanisms against cyanide poisoning, such as cyanide-insensitive oxidases or chemical conversion of HCN to thiocyanate by the rhodanese enzyme (Cipollone et al. 2007; Frangipani et al. 2014; Cunningham et al. 1997).

Iron is a vital element required by all living organisms for many cellular processes such as electron transport chain and as a cofactor for many enzymes (Litwin and Calderwood 1993). Microorganisms growing under aerobic conditions need iron for a variety of functions including reduction of oxygen for the synthesis of ATP, the formation of heme, and other essential purposes. Siderophores are the low-molecular-weight iron binding substances secreted by endophytes for the acquisition of iron present in the environment, thereby limiting the availability of iron to the plant pathogens present in the same niches. Thus, endophytes suppress the pathogens by limiting the availability of iron in the environment. Under iron-limiting conditions, siderophores—low-molecular-weight ferric iron-chelating compounds—are secreted extracellularly, with their main objective being to provide iron to iron-deficient cells (Sessitsch et al. 2004). Three primary forms of siderophores—catecholate, hydroxymates, and carboxylates—are formed

depending on the functional group. The use of radio-labeled ferric siderophores as a sole source of iron demonstrated that plants could take up labeled iron by PGPB. Some PGPR strains form siderophores that bind Fe^{3+}, reducing its availability to specific local microflora species (Kloepper et al. 1980). When grown on Chrome Azurol S with an iron deficiency, *P. fluorescens* was reported to produce extracellular siderophores (Suryakala et al. 2004). The other genera reported are *Aeromonas*, *Azadirachta*, *Azotobacter*, *Bacillus*, *Burkholderia*, *Pseudomonas*, *Rhizobium*, *Serratia*, and *Streptomyces*. Siderophores, which provide iron to plants, also may assist in reducing the stresses that high soil levels of heavy metals place on plants. Soil bacteria enhance plant iron intake (Diels et al. 2002). According to O'Sullivan and O'Gara (1992), chemicals like siderophores are mostly produced during the exponential growth phase, when the population needs more nutrients for cell division. Similar to how most secreted pseudobactin molecules bind to Fe in the media, the pseudobactin Fe complex has a high stability factor (Chen et al. 1994; Loper and Henkels 1999). The antagonistic bacteria may scavenge most of the available iron and prevent the growth of fungal pathogens since the siderophores produced by biocontrol bacteria have a higher affinity to iron than the siderophores produced by fungal pathogens. Recently, Ji et al. (2014) reported that the endophytic diazotrophs *Klebsiella pneumoniae*, *B. subtilis*, and *Microbacterium* sp. displayed antagonistic behavior toward *R. solani* as a siderophore producer.

8.7 Conclusion and Way Forward

In most instances, the screening of bacterial endophytes and rhizobacteria for the development of microbial inoculants in agricultural applications is often encountered with the genera *Bacillus* and *Pseudomonas*. Bacterial endophytes are the rhizobacteria that have the unique capability to enter the host plant and reside inside different plant parts of the host. *Bacillus* and *Pseudomonas* offer benefits to the host plant directly and indirectly through multiple plant growth-promoting traits. The direct benefits include fixation of atmospheric nitrogen, solubilization of nutrients, secretion of phytohormones and antimicrobial metabolites, synthesis of ammonia, siderophore, HCN, etc., which are helpful in plant growth and yield and also provide protection against invading pathogens. The indirect benefits offered by the bacterial endophytes to the host plant include induction of systemic resistance through the synthesis of PR proteins and phenols, PAL, PPO, PO, etc., thus offering resistance against biotic stress. The endophytic *Bacilli* are reported for the synthesis of novel cyclic and acyclic lipopeptides, which are receiving much attention in biotechnological applications in agriculture and beyond, especially in medical fields, due to increasing resistance of pathogens against conventional pesticides and chemicals.

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Chapter 9
Endophytes: Rendering Systemic Resistance to Plants

Diptanu Datta, Lopamudra Behera, Vallabhaneni Tillak Chaudhary, Sumit Kumar, and Kartikay Bisen

Abstract Endophytes are those microorganisms that live throughout the life cycle of a plant without causing noticeable negative signs. As endophytes are known to induce resistance in plants and can also suppress phytopathogens, they can be used as a replacement for harmful chemicals. Mostly endophytes establish a symbiotic or commensal relationship with plants, thereby benefitting the latter in terms of nutrition, growth, and disease resistance. Endophytes are known to induce plant hormones, the key chemical constituents that provide tolerance against a number of abiotic and biotic stresses. Endophytes induce resistance in plants and also inhibit phytopathogens by producing several bioactive molecules, defense-related enzymes, PR proteins, siderophores, hyper parasitism, antibiosis, and through induced systemic resistance. Different bioformulations on endophytic fungi and bacteria have yielded promising results in the management of diseases. After reviewing the work done on endophytes, it could be concluded that in the era of sustainable agriculture, endophytes opened a new venture in terms of eco-friendly disease management.

Keywords Endophytes · Systemic resistance · Disease management · Bioformulation · Sustainable agriculture

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9.1 Introduction

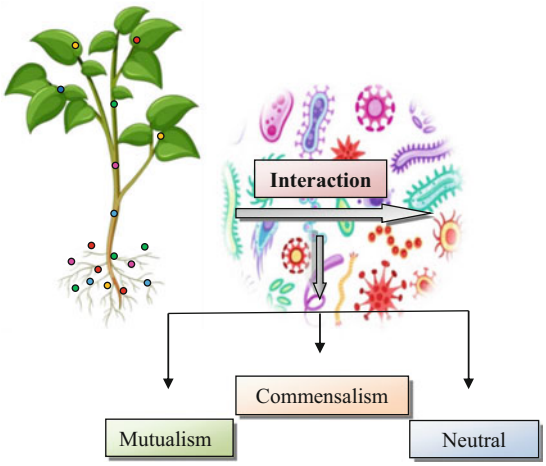
Endophytes ("endo" = inside, "phyte" = plant) are microorganisms (both fungi and bacteria) that live, or complete their life cycle in the living tissue of the plant, without causing obvious negative symptoms. The German botanist Heinrich Friedrich Link (1989) described endophytes for the first time and named them as "endophytae," but the word "endophyte" was coined by Bary (1886). Vogl (1898) was the first to record the existence of the mycelium in the *Lolium temulentum* grass seed. Perotti (1926) was the first person to identify nonpathogenic flora occurring in root tissues. Every plant that has been studied to date has a minimum of one species of endophytic fungus, and several plants, in particular woody plants, can literally have hundreds or thousands of endophytic species (Gaylord et al. 1996; Arnold et al. 2000). Plant tissues are usually inhabited without any harmful impact by bacterial or fungal endophytes. The ability of endophytes to impart resistance to different abiotic stress conditions such as light intensity, cold, temperature, salinity, droughts, etc., and other several biotic stress conditions such as pathogenic and insect attack by various plant growth processes has taken on greater significance in the field of agriculture (Arnold et al. 2003; Bae et al. 2009). It is possible to render the era from 1981 to 1985 a historical one as the research on endophytes in plant defense against herbivorous insects was popularized during this period. Webber (1981) for the first time demonstrated the role of the endophytic *Phomopsis oblonga* to protect elm trees against the beetle *Physocnemum brevilineum*. Endophytes are now drawing considerable interest from researchers in the management of plant and human pathogens. The reduction in the application of hazardous pesticides and chemical fertilizers can only be achieved through the generous use of endophytic microbes. Bargabus et al. (2002) and Mishra et al. (2006) studied the character of endophytes in strengthening the defense mechanism of crops for different plant diseases. Endophytes provide a heterogeneous chemical composition of tissues and organisms in plants and protect the host plant by producing biologically active compounds (Zamioudis and Pieterse 2012). This induced systemic resistance delivers the plant defense in an indirect manner. The essence of endophytes in plants and their impact on agriculture are determined by both climate and edaphic factors. It is required to understand the commonly related microbial symbols of a specific crop in order to optimize the benefits of endophytes in agricultural crops for effective plant physiological system establishment, function in promoting plant growth, and control disease and the ability to produce all bioactive metabolites (Varma et al. 2017). In this book chapter, we have explored in depth the relationship between endophytes and other microbes, their implementation, and their prominent role in systemic plant resistance.

9.2 Brief Overview of Fungal and Bacterial Endophytes

Endophytic fungi are divided both into class I and class II endophytes. Class I endophytes are called clavicipitaceous endophytes, whereas class II endophytes are referred to as non-clavicipitaceous. Class I endophytes are confined to grasses species, establishing a nonpathogenic, systemic, and typically intercellular connections during their life cycles (Arora 1991). They have been found to confer resistance against drought and help in increasing plant biomass (Clay 1988). On the other hand, class II fungal endophytes are mutualistic or symbiotic and provide hosts with benefits during abiotic stress and nutrition for tissue growth and reproduction (Rodriguez et al. 2009). *Trichoderma* spp. is among the most studied fungal endophyte species in the case of agricultural crops (Uppala 2007; Romão-Dumaresq et al. 2012). The other beneficial fungal endophytes reported are *Epicoccum nigrum* (Fávaro et al. 2012), *Penicillium* spp., *Alternaria*, *Xylaria*, *Cladosporium* (Paul et al. 2012), *Chaetomium globosum* (Naik et al. 2009), *Aspergillus*, *Curvularia* (Zakaria et al. 2010), *Aspergillus flavus*, *Alternaria alternata*, *Acremonium zeae*, *Fusarium verticillioides*, *Trichoderma* spp. (Orole and Adejumo 2011), etc. Romão-Dumaresq et al. (2012) reported the role of endophyte *Trichoderma virens* in inhibiting infection of *Ceratocystis paradoxa*, known to cause pineapple disease of sugarcane by secretion of endochitinases. *Lecanicillium lecanii*, *Beauveria bassiana*, and *Paecilomyces* spp. have been found to give protection against aphids in cotton (Sword et al. 2012). In black pepper, Sreeja et al. (2016) reported the antagonistic activity of *Ceriporia lacerate*, *Fusarium* spp., *Phomopsis* spp., and *Diaporthe* spp. and against *Radopholus similis* and *Phytophthora capsici*. The most common bacterial endophytes found in agricultural crops are *Acetobacter diazotrophicus*, *Bacillus* spp., *Serratia* spp., *Enterobacter* spp., *Agrobacterium radiobacter*, *Pseudomonas putida*, and *P. fluorescens* (Ramesh et al. 2009; Maheswari et al. 2013; Döbereiner et al. 1995; Gyaneshwar et al. 2001; Uppala et al. 2010; McInroy and Kloepper 1995). *Herbaspirillum seropedicae* and *Acetobacter diazotrophicus* were found to fix nitrogen in sugarcane plants (Döbereiner et al. 1995). In banana, the antagonism properties of *Bacillus amyloliquefaciens*, *B. thuringiensis*, and *B. subtilis* against *Colletotrichum* spp. and *Fusarium oxysporum* f. sp. *cubense* causing anthracnose and panama wilt, respectively, were explored by Souja et al. (2014). In sunflower, *Bacillus pumilus* and *Achromobacter xylosoxidans* help in seedling development under water stress conditions and also induce the production of salicylic acid (Forchetti et al. 2010). In brinjal, *Pseudomonas fluorescens* was found to enhance the IAA and siderophore production. It was also found to give protection against *Ralstonia solanacearum*, the bacterial wilt pathogen in brinjal. According to Uppala et al. (2010), both *Bacillus* spp. and *Pseudomonas* spp. enhance the production of defense-related enzymes, i.e., phenylalanine ammonia lyase, peroxidase, and polyphenol oxidase, thereby inducing systemic resistance. The chilli endophyte *Pseudomonas fluorescens* EBS 20 gives protection against *Pythium aphanidermatum* (Muthukumar et al. 2010).

9.3 Plant Endophyte Interaction

Endospheres of the plant community contain various microbial endophytes that constitute a complicated micro-ecosystem. Several types of interactions can be built up between endophytic microbes and plants that can be widely categorized as beneficial, harmful, and neutral. Nevertheless, numerous types of interactions exist between plants and endophytic microorganisms in ecological terms (Schulz and Boyle 2005). However, a greater number of endophytic microbes are commensal, which means they get benefits from the host plants in terms of nutrition or any other means of survival. But some other microbes like fungal and bacterial endophytes show a symbiotic relationship with host plants (Brader et al. 2014). In this type of interaction, the host supplies nutrition to microbial endophytes and in return endophytic microbes provide systemic resistance to plants to fight against invading pathogens (Shimizu 2011; Dochhil et al. 2013). Besides this, the endophytic microbes help in the physiological processes of plants and promote growth and development. Some authors (Mostert et al. 2000; Wani et al. 2015) have categorized endophytic microbes as systemic and nonsystemic endophytes since endophytes may have associations with the host plants in the short or long term. Nonsystemic endophytes are facultative and temporary and vary over time in population size based on host factors such as plant development, environmental status, and other biotic factors such as pathogen invasions. These nonsystemic endophytes can become parasites from symbionts eventually after residing some period in the host and damage the host in certain ways. Systemic endophytes have mutualistic relationship with plants. Hence, systemic endophytic microorganisms can be transferred vertically among the host plants. However, some systemic endophytes colonize their host and form part of the plant endobiome through horizontal transfers (Wani et al. 2015; del Carmen and Santoyo 2020). This symbiotic relationship enables endophytes to acquire some genetic information to generate a particular bioactive compound close to the host plant via horizontal gene transfer. The type of interaction between host plants and endophytes varies depending upon the host genotypes. Plants like sugarcane, rice, and maize give the endophytes adequate living conditions to promote nitrogen fixation and provide plants with nutrition (Boddey et al. 1995; Engelhard et al. 2000; Iniguez et al. 2004).



9.4 Role of Endophytes in Contributing Systemic Resistance to Plants Diseases and Mode of Action

Endophytes store a large number of bioactive metabolites, including phenolic acids, alkaloids, quinones, hormones, tannins, saponins, and terpenoids, thereby fostering plant tolerance to biotic and abiotic stresses (Mousa and Raizada 2013; Lugtenberg et al. 2016). Endophytes provide the plants with resistance to pathogens either directly (endophytes/pathogens interactions) or indirectly (enhanced plant defense). In the case of direct mechanism, endophytes secrete antibiotics that help inhibit pathogens (Arnold et al. 2003). Commonly, endophytes are known to produce many secondary metabolites, and a few of them show antifungal and antibacterial properties, which in turn effectively inhibit the development of plant pathogens (Gunatilaka 2006).

9.4.1 Different Modes of Action of Endophytes in Combating Plant Pathogens

Endophytes have multiple pathways to counteract the harmful effects of different plant pathogens on the host plants.

9.4.1.1 Competitive Root Colonization

The rhizospheric PGPB (plant growth-promoting bacteria) have been described as plant defenders against various diseases. It has been found that the root epidermis has a lot of supplements that draw a wide variety of microorganisms, including those that cause diseases. Reports have shown the role of flagella in PGPB migration to nutrient-rich root surfaces, and these PGPB have been able to make use of nutrients, i.e., root exudate that oozes from the surface of plant roots (Turnbull et al. 2001; Duffy 2001).

9.4.1.2 Hyperparasitism

Endophytes specifically target known microorganisms or their propagules during hyperparasitism (Tripathi et al. 2008). Pathogens are attacked by endophytic fungi by twisting and penetrating the hyphae and the development of lyase enzyme that breaks the pathogen's cell wall. *Trichoderma* sp. was found to invade into hyphae of *Rhizoctonia solani* (Grosch et al. 2006). In nutrient-deficient environments, most endophytes reveal their predatory features.

9.4.1.3 Competition for Ferric Iron Ions

Iron is an essential survival factor of microorganisms that often does not occur in the root region. The application of endophytic bacterium *P. fluorescens* helps to inhibit *Erwinia carotovora* pathogen as it competes actively with the pathogen to acquire the organic iron. Many endophytic bacteria are found to overcome the growth of harmful microbes by secretion of siderophore (Husen 2003). Siderophores are known to inhibit many plant pathogenic fungi, such as *Phythium ultimum*, *Phytophthora parasitica*, *Sclerotinia sclerotiorum*, and *Fusarium oxysporum* (McLoughlin et al. 1992; Buysens et al. 1996). The role of siderophores produced by several PGPB in biological control of *Erwinia carotovora* was confirmed by Kloepper et al. (1980).

9.4.1.4 Competition for Nutrients and Niches (CNN)

The mechanism of competition for nutrients and niches created numerous advantages for those endophytic bacteria that help in the management of pathogens. This mechanism was found to inhibit root rot in avocados (Pliego et al. 2008).

9.4.1.5 Antibiosis and Antibiotics Suppressing Pathogens

Antibiosis is an important mechanism for controlling disease-causing microorganisms, which has been documented in crops. It involves the release of similar secondary metabolites and other volatile compounds by the biocontrol agent (Fravel 1988). *Bacillus* species could secrete peptide antibiotics and several lipopeptides in plenty with specific activities against plant pathogenic fungi. Bais et al. (2004) reported that surfactin, an antibiotic used to control plant pathogens, has been seen to be effective in *Arabidopsis* against *Pseudomonas syringae.* Benzothiazole, pyrazine (2,5-dimethyl), phenol (4-chloro-3-methyl), and phenol-2,4-bis (1,1-dimethylethyl), the volatile organic compounds from Bacillus velezensis ZSY-1, showed substantial antifungal activity against Botrytis cinerea, Alternaria solani, Monilinia fructicola, and Fusarium oxysporum (Gao et al. 2017).

9.4.1.6 Production of Defense-Related Enzymes

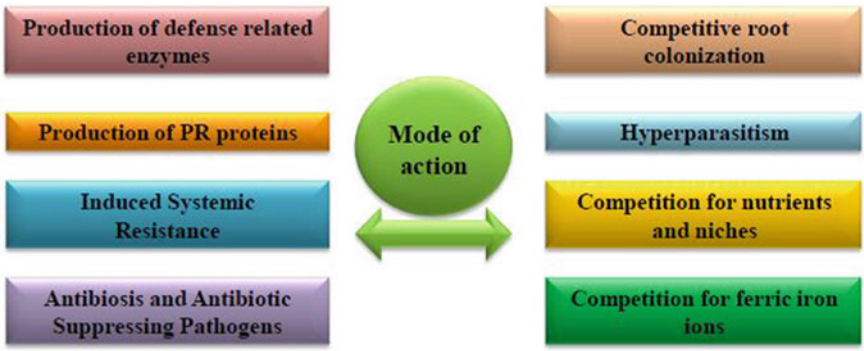
Plant disease management is mainly focused on the use of bactericides, fungicides, and pesticides toxic to plant pathogens, causal gents, or plant disease vectors. Defense enzymes such as peroxidase, PAL, chitinase, polyphenoloxidase, and β-1,3-glucanase are related to resistance induction in plants (Gajanayaka et al. 2014; De Costa 2015). Chitinases are a large group of defense-related enzymes and are also one of the important plant pathogenesis-related proteins (PRPs), which degrade chitin and improve the plant defense against the pathogens having chitin in them (Jalil et al. 2015). Haggag and Abdallh (2012) stated the production of chitinase enzymes by endophytic *Streptomyces hygroscopicus*, which has been identified as an inhibitor of *Rhizoctonia solani*, *A. alternata*, *F. oxysporum*, *Aspergillus flavus*, *Aspergillus niger*, *Sclerotinia scleotiorum*, and *B. cinerea.* Endophytic strain *B. cereus* 65 isolated by Pleban et al. (1995) has been documented to produce chitinase enzymes, which when applied directly to the soil substantially protected the cotton seedlings from *R. solani*, the causal agent of root rot disease.

9.4.1.7 Production of Pathogenesis-Related Proteins

PRPs are a range of novel host defense proteins that mainly inhibit pathogen progress in compatible interactions. Cai et al. (2010) isolated endophytic bacteria *B. amyloliquefaciens* strain TB2, which has been found to be effective against downy mildew disease of litchi caused by *Peronophthora litchi* where the treated plants showed higher levels of PRPs, i.e., 1,3-glucanase and chitinase 6 days after the inoculation of bacteria.

9.4.1.8 Induced Systemic Resistance

Induced systemic resistance (ISR) and systemic acquired resistance (SAR) are two types of resistant mechanisms dependent on external stimuli, which can be distinguished based on the existence of the elicitor and the regulatory pathways involved. SAR advances on plants following a necrotic pathogen infection and is more effective second time during pathogen attacks in the development of defense mechanisms. Another infection-induced mechanism is ISR, which raises the chemical or physical barrier of the host plant rather without killing the harmful pathogen. ISR protects the plant from further attacks by herbivores and pathogenic microbes. Rashid et al. (2017) examined that treatment with an endophytic *B. velezensis* YC7010 significantly induced resistance in green peach aphid leaves significantly through senescence promoting gene PAD4 (phytoalexin deficient 4) by suppressing the expression of BIK1 (botrytis-induced kinase1). Likewise, *P. fluorescens* PICF7, a native olive endophyte, was able to cause a wide range of defensive reactions in root tissue, antagonistic to *Verticillium* wilt of olives. Zhang et al. (2004) stated that the endophyte strain *Bacillus pumilus* SE34 decreased tobacco mold severity when used.



9.5 Applications of Endophytes

There is an increased interest in the use of endophytes for their applications in agriculture for promoting plant growth and combating plant pathogens. Endophytes were found to accelerate plant growth via various mechanisms, viz., phytostimulation (e.g., by hormone production), biofertilization (e.g., by fixation of atmospheric nitrogen), solubilization of minerals such as phosphorus and formation of siderophores to scavenge Fe_3^+ ions under Fe_3^+ limiting conditions, induction of stress tolerance (e.g., by regulation of the release of stress hormone by the enzyme

1-aminocyclopropane-1-carboxylate deaminase), and rhizoremediation (i.e., protection of plants by rhizobacteria against environmental pollutants).

9.5.1 Application of Endophytes in Plant Growth Promotion

Plant hormones are the key chemical constituents, which provide tolerance against a number of abiotic and biotic stresses. They serve as the effector molecules and are known to be responsible for signal perception, transduction, cellular homeostasis, and gene expression. Lugtenberg et al. (2016) reported the production of hormones such as ethylene, cytokinins, gibberellins, and auxins by bacteria, which enhanced the plant growth. Endophytic bacteria ARBR3, AM65S2, IR64R1, AM65R1, IR64L1, ARBS2, and JERR2 isolated from aerobic rice varieties have been reported to have the possibility to produce phytohormones like IAA, gibberellins (GA), and cytokinin (Shylla et al. 2016). The majority of rhizospheric bacteria are found to produce auxins, which are essential for lateral root formation (Pliego et al. 2011). Shcherbakov et al. (2013) reported the capability of endophytic bacteria to fix atmospheric nitrogen in plants.

9.5.2 Application of Endophytes in Disease Management

With the expanding social concern in avoiding or reducing the application of pesticides and chemical fertilizers for sustainable eco-friendly alternatives, the search for beneficial microorganisms and microbial-derived compounds against several pathogens in various crops has been given impetus (Table 9.1).

9.6 Bioformulation of Endophytic Microorganisms

Application of plant endophytic microorganisms in field crops has been shown to have a positive effect on crop production. Field application of crop-specific endophytes and microorganisms in an appropriate carrier system is supposed to be a value-added assignment (Mastan et al. 2019). Regardless of the wide range of beneficial activities of bacterial endophytes, the latest investigations on the bacterial endophytes lack concern for their formulation strategies (Barrera et al. 2020). Application of beneficial microorganisms in fields has a constraint due to vulnerability of living cells to extreme environmental conditions. Thus, microorganisms showing impressive plant growth and biocontrol potential in the lab or controlled conditions often fail to perform in field conditions. Various studies have shown that the poor performance of microorganisms is due to their deprived population in soil resulting from the highly competitive native microbe population. In this regard,

Table 9.1 List of endophytes detailed for disease management in crops

S. No.	Crop	Pathogen	Endophytic microbes	Reference
1	Bean	Anthracnose	*Bacillus subtilis* *Bacillus atrophaeus* *B. tequilensis* *B. subtilis* subsp. *spizizenii* *Streptomyces cyaneofuscatus* *S. flavofuscus* *S. parvus* *S. acrimycini*	Gholami et al. (2013)
2	Bell pepper	Phytophthora blight	*Serratia* strainB17B *Enterobacter* strain E *Bacillus* strains IMC8, Y, Ps, Psl, and Prt	Irabor and Mmbaga (2017)
3	Black pepper	Black pepper root rot disease	*Pseudomonas putida* Pt12 *Pseudomonas* sp. Pt13	Nascimento et al. (2015)
		Phytophthora capsici and *Radopholus similis*	*Annulohypoxylon nitens* *Daldinia eschscholzii* *Fusarium* spp. *Ceriporia lacerate* *Diaporthe*s spp. *Phomopsis* spp.	Sreeja et al. (2016)
4	Cauliflower	Black rot disease	*Pseudomonas fluorescens* PF-1 *Bacillus subtilis* strain BS-7	Singh et al. (2010)
5	Chilli	Bacterial wilt (*Ralstonia* solanacearum)	*Lysinibacillus* sp. *Bacillus subtilis* *Azotobacter chrococcum* *Pseudomonas cepacea*	Istifadah et al. (2017)
6	Chili pepper	Bacterial wilt (*Ralstonia syzygii*)	*Bacillus pseudomycoides* NBRC 101232 *B. thuringiensis* ATCC 10792 *B. mycoides* strain 273	Yanti et al. (2018)
7	Cucumber	Angular leaf spot	*Ochrobactrum pseudintermedium* (CB361-80) *Pantoea agglomerans* (CC372-83)	Akbaba and Ozaktan (2018)
		Downy mildew	*Bacillus* sp.	Sun et al. (2013)

(continued)

Table 9.1 (continued)

S. No.	Crop	Pathogen	Endophytic microbes	Reference
		Fusarium wilt	*Pseudomonas* spp.	Ozaktan et al. (2015)
8	Oilseed	Soil-borne diseases	*Pseudomonas* spp. *Serratia* spp.	Nejad and Johnson (2000)
9	Tomato	Fusarium wilt	*Alcaligenes faecalis* subsp. *Faecalis* str. S8	Abdallah et al. (2016)
			Bacillus amyloliquefaciens RWL-1	Shahzad et al. (2017)
			Bacillus amyloliquefaciens FBZ24	Elanchezhiyan et al. (2018)
		Bacterial wilt	*P. fluorescens* 63-28	Vanitha and Umesha (2011)
			Staphylococcus epidermidis BL4 *Bacillus amyloliquefaciens* BL10	Nawangsih et al. (2011)
		Botrytis cinerea	*Brevibacillus brevis*	Yang et al. (2011)
10	Spinach	Fusarium wilt	*Enterobacter cloacae* SM10	Tsuda et al. (2001)
11	Black gram	Dry root rot	*Pseudomonas fluorescens* strain Endo2 and Endo35	Karthikeyan et al. (2006a, b)
12	Maize	Southern corn leaf blight	*Bacillus subtilis* DZSY21	Ding et al. (2017)
13	Rice	Bacterial leaf blight, sheath blight	*Bacillus subtilis B. amyloliquefaciens* (FZB 24), EPB 9, EPB10, EPCO 29, and EPCO 78	Nagendran et al. (2013, 2014)
		Bacterial leaf blight	*Streptomyces* spp. (AB131-1 and LBR02)	Hastuti et al. (2012)
		Rice blast	*Bacillus tequilensis* GYLH001	Li et al. (2018)
		Sheath blight	*Pseudomonas fluorescens* GRP3	Pathak et al. (2004)
14	Wheat	Fusarium head blight	*Bacillus megaterium* (BM_1) *Bacillus subtilis* (BS_{43}, BSM_O y BSM_2)	Pan et al. (2015)

(continued)

Table 9.1 (continued)

S. No.	Crop	Pathogen	Endophytic microbes	Reference
			Bacillus velezensis LM2303	Chen et al. (2018)
		Powdery mildew	*Bacillus subtilis* strain E1R-J	Gao et al. (2015)
15	Coffee	Coffee leaf rust	*Bacillus lentimorbus* DutkyTG4-Ia *B. cereus* TF9-Ia	Shiomi et al. (2006)
16	Olive	Verticillium wilt	*Pseudomonas fluorescens* PICF7	Cabanas et al. (2014)
17	Sunflower	*Alternaria alternata*	*Penicillium citrinum* LWL4 and *Aspergillus terreus* LWL5	Waqas et al. (2015)
18	Soyabean	R. *solani*, *F. oxysporum*, *S. rolfsii*, *C. truncatum*, *A. alternata*, *Macrophomina phaseolina*	*Bacillus* spp. *Pseudomonas* spp.	Dalal and Kulkarni (2013)
19	Grapevine	*Botrytis cinerea*	*Burkhloderia phytofirmans* Ps JN	Compant et al. (2008)
20	Pea	*F. oxysporum* f. sp. *pisi*	*B. pumilus* SE34	Chaudhary et al. (2009)
		Pythium ultimum and *F. oxysporum* f. sp. *pisi*	*P. fluorescens* 63-28	Ardebili et al. (2011)
21	Banana	*F. oxysporum* f. sp. *cubense*	*Pseudomonas* and *Burkholderia*	Fishal et al. (2010)
22	Peanut	*Sclerotinia sclerotiorum*, *S. minor*, *S. rolfsii*, *Fusarium solani*	*Bacillus* spp. *Pseudomonas* spp.	Tonelli et al. (2010)
23	Sugarcane	*Ceratocystis paradoxa*	*Trichoderma virens*	Romão-Dumaresq et al. (2012)
		Fusarium verticilllioides, *Colletotrichum falcatum*, *Ceratocystis paradoxa*, and *Xanthomonas albilineans*	*Epicoccum nigrum*	Fávaro et al. (2012)
24	Tobacco, corn, wheat, soybean	Entomopathogen	*Beauveria bassiana*	Russo et al. (2015)

formulating potent bioformulations is a vital part of exploiting microbial formulations in field conditions. Therefore, to ensure the longevity of the endophytes, we first confirm their protection in field. This security can only be obtained by developing appropriate formulation with proper carrier supported bioformulation (Bashan et al. 2014).

For developing a potential bioformulation, strain selection is one of the most vital steps. Due to their host specificity, it is essential to choose the right endophyte for

formulation development and targeted microbes should be specific to the crop to ensure the targeted result (Berg et al. 2005). These endophytic microorganisms remain safe in the plant and face lesser competition (Hardoim et al. 2008; Gaiero et al. 2013). The microbes with endospore-producing ability and temperature tolerance should be preferentially used for making a formulation (Senthil kumar et al. 2007).

After the selection of correct strain, the following step is to augment the procedure for its industrial production including multiplication and metabolite production. Optimization of several parameters such as cell counting, pH, temperature, oxygen, moisture, and nutrition is a vital part of the mass production of agriculturally important microorganisms. Zahir et al. (2010) reported an increase in the number of *Rhizobium* by the addition of tryptophan in the media. Enhanced yield in mung bean under field conditions was reported when the crop was treated with *Rhizobium* formulation containing tryptophan. However, the same parameters for some other endophytes may not work, and they may need a few specialized conditions and nutrition. Industrial-level production of endophytes should be cost-effective, which will improve the product applicability and enhance the farmer's interest.

Various carriers have been assessed for shelf-life analysis and development of bioformulation of endophytic microbes (Bashan et al. 2014). For the successful commercialization of microbial formulations, the good viability for a longer period of time is very critical (Bazilah et al. 2011). Bioformulations having CFU 10^9 with extended shelf life of up to two years have effective performance in the field (Deaker et al. 2004; Schulz and Thelen 2008). Cell viability of endophytic *Pseudomonas corrugata*, *Pseudomonas fluorescens*, *Bacillus subtilis*, and *Azospirillum brasilense* in alginate beads was found to be 14 years (Bashan and González 1999). Endophytic *Bradyrhizobium japonicum* applied in soybean was reported to have a shelf life up to 8 years (Bashan et al. 2014). The application of endophytic microorganisms can offer an ecological alternative to deal with challenging eco-friendly crop production. The bioformulation of endophytes may play a vital role in enhancing the exploration of this rising low-cost technology. Various bioformulation techniques for field application were studied. These practices include wettable powder, pellets, gel-based inoculants, and foliar spray (Cheng et al. 2015; Hu et al. 2011; Bejarano et al. 2017). However, very few studies have reported the entrapment and encapsulation of endophytes by using seed coating by a bacteria-calcium alginate mix (Otieno et al. 2015; Lally et al. 2017).

Barrera et al. (2020) studied the encapsulation of endophytic bacteria into an amidated pectin hydrogel, which is a biopolymeric substance. In their study, they aimed to enhance the capability of a PGPR, *Kosakonia radicincitans* DSM16656^T to colonize the plant seedling endophytically. They found that the pre-osmoadapted bacterial cell formulation in amidated pectin beads increased the endophytic activity of bacteria by 18.9%. Furthermore, confocal microscopy investigations with GFP-tagged bacterial cells revealed that bacterial aggregates formed during bead activation played a vital role in colonization. Bensaci et al. (2015) tested the efficacy of two bioformulations of cultural filtrate of endophytic fungus *Cladosporium oxysporum* Berk. & M.A. Curtis isolated from the *Euphorbia bupleuroides* subsp.

luteola (Kralik) Maire against black bean aphid *Aphis fabae*. In his study, he found that the invert emulsion formulation was more effective than the formulation with aqueous suspension.

Mastan et al. (2019) investigated the efficacy of bioformulations of endophytic fungus *Coleus forskohlii* with two different carriers including wheat bran and talc. They had initially grown the fungal endophytes on sterilized wheat bran, which was analyzed under a scanning electron microscope. Thereafter, ten-day grown culture was used for bioformulation using wheat bran and talc. They have found that the endophytic formulation with wheat bran considerably enhanced the height, number of branches, and root biomass of crop plants in comparison to talc-based formulation. Shelf life of endophytic fungi was also higher in wheat bran-based formulation by six months. Hu et al. (2011) evaluated the two formulations of endophytic *Bacillus subtilis* Tu-100 for suppression of *Sclerotinia sclerotiorum* in mustard. In their study, they reported that the pellet formulation considerably checked the disease incidence and increased the plant biomass. These bioformulations provided steady *B. subtilis* Tu-100 biomass up to $\geq 10^5$ CFU g^{-1}. Cheng et al. (2015) investigated the bioformulations of *Bacillus cereus* CE3 for shelf life and efficacy against chestnut and fruit rot pathogens. After a series of experiments, they confirmed a formulation that contained 60% *B. cereus* freeze and dried powder, 4% sodium lignin sulfonate as disperse, 28.9% diatomite as a carrier, 1% K_2HPO_4 as a stabilizer, 6% alkyl naphthalene sulfonate as a wetting agent, and 0.1% β-cyclodextrin as an ultraviolet protectant. This formulation exhibited 100 times dilution of 60% *B. cereus* wetting powder and had 79% corrosion rate to test pathogen.

9.7 Future Prospects

Exploitation and deployment of new solutions for the successful establishment of sustainable agriculture are very vital to overcoming the heavy use of agrochemicals. Agriculturally important microorganisms are now being used in the integrated pest management programme and expected to be harnessed more in plant disease management systems in the future. There is an ever-increasing demand for exploiting ecological compatible and eco-friendly practices in agriculture. After analyzing the work done on endophytes, it could be concluded that endophytes have unlocked a new venture in the era of sustainable agriculture and eco-friendly management of plant diseases. Endophytic microorganisms are considered plant probiotics as they exist inside the plant body. Use of beneficial microorganisms including endophytic microbes in agriculture offers various benefits and proved to be a potential alternative for a long. These microorganisms could be applied directly to the plants to improve health and to provide resistance against plant pathogens. The current efforts and studies in microbial biostimulants are a beginning that could lead to a significant increase in the application of microbe-based products and a reduction in the use of agrochemicals. Endophytes could help in crop production with limited fertilizers,

pesticides, and any other hazardous chemicals. Various bioformulations derived from endophytic bacteria and fungi have delivered promising results in terms of disease suppression. Bioformulations developed from pure organic substances and in combination with various metabolites are a new topic that needs to be investigated. Recently, endophytes are being explored for the synthesis of nanoparticles which are supposed to be used against various plant diseases. These innovations suggest an unlimited role for endophytes in the future for developing more potent and cost-effective bioformulations. Hence, the recommendation is to encourage research on the exploration of beneficial endophytic microorganisms and their isolation from untouched wild areas. Comprehensive information and research on this subject will provide a better indulgence of these microbes and their application in crop production to safeguard food production and protect the environment.

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Chapter 10
Arbuscular Mycorrhizal Fungi (AMF) as Potential Biocontrol Agents

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Abstract Arbuscular mycorrhizal fungi (AMF) belong to the phylum *Glomeromycota* and form a symbiotic relationship with more than 80% of land plants. They are beneficial for plants in many ways and extensively researched for their potential as biocontrol agents (BCA). First, we outline the origin of the concept, taxonomy and ecological distribution of AMF. Afterwards, current concepts of AMF as BCA against different types of plant pathogens and pests, e.g. nematode, fungi, bacteria, virus and insect along with their mode of action and mechanisms and factors regulating the effects and biochemical and molecular mechanism that regulates plant response to a pathogen, are presented. We further discuss key findings about AMF as BCA. Finally, the best approaches to incorporate this knowledge into sustainable agriculture, as well as the possible benefits of AM, are compiled.

Keywords Arbuscular mycorrhizal fungi (AMF) · Biocontrol agents · Plant pathogens · Plant response · *Glomeromycota*

10.1 Introduction

Biological control agents (BCAs) can be defined as the use and management of biological microorganisms, natural or genetically manipulated that can reduce the frequency or intensity of diseases caused by plant pathogens. In the process of controlling plant diseases and insect pests, chemicals are applied to the agricultural

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lands which have exhausted and distressed the soils. Reducing the chemical input and restoration of soil health for sustainable biocontrol and agricultural practices, BCAs have become an attractive field of research among the scientific communities across the globe. Several naturally occurring or genetically modified microorganisms have been studied as BCAs and found effective against various types of plant pathogens. There is a developing understanding of sustainable agriculture, including ways to limit environmental harm, reduce chemical inputs and offer environmental stress protection. The use of beneficial microorganisms could be an essential strategy for increasing productivity and plant immunity to biotic and abiotic challenges in an eco-friendly way.

To restore soil health, enhance plant growth and crop yields and deal with biotic stress, arbuscular mycorrhizal fungi (AMF) are found very helpful (Rillig and Mummey 2006). Because of their abundance in natural and agricultural terrestrial ecosystems, arbuscular mycorrhizal fungi (AMF) are considered as a potential tool for biocontrol (Barea and Jeffries 1995). It is a unique symbiotic connection between plants and arbuscular mycorrhizal fungi and is found approximately in all terrestrial plant habitats (Smith and Read 2010). This mycorrhizal link can be found practically in all ecological contexts, including natural habitats, mainly in those sustaining high species variety plant communities and regular agricultural systems, especially when managed with sustainable approaches (Bethlenfalvay and Schüepp 1994). AMF play an important role in controlling plant pathogens.

These fungi are important players in the nutrient cycle, as well as in protecting plants from environmental and biotic stresses. The presence of AMF in the plant root helps compensate for the damage caused by soilborne pathogens or herbivores while also increasing immunity in mycorrhizal plants. The efficiency of AM fungi in biocontrol is determined by the AMF, the nutrient availability and the host plant. AMF reduce the severity of plant diseases in a variety of crops, suggesting that it could well be employed as a tool for plant disease management. AMF can modify the quality and abundance of rhizosphere microflora and alter overall rhizosphere microbial activity. These fungi can affect the microbial balance in the mycorrhizosphere by modulating host root exudation patterns. AMF provide significant benefits in the favour of sustainable agriculture by contributing to the plant nutrition and biocontrol of plant pathogens, thereby reducing the quantum of synthetic fertilizers/plant protection agrochemicals substantially and the proportionate environmental/health hazards associated with.

10.2 History, Classification and Taxonomy

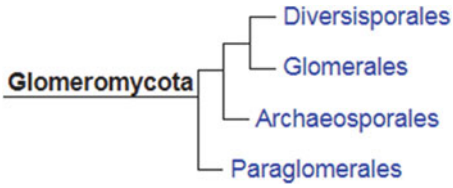
The features of mycorrhizal associations are regulated by the host and associated fungal species and host plant interaction pattern; these associations can be classified into six types of mycorrhiza: (1) ectomycorrhiza (EM), (2) arbuscular mycorrhiza (AM), (3) monotropoid, (4) arbutoid, (5) orchid and (6) ericoid (Bonfante and Anca 2009). EM fungi form associations with plants of the pine family (Pinaceae) and

angiosperms by a hyphal mantle that encloses the root, and a Hartig net formation can be observed that penetrates the root cells. Glomeromycetes form AM association with most of the terrestrial plants usually with arbuscules and often with vesicles. The monotropoid association is found in mainly *Fagus, Pinus* and *Salix* spp. The hyphal penetration is restricted by epidermal layers. The orchid association is very similar to monotropoid, where coils of hyphae (pelotons) penetrate within cells in the plant family, Orchidaceae. Ericales type of association is found in epidermal cells of Ericaceae members. Arbutoid association is found in autotrophic plants of Ericaceae family, where multiple hyphae penetrate epidermal Hartig net cells (Brundrett 2004). In this chapter, we will focus on AMF, the most common types.

AMF are numerous and ubiquitous and are known to undertake many roles, including that of a partner in obligate symbiotic association with plant roots. A profound study on mycorrhizal fungi was initiated by Franciszek Kamienski (Kamienski 1881), a Polish mycologist, who described this symbiotic association of a fungus and roots in *Monotropa hypopitys L.* Later, coined the term 'mycorrhiza' to the association. Historical development of taxonomy and our understanding of AMF can be categorized based on the timescale for a better overview of the research background. During the period of the Golden Age of Microbiology (1800–1900), important AM fungal genera, such as *Glomus*, *Rhizophagus* and *Sclerocystis*, were described (Schüßler and Walker 2010, and the first attempt was made for the taxonomical classification (Tortora et al. 2003). For the next 80 years, extensive morphological studies led to the transfer of many AMF to a different genus. Three new genera, *Acaulospora*, *Geosiphon* and *Gigaspora*, were proposed. More precise details about taxonomical classification lead to the reorganization of many AMF species being transferred to another genus. Genus *Geosiphon* (monophyletic, *Geosiphon pyriforme*) was described by Wettstein. The establishment of identification keys for *Endogonaceae* species, which served as the foundation for present morphological classifications, was a more important task during this phase (Gerdemann and Trappe 1974; Kehri et al. 2018).

The late 1980s to 1990s witnessed an explosion in AMF taxonomy studies. Numerous new species were defined, mainly from the genus *Gigaspora* and *Glomus*, and new genera *Entrophospora* and *Scutellospora* were established. Walker established standard terms for concepts of spore wall properties and murographs during this time period, and a much sophisticated classification was proposed with the addition of a new order *Glomales* by Morton and Benny (1990). The suborders *Glomineae* and *Gigasporineae* and families *Acaulosporaceae*, *Glomaceae* and *Gigasporaceae* were established. The studies during early 1991 to 2000, regarding the AMF taxonomy, were mainly based on the details of spore structures and were unable to resolve many new species characteristics and their evolutionary phylogenetic positions. As a result, different pieces of work were found contradictory to one another and some cryptic taxa were misclassified. With the invention of the first PCR primer, the demand for more tangible and objective instruments for classification was fulfilled (VANS1) (Simon et al. 1992). Certainly, this was a significant step forward in the field of AMF taxonomy, allowing for the discovery and identification of new species. From 2001 to 2010, molecular approaches accelerated the taxonomy

studies and many new orders, families, and genera of AMF were discovered. Schüßler et al. (2001) proposed AMF monophyletic phylum and all AMF species were transferred from *Zygomycota* to *Glomeromycota*. Until 2009, their findings served as the foundation for a number of subsequent investigations. From early 2011 to the present day, a lot of serious problems about AMF taxonomy were solved using advanced approaches. Availability of less molecular data and a high number of publications created confusion among researchers; however, a deeper insight of compiled data along with new systematics and phylotaxonomy was provided by Krüger et al. (2012). Subsequently, a stable and widely accepted classification of Glomeromycota was published by Redecker et al. (2013). Nowadays it is widely accepted that Glomeromycota consists of four orders.



10.3 Distribution and Ecology

AMF are found in more than 90% of terrestrial plants, and the structure and functions of their communities vary depending on the host plant (Öpik et al. 2006). *Funneliformis mosseae* is a good example of cosmopolitan AMF that can be found in North America, South America, Europe, Africa, Asia and Australia (Al-Qarawi et al. 2013). Some of the AMF are locally distributed like *Glomus brasilianum* (Spain and Miranda 1996). Data from Institute of Culture collection and publication from 1960 to 2012 were collected to understand the biogeographic distribution of *Glomeromycota*. All the seven continents, 87 countries, 11 biogeographical domains and 14 biomes were found to have AMF species. Almost all the genera were cosmopolitan in nature. The distribution of AMF species decreases slightly from low to high latitudes, but it is steeped in the southern hemisphere than in the northern (Stürmer et al. 2018). Habitats influenced AMF communities more than host preference, according to molecular evidence and morphological assessments (Li et al. 2010). People's anthropogenic and land operations have been shown to have a substantial impact on AMF diversity (Bordoloi et al. 2015). AMF are dominant in soil microbial communities in most of the agroecosystems. AMF immobilize mineral nutrients and make them available to plants, allowing them to continue to develop and reproduce normally. The distribution of the AM fungal species is influenced by the amount of ground vegetation and the intensity of disturbance. Interactions between mycorrhizal fungi and plants can be impacted by climate change (Goicoechea 2020). During the last two decades, a boom can be seen in

commercial products based on mycorrhizal fungi inocula and related services. Positive evidence and data from scientific studies on the benefits and application of AMF as BCA have created awareness among the general public and government bodies. There are a lot of reasons for this growth in the mycorrhizal industry. Economic effectiveness, sustainability and environmentally friendly are some general features that make AMF-based products attractive to public and commercial companies.

10.4 Defence Mechanism of AMF

AMF's ability to mitigate the harmful impacts induced by root infections in a variety of plant hosts has been demonstrated. It would not be exaggeration if we say plants do not have roots, they have mycorrhizas; more than 90% of flowering plants form a symbiotic association with these AMF (Harley and Smith 1983). Mycorrhizal association of AMF and plants is the most prevalent type in regular cropping systems and natural ecosystems (Gianinazzi and Schüepp 1994). AMF, in order to complete the life cycle, colonize the root cortex and form an extraradical mycelium which creates a microenvironment in soil that helps the plant to obtain minerals and water. AM symbioses are important in the ecological nutrient cycle, and extraradical mycelium also interacts with other soilborne microorganisms and modulates the microbial communities which improve soil health (Bethlenfalvay and Schüepp 1994).

The beneficial functions of AMF that help in damage compensation (see Table 10.1) and defence mechanism are discussed in this section. Table 10.1 summarizes some of the beneficial functions of AMF that could be involved in the reduction of damages due to phytopathogens and pests.

10.4.1 Enhancement of Nutrient Uptake

Several studies have found that enhanced mineral uptake in AMF-infected plants is linked to greater disease resistance (Li et al. 2013). Farzaneh et al. (2011) also reported enhanced uptake of P, Mn, K, Cu and Fe in AMF-infected plants of chickpea. As the nutrient concentrations were elevated, a simultaneous increase in plant biomass was observed. But, that is not always the case; reported that phosphorus content increased wheat sensitivity to the biotrophic fungus *B. graminis* f. sp. *tritici*.

Table 10.1 Some beneficial functions of AMF that help in reducing damages caused by pathogens

S. N.	Functions of AMF	Effect	Reference
1.	Enhancement of nutrient uptake	Exchange between N and carbon source increased	Li et al. 2013
2.	Damage compensation	Root functional and biomass loss caused by infections or abiotic stress can be mitigated	Linderman 1994; Mathur et al. 2019; Maya and Matsubara 2013
3.	Interaction with microbial communities present in mycorrhizosphere	CMNs confer resistance in healthy nearby plants against the invasion of a pathogen	St-Arnaud et al. 1997
4.	Competition for colonization	AMF can outcompete pathogens for the space of colonization	Davis and Menge (1980)
5.	Physiological or histological changes	*G. Mosseae* affects root size and root branching, number of root tips, length, surface area and root volume	Tahat et al. 2008
6.	Competition for photosynthates	AMF and root pathogens both depend on the carbon source provided by the root	Smith and Read 1997
7.	Changes in root exudates	To counter infection, AMF can increase allelopathic content while decreasing common root exudate secretion	Ren et al. 2015
8.	Activation of plant defence mechanisms	AMF interaction with roots can boost the immune systems of the plant when exposed to a pathogen	Molinari and Leonetti (2019)
9.	Mycorrhizal networks	Common mycorrhizal networks (CMNs) between the same and other species of plant roots can increase disease resistance in healthy neighbouring plants	Song et al. 2010
10.	Enhancement in pollution tolerance	(a) Yield was increased under elevated O_3 stress (b) Acquisition capabilities of AMF can enhance plant fitness in polluted sites	Wang et al. 2017 Clark and Zeto 2000

10.4.2 Damage Compensation

AM fungi are thought to have improved P absorption in plants and their tolerance to soilborne diseases by improving root function and biomass (Linderman 1994). AMF colonization can indirectly compensate the damage by promoting the growth of root hairs and increasing the absorptive capacity of roots. Damage caused by abiotic factors, e.g. water depletion or drought-like conditions, can be overcome with AMF. It was experimentally demonstrated that AMF colonization can help in the restoration of structure and functionality of PSII and PSI under water depletion (Mathur et al. 2019).

10.4.3 Interaction with Microbial Communities Present in Mycorrhizosphere

The mycorrhizosphere is the zone of soil influenced by roots colonized by mycorrhizal fungi, and in comparison to the rhizosphere, the mycorrhizosphere enhances microbial activity and aids in nutrient absorption. Reduction in the pathogen development within the mycorrhizosphere has been reported in various reports. A comparative study between mycorrhizal plants *T. patula* and non-mycorrhizal plants *Dianthus caryophyllus* was conducted to examine the effects of disease caused by *Fusarium oxysporum* f. sp. *dianthi* in the presence of AM fungi. The survival of *T. patula* was increased nearly twofold in comparison to *Dianthus caryophyllus*. A significant reduction in symptoms of disease was also observed (St-Arnaud et al. 1997). Interestingly, this type of disease reduction was found unrelated to plant nutrition level.

10.4.4 Competition for Colonization

AMF and soilborne plant diseases use similar root tissues, and if colonization occurs at the same time, there may be direct competition for space. Because both AM fungi and plant diseases normally develop within separate cortical cells of roots, there may be a rivalry for space if they colonise the same host tissues. Localized competition between AM fungus and *Phytophthora* sp. was found by Davis and Menge (1980). *Phytophthora* development was reduced in AM-colonized and nearby non-colonized root systems, and the pathogen was unable to penetrate the pre-colonized root cells, according to the findings.

10.4.5 Physiological and Histological Changes

When tomato roots (*Lycopersicum esculentum* Mill.) were colonized with AMF species *G. mosseae*, positive impacts were seen. It enhanced the root size, branching of roots, root tips, its length, surface area and volume (Tahat et al. 2008). Plants associated with AMF were found with a better vascular system, which contributes to the general mechanical strength of the plant to suppress the effects of pathogens. Increased lignin content in the endodermal layer of mycorrhizal tomato and cucumber plants is supposed to overcome the effects of Fusarium wilt (*Fusarium oxysporum* f. sp. *lycopersici*) (Schonbeck 1979; Dehne et al. 1978). Mycorrhizal plant onion infected with pink rot pathogen (*Pyrenochaeta terrestris*) produced wound barriers at a higher rate than non-mycorrhizal plants (Becker 1976). In another example, increased wound barrier formation can inhibit *Thielaviopsis* black root rot of mycorrhizal holly (*Ilex crenata*) plants (Wick and Moore 1984).

10.4.6 Competition for Photosynthates

The growth of AMF and root pathogens is dependent on their common host (plant) for sugar or photosynthetic substance, and both have competition for the carbon source received from the root. If AMF achieved dominance over pathogen(s) for sugar source partition, it can restrict the growth of pathogen(s). The carbon source for AMF is provided by the host plant. According to estimates, some 4–20% of the host's net photosynthates are transmitted to the AMF. However, this aspect has to be explored in more detail (Linderman 1994; Smith and Read 1997).

10.4.7 Changes in Root Exudates

Fluids emitted through the roots of mycorrhizal plants are called root exudates. These secretions affect the rhizosphere inhibiting harmful microorganisms and encouraging the growth of self and kin plants. Research findings by Wang et al. (2012) suggest that soybean (*Glycine max*) root exudates are important in modifying the soil microbial population and that interactions across microbial functional groups have an impact on soil ecosystem functioning. When the watermelon plant is under AM colonization, a change in the composition of root exudates, increasing allelopathic content of p-coumaric acid and malic acid exudation while decreasing common root exudate secretion, was noticed upon infection with *Fusarium oxysporum* f. sp. *niveum* (FON) (Ren et al. 2015).

10.4.8 Activation of Plant Defence Mechanisms

When arbuscular mycorrhizal fungi (AMF) engage with roots to create mutualistic connection, they stimulate systemic acquired resistance (SAR) defence mechanisms of plant. This process is known as induced systemic resistance (ISR) (Métraux et al. 2002). These processes thought to be involved in plant protection by the fungi are partial stimulation of plant defence reactions during root colonization. This process is discussed in detail in Sect. 4.11.

10.4.9 Mycorrhizal Networks

AMF can spread from a specific mycorrhizal plant's root system, colonize the roots of plants in certain proximity and establish common mycorrhizal networks (CMNs) that interconnect plants of the nonspecific or different species. Song et al. (2010) opine that CMNs can share information about health and infection, and it was

successfully demonstrated in tomato plants. AMF and *F. mosseae* can form CMNs with tomato plants, and when one plant is infected with *A. solani,* an increase in disease resistance in healthy neighbouring plants was recorded.

10.4.10 Enhancement in Pollution Tolerance

AMF can be helpful in the alleviation of stress due to different types of pollution. In controlled conditions, with O_3 concentration higher than 80 ppb, AM symbiosis was able to increase up to 68% in shoot and 131% in root biomass. AM symbiosis resulted in higher production under O_3 stress in comparison to non-mycorrhizal plants, and the AM effects were more profound as O_3 concentration was increased (Wang et al. 2017). Heavy metal (e.g. Cu, Ni, Fe, etc.) acquisition capabilities of AMF can help plants survive in polluted soils (Clark and Zeto 2000).

Studies based on pot culture and field trial on beneficial effects of AMF and their role in conferring resistance to pathogens have been investigated extensively. The regulation and maintenance of growth and response to a pathogenic infection need biochemical and molecular cross-communication between the plant root and the AMF. Being obligate symbiont and the non-culturable nature of the fungus, it becomes very difficult to study biological interaction with traditional methods. However, approaches based on multi-omics provided a significant understanding of this interesting relationship between plants and AMF. Plant roots release chemical signals that are detected by AMF, as well as AMF signals and volatile compounds that cause changes in plant machinery. Various plant-fungal signalling networks and many new nutrient transporters have been discovered and identified to explain physiological processes that influence pre-symbiosis, symbiosis and staging of infection in relation to the biocontrol of pathogen with AMF.

10.4.11 Molecular Mechanism of Interaction Between AMF Plant and Pathogen

The mycorrhizal symbiosis is beneficial to the plants in term of nutrient exchange, and their colonization confers increased resistance against different biotic and abiotic stresses. However, plants are naturally equipped to recognize biotic invasion and combat its negative impacts on growth, productivity and survival. The inherent innate immunity in plant is mainly two types; first is pathogen-associated molecular patterns or microbe-associated molecular patterns (PAMPs/MAMPs)-based triggered immunity (PTI or MTI). A vast family of pattern recognition receptors (PRR) and toll-like receptor (TLR) can detect the presence of PAMPs/MAMPs and initiate defence response. PTI grants baseline of resistance (Monaghan and Zipfel 2012). The second type of immunity is called effector-triggered immunity

(ETI); it is activated when resistance protein of host plant recognizes pathogen or molecules secrete by pathogen, leads to the activation of transcriptional processes and provides resistance for a long time. When the relevant receptor resistance (R) proteins in plants recognize virulence effectors in a certain gene-to-gene relationship, this results in the establishment of ETI (Zhang et al. 2018). The ETI induction pathway in plants is activated after a pathogen attack, increasing the synthesis of pathogenesis-related proteins (PRs) (Mazumder et al. 2013). In order to protect plants from reinfections, the accumulating PRs aid in the establishment of systemic acquired resistance (Zhang et al. 2013).

Plants also have acquired immunity known as systemic acquired resistance (SAR). Salicylic acid (SA) and its derivatives mediate this class of immunity, which is linked to the buildup of pathogenesis-related (PR) proteins. The regulatory protein NPR1 travels to the nucleus in response to SA and interacts with transcription factors to activate SAR by inducing the production of defence genes (Durrant and Dong 2004). Both PTI and ETI can lead to systemic acquired resistance (SAR), an SA-dependent induction of resistance in distal unaffected plant sections that work against a wide spectrum of pathogen (Wenig et al. 2019).

In response to a soi-borne microorganism such as arbuscular mycorrhizal fungus infection, plants may generate a stronger systemic resistance mechanisms, and this process is known as ISR (Muthamilarasan and Prasad 2013). In case of AMF, it is called as mycorrhiza-induced resistance (MIR); this induced resistance is seen against a wide range of pathogens, including fungi, bacteria and viruses (Fritz et al. 2006; Sanmartín et al. 2020, Sanmartín et al. 2020), leaf feeder insect (Song et al. 2013) and aphids (Maurya et al. 2018).

Mycorrhizal fungus first induces a biotrophic pathogen-like response in plants, which they subsequently modify to effectively colonise the plant roots. Once the symbiosis is developed, the responses controlled by the JA and ET pathways against necrotrophic infections are triggered, as well as the microbe-induced resistance and priming (Hohmann and Messmer 2017).

In response to infection of AMF, plants initiate SA-dependent defence mechanism to counter the infection, a response that is thought to be activated against biotrophic pathogens, enhanced by AMF, but gradually down-regulated, which finally allows the symbiotic interaction. JA/ET-dependent defence mechanism is effective against necrotrophic pathogens. During plant-AMF interactions, the successful execution of one pathway over another due to cross-talk between the SA and JA/ET signalling pathways allows the plant to efficiently fine-tune its defence response to the invading organism (Fig. 10.1). Over the past few decades, there has been extensive research on the ability of mycorrhizal fungi to activate ISR in plants to protect them from potential pathogen and/or insect attacks (Campos-Soriano and Segundo 2011). MIR response is a cumulative effect of phytohormones, metabolites and rhizosphere (Kadam et al. 2020). The arbuscular mycorrhizal (AM) fungus first detects the signals released by the host plants before secreting the appropriate Myc components to communicate with the plants. The host plant recognizes the Myc factors through superficial receptors on the epidermal cells and then starts the symbiotic process that causes the nuclear membrane to release

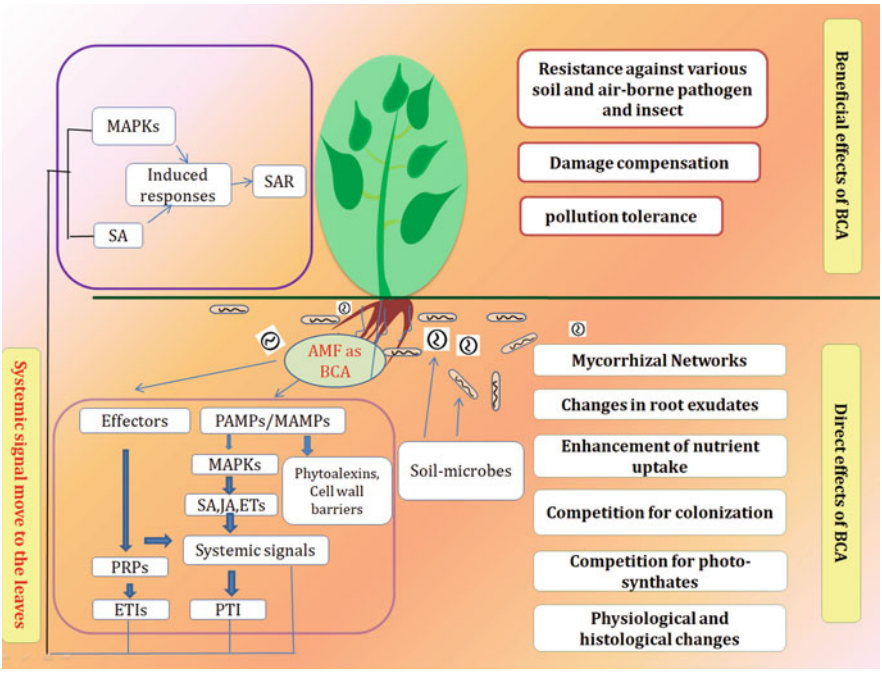


Fig. 10.1 Schematic representation of mycorrhizal-induced resistance (MIR) mechanism and associated beneficial effects. PAMPs/MAMPs, pathogen-associated molecular patterns or microbe-associated molecular patterns (PAMPs/MAMPs); SA, salicylic acid; JA, jasmonic acid; ET, ethylene; ETI, effector-triggered immunity (ETI); PTI, PAMPs-triggered immunity; PRPs, pathogenesis-related (PR) proteins; MAPKs, mitogen-activated protein kinases

calcium, causing oscillations in the perinuclear region of the cell. A nuclear-localized calcium and calmodulin-dependent protein kinase (CCaMK) decodes calcium oscillations, phosphorylates the appropriate substrates, activates transcription factors and causes the production of genes related to symbiosis (Fu and Xiang 2012).

AMF and plant initially interact chemically, which results in the development of symbiosis. The implicated signals, though, can also act as defence priming stimuli. The very first chemical stimuli exchanged to start the symbiosis are hormones, flavonoids and strigolactones from the host and nodulation factor (Myc) from fungi (Steinkellner et al. 2007; Mauch-Mani et al. 2017). Mycorrhizal-induced resistance (MIR), a sort of priming that could explain why colonized plants are more resistant to this diverse group of soil pathogens, is established as a result of the presence of mycorrhizal fungus in the roots. There are several ways that AMF may reduce the incidences of disease, including enhanced nutrition, competition for nutrients and infection sites, change in the root morphology and architecture, chemical changes in the plant, for example, root exudates, easing of the plant stress and change in microbial community in rhizosphere.

10.4.12 Potential Application of AMF as a Biocontrol Agent

This section provides an overview of the potential application of AM fungi as BCAs against nematodes, fungi, bacteria, viruses and pests. The studies related to the interactions between AMF symbiosis and these pathogens are summarized in Table 10.2.

10.4.12.1 Nematode

Plant-parasitic nematodes (PPN) and AMF both depend on plant roots for their food and shelter needs. Many studies on AMF-nematode interactions have been undertaken to see if there is a way to increase the resistance or tolerance of plants against nematodes by applying AMF. PPN are economically important crop pests in several cases. Because most effective nematode control methods do not meet the requirements for addressing contemporary environmental issues, they must be replaced by other nematode control methods that have a lower impact on non-target organisms. AMF could be a promising alternative for increasing host resistance and/or tolerance. Some examples and the effects of AMF on nematode interactions are given in Table 10.2. *Glomus fasciculatum*, (Labeena et al. 2002**;** Siddiqui and Akhtar 2006**;** Nehra 2004) and *Glomus* sp. are good examples of AMF that were found effective against *Meloidogyne incognita*, often known as the southern root or cotton root-knot nematode. The interaction of BCA with roots can protect the plants against nematode diseases. BCA-mediated resistance appears to be dependent on salicylic acid (SA)-mediated systemic acquired resistance (SAR) and is linked to the activation of chitinase and glucanase enzyme activity, as well as the suppression of the plant antioxidant enzyme system. Immunity is triggered when invading nematode juveniles penetrate and travel into the roots, but it most likely acts during the nematode's feeding site development stage (Molinari and Leonetti 2019).

10.4.12.2 Fungus

Numerous plant pathogenic fungi are well known for wreaking havoc on plants and posing a severe threat to many crops. The potential of AMF as a BCA against fungus, as well as their action mechanisms, is an intriguing study area. The negative impact of nitrogen deposition on AMF root colonization is well known (Lin et al. 2020). Soybean plants were grown under greenhouse and supplemented with nitrogen and infected with the pathogen *Macrophomina phaseolina* (charcoal root rot). Plants' health status was far better in AMF-colonized plants than non-AMF even at elevated nitrogen levels. These findings suggest that while N fertilization may raise the risk of diseases in soybeans, mycorrhiza may help reduce soybean charcoal root rot even when the crop is supplemented with N (Spagnoletti et al. 2020).

Table 10.2 List of some AM fungi and different types of pathogen and plant growth

Pathogen	Species/strain	Disease	AMF	Effect/mechanism	Reference
Nematode	*Radopholus similis* and *Pratylenchus coffeae*	Endoparasite that forms cankers in banana and coffee	*Glomus intraradices*	AMF suppressed the *R. similis* population by almost 50% split-root compartmental set-up	Elsen et al. (2008)
	Pratylenchus penetrans	Necrotic lesions on the roots	Native inoculum of AMF	Infection and multiplication of *P. penetrans* reduced significantly and preinoculation with AMF further decreased nematode colonization and reproduction	
	M. javanica	Root knot	*G. intraradices* *G. mosseae* *G. etunicatum*	On peach almond hybrids, AM fungus had a negative impact on the nematode population	Calvet et al. (2001)
	M. incognita	Root knot	*Glomus* sp.	Reduced nematode population on brinjal	Ray (2020)
	M. incognita	Root knot	*G. fasciculatum*	On tomato plants, the population of nematodes was declined	Pradhan et al. (2003)
Fungus	*Macrophomina phaseolina*	Root rot and charcoal rot in many plant species	*Rhizophagus intraradices*	Reduced severity of disease in nitrogen-fertilized soybean	Spagnoletti et al. (2020)
			Glomus deserticola, Gigaspora gigantea	AMF shows its inhibitory property against the virulent *M. phaseolina* in cowpea	Oyewole et al. (2017)
	Fusarium spp.	Fusarium wilt in tomato, banana, pepper, date palm, etc.	*Glomus monosporus*, *Glomus clarum*, *Glomus deserticola*	As consortium AMF increased crop production	Meddich et al. (2018)
			Glomus intraradices	AMF in combination with rhizobacteria reduced disease severity	Akköprü and Demir (2005)

(continued)

Table 10.2 (continued)

Pathogen	Species/strain	Disease	AMF	Effect/mechanism	Reference
	Fusarium udum	Vesicular wilt in pigeon pea	*G. fasciculatum*	Reduced disease severity in pigeon pea	
	Rhizoctonia solani	Brown patch, damping off, black scurf, bare patch, root rot, belly rot, sheath blight in different plants species	*Claroideoglomus* sp., *Funneliformis* sp., *Diversispora* sp., *glomus* sp. *and Rhizophagus* sp.	Alleviate oxidative stress in watermelon and improved resistance against *Rhizoctonia solani*	Wu et al. (2021)
	Cylindrocladium Spathiphylli	On legume crops and ornamental trees, root rot, crown and lower stem blight and leaf spots are common	*Glomus* sp. *G. proliferum* *G. Intraradices* *G. Versiforme*	In bananas, AM fungus greatly increased growth and decreased disease characteristic	Declerck et al. (2002)
Bacteria	*Ralstonia solanaceraum*	Bacterial wilt	*Glomus mosseae*, *Scutellospora* sp. and *Gigaspora margarita*	Improvement in health of banana plant	Tahat et al. (2008)
	Spiroplasma citri	Citrus stubborn disease	*G. mosseae*	Bioprotective effect of *G. mosseae* on *S. citri* was effective	Tahat et al. (2014)
	P. Syringae	It infects a wide range of woody plants particularly those that have been damaged by frost or injury	*Glomus mosseae*	Prohibited the infection of soybean plants	
	Pear decline (PD) phytoplasma	Shoot mortalities, early reddening and upper rolling of leaves, reduced leaf and fruit size, premature leaf drop and poor shoot and spur growth	*Glomus intraradices*	In both non-PD and PD-infected pear trees, a greater shoot length was observed. Mycorrhiza's long-term benefits have been confirmed	García-Chapa et al. (2004)
	Xanthomonas campestris pv. *alfalfae*	Bacterial leaf spot	*G. Intraradices, Gigaspora gigantea*	In the shoots, there are changes in gene expression and an increase in disease resistance	Liu et al. (2007)
Virus	Tomato yellow leaf curl Sardinia virus (TYLCSV)	A most destructive disease of tomato, found in tropical and subtropical regions	*Funneliformis mosseae*	Reduction in infection	Maffei et al. (2014)

	Cucumber mosaic virus	Cucumber and other cucurbits, occurs worldwide	*Funneliformis mosseae*	Enhanced tolerance to viral infection	Miozzi et al. (2020)
	Xanthomonas campestris pv. *Vesicatoria*	BAC	*R. irregularis*	Increased resistance	Cervantes-Gámez et al. (2016)
Pest	*Arctia caja*	Caterpillars feed on a wide variety of low growing herbaceous plants	Field AMF	Increased levels of the carbon-based feeding deterrents, aucubin and catalpol, were observed	Gange and West (1994)
	Phlogophora meticulosa	Feed on flowers of commonrReed and other grasses (e.g. *Lolium perenne*)	*F. mosseae*	Foliar endophytes and mycorrhizae affected larval growth and survivorship	Vicari et al. (2002)
	Spodoptera littoralis	Feed on Plantaginaceae family plants	*R. irregularis*	Provided defence through increased level of sesquiterpenes	Fontana et al. (2009)

M. phaseolina belongs to the *Botryosphaeriaceae* family and causes damping off, seedling blight, collar rot, stem rot, charcoal rot, basal stem rot and root rot in a variety of plant species. Another study on biocontrol of *M. phaseolina* in cowpea with AMF was conducted to demonstrate the inhibitory potential of AMF. *Glomus deserticola* and *Gigaspora gigantea* were able to enhance tolerance to drought and charcoal rot disease in cowpea. Cowpea with single-strain *G. deserticola* was found against water stress and yield was increased, but dual inoculation (*G. deserticola* and *G. gigantean*) was most effective for water stress and yield and overcomes the disease severity (Oyewole et al. 2017).

No single universal phenomenon can explain the mechanism of AMF protection against pathogens, and it is suggested that a range of different mechanisms are operated together. Efficient biocontrol by AMF is the result of biochemical and molecular interactions. AMF were found effective as biocontrol agents against *P. parasitica* in tomato. In a study the severity of disease was found to be correlated with the abundance of infection sites, rate of transmission in the root and time. Undoubtedly, a biocontrol agent can modulate infection sites and rate of spread mechanisms (Vigo et al. 2000).

Effect of *Glomus monosporus, Glomus clarum, Glomus deserticola* and indigenous AMF strain on morphology and physiology of date palm (*Phoenix dactylifera* L.) was investigated under the stress of *F. oxysporum* infection, drought and salinity. Colonization of AMF was mildly affected by water stress although the rate of colonization was higher. Despite pathogen inoculation and water stress, the indigenous AMF consortia with *G. monosporus* or *G. clarum* had a favourable effect on date palm biomass production. AMF allowed maintaining high-level leaf water parameters in plants under drought conditions regardless of their inoculation with *F. oxysporum* . The mortality rate of plants infected by *F. oxysporum* was lower in AMF plants than non-mycorrhizal plants. The native AMF community, named *Aoufous*, was found to confer best crop protection under severe biotic and abiotic stress (Meddich et al. 2018).

Single treatment of *G. intraradices* and in combination with four rhizobacteria was able to reduce disease symptoms of *Fusarium oxysporum* f. sp. *lycopersici* (Fol) by 8.6 to 58.6% in tomato plant. Single inoculations of bacteria were more efficient than single AMF or combined *G. intraradices* and rhizobacteria inoculations. Colonization with rhizobacteria was maximum in triple inoculations (Fol + *G. intraradices* + RB), while the association of *G. intraradices* was sharply down in treatment of FOL + *G. intraradices* in comparison to the triple inoculations. This type of study can open further avenues for the development of formulations of efficient combinations of biocontrol agents for sustainable agricultural practices (Akköprü and Demir 2005).

A total of eight commercial AM formulations were tested in combination with watermelon plants. One of them, mycorrhiza for vegetables (VT), was most effective, and its colonization in plant roots increased biomass (63.4%) and photosynthetic rate (68.6%) in comparison to non-mycorrhizal plants. The optimum inoculum density was 300 spores/plant which strikes maximum to shoot up in plant hormone (abscisic acid (ABA), indole acetic acid (IAA) and gibberellic acid). When

pathogenic fungus *Rhizoctonia solani* infects VT-colonized plants, the host plant performs better than the control non-mycorrhizal plant. VT helps in the reduction of electrolyte loss, H_2O_2 formation and lipid peroxidation and simultaneously increased root activity and antioxidant enzyme activities, suggesting alleviation of oxidative stress caused by *R. solani*. VT has the potential to enhance resistance against *R. solani* in watermelon and cucurbits, and it was recommended as a biological control agent (Wu et al. 2021). The effects of AMF *Glomus intraradices* on banana (*Musa* spp. c.v. Grand Naine) propagates were tested in sterilized and non-sterilized vertisol to incite the competitive nature of AMF against soilborne microorganisms including the native AM fungi community. AMF-colonized plants displayed higher dry weight and phosphate content in their shoots and roots than non-mycorrhizal plants (Declerck et al. 2002).

Response of mycorrhizal plants to pathogen *Verticillium dahliae* causing verticillium wilt in strawberry plants was evaluated by examining the visual morphological symptoms, water conditions and photochemical activity. Resistance in plants was associated with increased stomatal conductance, transpiration rate and, as result, leaf water potential. Plants resistant to *V. dahliae* were not showing photochemical activity and no significant effect of AMF was observed. A combination of bacteria *P. monteilii* (strain CRC1) and AM fungus (*G. intraradices*) was formulated for effective biological management of complex root disease in *C. forskohlii* under organic conditions (Singh et al. 2018).

Blackleg disease in potato is caused by pathogenic bacterium *Pectobacterium carotovora* subsp. *atrosepticum* PHY7 (Pca). AMF and fungus *Epicoccum nigrum* ASU11 were evaluated to eradicate the Pca population. The combination of AMF and *Epicoccum nigrum* ASU11 was found most efficient to reduce the population of bacteria and suppress the disease symptoms. This dual inoculation of AMF and *Epicoccum nigrum* ASU11 increased the biomass of the plant in comparison to the control plant. The effects of dual inoculation in the infected plant were quantified in terms of various biochemicals and enzymes. These findings revealed that AMF and *E. nigrum* have the capacity to boost potato development while also reducing the severity of the blackleg disease. These inoculants could have potential roles to play in designing future tactics for potato crop protection and increasing the viability of the crop. These findings can lead to improvised bio-formulation of BCA for long-term crop protection systems (Bagy et al. 2019).

10.4.12.3 Bacteria

Plant pathogenic bacteria, like fungi, cause a wide range of diseases in the plants. Pathogenic bacteria can cause different types of blights, spots, wilts, cankers and abnormalities in roots fruits. Several studies have been conducted to evaluate AMF potentials to deal with bacterial plant diseases. An experiment was designed to see the effects of plant oils and AMF on *R. solanacearum* infection in plants. *R. solanacearum* causes wilt disease in tomato plants. AMF were applied single or in combination with plant oils. This test was conducted in vitro and under

greenhouse and field conditions. *G. mosseae* seemed to have the lowest rate of disease reduction in comparison to plant oils, but it has the highest percentage of yield gain in all the conditions (Abo-Elyousr et al. 2014).

The ability of *Glomus mosseae* against aster yellows disease and citrus stubborn disease caused by phytoplasma and *Spiroplasma citri*, respectively, was investigated and found potent enough to reduce the symptoms associated with *Spiroplasma citri* infection. *G. mosseae* positively affected the health status of *Catharanthus roseus* plants infected with *S. citri*. It acts as a biocontrol agent against *S. citri* and boosted resistance in plants against *Spiroplasma* infection. However, *G. mosseae* was ineffective when plants were infected with phytoplasma and no positive effect was observed (Tahat et al. 2008). The effects of dual inoculation of *G. mosseae* and bacteria *Pseudomonas syringae* with or without *Bradyrhizoboum japonicum* were assessed in soybean plants. The mycorrhiza in combination with *B. japonicum* was able to enhance the resistance more significantly in soybean plants to alleviate detrimental effects prominently in the presence of *P. syringae*.

A comparative study of *Glomus mosseae* and *Scutellospora* sp. and non-indigenous *Gigaspora margarita* was performed with tomato plant (*Lycopersicum esculentum*) under glasshouse conditions to test their colonization abilities and their effects on plant health. All the tested species were able to colonize tomato root, but *G. mosseae* (80%) was most efficient. It also increased biomass and number of flowers. The colonization also affects root size, branching, root tips, length, surface area and root volume in a positive way. The pore count of *Glomus mosseae* (455/100 g) was the highest among all the species tested (Tahat et al. 2008). Several studies have shown that AMF protect alfalfa from *Xanthomonas campestris*, resulting in less necrotic lesions in AMF-colonized alfalfa (Liu et al. 2007). There are several other examples of AMF's protection against phytopathogenic microorganisms (Cervantes-Gámez et al. 2016; Yuan et al. 2016; Bagy et al. 2019).

10.4.12.4 Virus

Plant viral infections are a major threat to global food security, and crop management practices and climate change are contributing to the problem. Tomato yellow leaf curl Sardinia virus (TYLCSV), belonging to the genus *Begomovirus*, family *Geminiviridae*, is one of the most serious threats to tomato production in several countries. The harmful effect of TYLCSV was reduced when tomato plants were colonized by AM fungi *Funneliformis mosseae*, and the expression level of the responsive gene of AMF was not affected. The development of mycorrhiza and expression profile was least affected by viral infection. Mycorrhizal plants were having a lower load of viral DNA in root and shoot and were showing milder symptoms in comparison to non-mycorrhizal plants (Maffei et al. 2014).

The infection of cucumber mosaic virus (CMV), family *Bromoviridae* in the mycorrhizal tomato plant, was assessed in the correlation of phenotypic, physiological, biochemical and transcriptional profiles. Researchers proposed the 'mycorrhiza-induced susceptibility' (MIS) term for AMF colonization mediated detrimental

effect on plant defences against viruses. Through this study, the defensive mechanism of AMF plant against the virus was explored. It was concluded that the established AMF association can efficiently limit symptoms developed by viral infection in plants. The change in expression of genes related to photosynthesis and CO_2 assimilation rate due to viral infection was down-regulated in AM plants. Transcriptional data revealed upregulation of salicylic acid (SA) and down-regulation of ROS-related genes in virus-infected AM plants. It was concluded that the AMF association potentially influenced the development of CMV infection and produced a stimulatory effect capable of increasing viral infection resistance in plants (Miozzi et al. 2020).

The difference between the transcriptional profile of leaves in *Rhizophagus irregularis*-colonized plant and the non-AM plant was compared using RNA-seq technology. A total of 742 out of 21,113 genes were found to be expressed differently in both types of leaves. Genes related to stress and hormone regulation were most likely to be affected. As expected, gene expression of mineral and sugar transporter in the mycorrhizal plant was noticed. Differences in expression pattern were correlated with systemic defence priming in AMF-induced resistance to counter *Xanthomonas campestris* pv. *vesicatoria* (Cervantes-Gámez et al. 2016). Genome-based studies had provided a better understanding of the gene expression and epigenetic changes in AM fungi-colonized plants. The dual culture (*Glomus mosseae* and *Fusarium equiseti* GF18-3) inoculation in cucumber plants was effective in the control of disease severity caused by cytomegalovirus (CMV); however, the single culture of *Glomus mosseae* was not so efficient in comparison to the dual culture. The dual culture was able to elicit responses based on salicylic acid, while alone treatment of *Glomus mosseae* activated responses mediated by jasmonic acid dependent on genes (Elsharkawy et al. 2012). The incidence and severity of necrotic lesions due to *Botrytis cinerea* or tobacco mosaic virus on the leaves of AM plants were higher than those of non-mycorrhizal controls.

The impact of AM fungi on viral infection is variable, and most studies are based on shoot virus infection. The contribution of AM fungi in plant and virus interactions is less investigated, but the promising findings are triggering more research into the AMF and viral interaction mechanisms in order to gain a better knowledge of plant immunity.

10.4.13 Pest Control

Pest insect infestation can be influenced by AMF symbioses. These effects strongly depend on the insect's lifestyle and level of specialization. AMF usually have a detrimental impact on generalist insects that are a predator of a variety of plants and vulnerable to the defensive responses of the plant (Gange and West 1994; Vicari et al. 2002). AMF may affect the metabolism of secondary metabolites, which are important in the plant's direct and indirect defences (Fontana et al. 2009). The extent of protection is also determined by the attacking pest insect's feeding pattern. The

formation of mycorrhizal symbiosis usually has a negative impact on leaf chewers and miners. Gange and West (1994) found that AM plant mycorrhiza *Plantago lanceolata* can reduce the population of *Arctia caja* larvae, and Vicari et al. (2002) noticed that the colonization of *F. mosseae* in *Lolium perenne* can reduce the survival and growth of *Phlogophora meticulosa* larvae. The fast activation of plant defences following their feeding is thought to be the cause of such reductions. Their feeding causes significant damage to leaf tissues, and mycorrhizal plants' defences are amplified as a result. Phloem-sucking insects like aphids cause less damage to the leaves and are hence able to escape from the host defence system. Although, some reports representing the opposite effect of AMF conclude that the sucking insect *Myzus persicae* performed better on mycorrhizal plants, possibly benefiting from their higher nutritional value (Gange and West 1994).

The following are the main conclusions that can be drawn from the studies discussed above: (1) The damage caused by different types of plant infections can be reduced by AMF. (2) Elicit and amplification of response against infection in roots are not the same; they may vary depending upon the applied fungal agent. (3) BCA protection is not universal for all the plants and different plants can respond differently. (4) Abiotic variables and other environmental circumstances can influence protection. As a result, interactions between different AM fungi and plant diseases are variable and dependent on the host plant and culture environment. The majority of BCA research has shown that AMF have a decent reaction to the fungal, bacterial, nematode, viral and insect disorders. But, it is also a fact that the effectiveness of AMF as BCA is not capable to perform protection against these pathogens under all circumstances.

10.5 Future Prospects and Conclusion

The association of plant-root and AMF forms mycorrhizosphere that influences the microenvironment of soil that favours the growth of other beneficial microorganisms antagonistic to soilborne pathogens and their proliferation (Lioussanne 2010). In summary, AMF used in biocontrol of a plant pathogen are an effective and sustainable method in agronomical practices and provide defence against various types of plant parasites, e.g. nematodes, fungi, bacteria, viruses and insects. AMF have a wide range of plant hosts that make them more suitable and instrumental to deal with a variety of plant pathogens. BCA can make heritable changes in their host. The mechanisms of these changes are not well understood and need further exploration.

The intricacy of the microorganism, soil composition and plant system is significantly influenced by existing environmental factors. It makes practical implications difficult and requires extensive experimental study to find optimal parameters to exploit the preventive potential of AM fungi. Despite the need for more research, current understandings are implied in biological control and management of plant diseases for substantial agro-systems, particularly with nursery and horticultural crops. Research studies recommend that suitable AM fungi inocula should be

applied when dealing with a pathogen or antagonistic members of soil micro-biota. Furthermore, the potential significance of AM in biological control must be explored and applied in crop breeding programmes for the selection of pathogen-resistant cultivars. High-throughput approaches, e.g. multi-omics, should be applied for better screening and investigation to understand dimensional interactions among AMF, microbial community, plant and soil in the environment. Because pathogenic inoculum density commonly reported in the field is difficult to replicate in a controlled nursery trial, more field experiments should be conducted to examine the efficacy of a BCA, which is the final stage of a BCA efficacy study. The results of studies on evaluating the biocontrol of plant diseases by using the antagonistic effects of arbuscular mycorrhizal fungi (AMF) are impressive. These studies suggest that AMF could significantly reduce the effect of different types of diseases through many mechanisms. Soilborne phytopathogens are hard to control, and the application of single biocontrol agent is often limited. These limitations can be overcome by integrated approaches and the consortium of microbes that can be more helpful than single inoculations. More elaborative studies are required keeping prime aim on (1) the biochemical and molecular strategies of AMF as biocontrol agents, (2) the feasibility of the AMF response to a wide range of biotic and abiotic stress, (3) incorporation of functional genomics, (4) the optimization and scale-up of process and techniques for application of AMF as BCA and (5) the formulation and exploration of commercial production of AMF as BCA. The pre-establishment of AM and substantial growth of the symbiosis before pathogen exposure is required for AM-mediated improvement in resistance or decrease in vulnerability. The virulence and inoculum potency of the pathogens present in the soil must also be considered when formulating a biological control agent. Any kind of biocontrol, including that mediated by an AM symbiosis, may be made irrelevant by a large pathogen inoculum density in the rhizosphere.

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Chapter 11
Rhizosphere Microbes and Wheat Health Management

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Abstract One of the major food crops farmed on a global scale is wheat. The microbial community of a soil affects its appropriateness for growing a crop in addition to its chemical and physical characteristics. The natural reservoir is called the rhizosphere, and it exhibits higher microbial activity. Mixed populations of saprophytic microbes, soilborne plant pathogens, plant growth-promoting microorganisms (PGPM), and microbes with antagonistic potential toward phytopathogens coexist in the rhizosphere soil. German microbiologist L. Hiltner coined the word "rhizosphere" in 1904 to refer to the layer of soil that covers and influences plant roots. Organic chemicals secreted by roots, also known as root exudates, are one of the most significant elements influencing the growth and development of microorganisms. Simple sugars, amino acids, organic acids, vitamins, and a variety of other substances are present in the exudates. Microalgae, fungus, bacteria, actinomycetes, protozoa, and other microbes live in soil. As part of their routine operations, they perform a variety of transformations, including as the addition of organic matter, nitrogen fixation, solubilization, and immobilization of various nutrients. In this regard, the soil microflora can be modified and safeguarded to enhance the bio-physio-chemical properties and control the soil's breakdown process.

Keywords Disease suppression · Microbiome · Rhizosphere · Soil health · Wheat health

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11.1 Introduction

Wheat (*Triticum aestivum* L.) is the second most important staple food crop being cultivated globally and therefore, wheat crop health is of prime significance (Giraldo et al. 2019). Wheat, however, is prone to both biotic and abiotic stresses that causes significant yield losses. Among the biotic stresses, diseases are the most important production constraints for wheat (Bonjean and Angus 2001). Wheat is host to an array of phytopathogens attacking the crop at its different growth stages and causing various degrees of yield losses owing to these phytopathogens (Gessese 2019).

The consistent application of chemical pesticides to crops with the intention of eliminating or reducing the severity of the diseases is the technique most frequently used to address the losses brought on by crop disease. But it is increasingly becoming evident that using chemical pesticides over an extended period of time can have a number of undesirable side effects (Viaene et al. 2016; Law et al. 2017). These chemicals build up in the soil, drain into the water, and are released into the air where they linger for years and pose a major hazard to the ecosystem as a whole (Hakim et al. 2021). Reduced diversity of beneficial microbial species in soil might liberate pathogen populations from competition and enhance the likelihood of pathogen invasion, particularly in the rhizosphere of the host plant, as a result of these chemical non-target effects (Jacobsen and Hjelmso 2014). The rhizosphere, which is made up of a complex web of plant roots, soil, and a wide variety of bacteria, fungi, eukaryotes, and archaea, is unquestionably the most complicated microhabitat. Crop growth and productivity are directly impacted by the rhizosphere conditions. Environments with abundant nutrients in the rhizosphere promote plant development and production and vice versa (Hakim et al. 2021). Plants gain from rhizospheric microorganisms' increased nutritional availability, production of plant growth hormones, and disease defense. But little is documented about how plants alter root microbial populations (Yin et al. 2021).

The microbial community of a soil affects its appropriateness for growing a crop in addition to its chemical and physical characteristics (Kumar et al. 2015b). The natural reservoir is called the rhizosphere, and it exhibits higher microbial activity. Mixed populations of saprophytic microbes, soilborne plant pathogens, plant growth-promoting microorganisms (PGPM), and microbes with antagonistic potential against phytopathogens coexist in the rhizosphere soil (Kallurmath and Rajasab 2000).German microbiologist L. Hiltner coined the word "rhizosphere" in 1904 to refer to the soil layer that envelopes and influences plant roots (Kumar et al. 2013; 2015a; Sinha et al. 2015). Organic chemicals secreted by roots, sometimes known as "root exudates," are one of the most significant factors contributing to the growth and development of microorganisms (Liljeroth and Baath 1988; Vishwakarma et al. 2020). Simple sugars, amino acids, organic acids, vitamins, and a variety of other substances are present in the metabolic byproducts (Singleton and Sainsbury 1991; Klein 1992). Microalgae, fungus, bacteria, actinomycetes, protozoa, and other microbes live in soil (Garrett 1981). As part of their routine operations, they perform a variety of transformations including the addition of organic matter, nitrogen

fixation, solubilization, and immobilization of various nutrients (Muller et al. 1988; Katayama et al. 1998; Brady and Weil 1999). In this regard, the soil microflora can be improved through manipulation and protection, and it also controls the soil's degradation process (Rezacova et al. 2007).

In addition to endangering the ecosystem through soil, water, and polluted air and pollution, inappropriate use of synthetic agrochemicals has also had a negative impact on soil microflora by changing the chemical and physical properties of the soil. The entire global life-support system is at jeopardy due to pesticide residues in the food chain (Singh et al. 2018). Various crop management practices have, however, been encouraged in subsequent years due to increased pesticide restrictions and rising application costs. However, the quick breakdown of crop residues, green manures, or organic bulks of agricultural wastes in the soil is critically required for crop intensification (Sinha et al. 2012). The disintegration of this material has been one of the main environmental concerns of today's globe because burning the crop residues is dangerous to soil health and severely contaminates our environment (Kumar et al. 2014a; b; Sinha et al. 2020). Both unmanaged and managed agricultural systems depend on the wide spectrum of processors carried by soil organisms. As the soil environment changes, the number, diversity, and activity of soil biota will alter (Kumar et al. 2010; Sinha et al. 2020). The rhizosphere dwelling microbes contribute differentially in agriculture. They impact crop production and productivity by influencing crop health through various mechanisms, so, keeping this in the view, we discussed about the rhizosphere microbial arsenal impacting wheat health in the present chapter.

11.2 Effects of Organic Manuring on Rhizosphere Microflora

Physical-chemical characteristics and soil microflora are crucial in understanding soil microbiology. Organic materials such as crop wastes, manures, and composts have a significant role in altering the soil environment (Anastasi et al. 2005). Microbial biomass may not be as sensitive to soil amendment with plant residues as microbial community composition. Compost, vermicompost, farm yard manure (FYM), green manures, and other types of soil amendments promote soil microbial activity and growth while gradually mineralizing soil nutrients (Randhawa et al. 2005). The application of FYM (Toyota et al. 1999) and spent mushroom compost (Piqueres et al. 2006) had a considerable impact on soil microbiology, according to the researchers' prior findings. However, the effects of compost were found to vary depending on both the type of soil and compost (Piqueres et al. 2006). Green manures have been explored as a potential management strategy for soilborne plant pathogens. They do this by increasing microbial competition and antagonism in the rhizosphere and non-rhizosphere soils (Manici et al. 2004; Ochiai et al. 2007;

Kamil et al. 2009; Kumar 2010) while at the same time lowering the inoculum potential of plant pathogens through the germination and lysis of their resting structures. According to research by Rodgers-Gray and Shaw (2001), Mogle et al. (2005), and Chamle et al. (2007), organic matter, such as green manures, has a significant impact on soil's physiochemical characteristics and population dynamics of the soil microflora (Anastasi et al. 2005).

During the application of green manures, the percentage of pathogenic fungal population, viz., *Fusarium oxysporum, Rhizoctonia* sp., *Sclerotium rolfsii*, *Sclerotinia sclerotiorum*, *Mucor racemosus, Pythium* sp., *Helminthosporium* sp., and *Macrophomina phaseolina*, was recorded to be decreased or disappeared while *Chaetomium globosum, Aspergillus* spp., *Penicillium* spp., and *Trichoderma* spp. were observed to be increased considerably which contribute to reducing the inoculum level in the rhizosphere and non-rhizosphere soils (Manici et al. 2004; Jha and Jalali 2006; Kumar 2010; Sinha et al. 2010). The application of green manures increases the amount and percentage of fungal species that are present, which helps to reduce the population of numerous phytopathogenic fungal species. Several researchers have obtained similar results (Jha and Jalali 2006; Ahmed and Upadhyay 2009; Kamil et al. 2009; Sinha et al. 2009; Kumar 2010; Kumar et al. 2017). The presence of beneficial microflora is encouraged by the addition of green manures (Kumar 2010; Kumar et al. 2010). These microflora exhibit various colonization patterns (Kumar et al. 2011a, b) due to their capacity to utilize their respective substrates in various ways. However, the mechanism by which they affect plant growth might differ from species to species and strain to strain. Green manuring affects the rhizosphere microbial population, which is important for the growth and development of a plant.

11.3 Plant Root Exudates and Rhizomicrobiome Interactions

The rhizosphere represents a highly dynamic front for interactions between roots and pathogenic and beneficial soil microbes (Hirsch et al. 2003). Plant roots exude an enormous range of potentially valuable small molecular weight compounds into the rhizosphere. Some of the most complex chemical, physical, and biological interactions experienced by terrestrial plants are those that occur between roots and their surrounding environment of soil, i.e., the rhizosphere (Bais et al. 2006). These interactions involving plant roots in the rhizosphere are plant root-plant root interactions, plant root-insect interactions, and plant root-microbe interactions. Among these plant interactions, plant root-microbe interactions are of major interest for the researchers for a long time (Sadasivan 1960; Rovira 1965; Bais et al. 2006). It is generally recognized that root exudates directly affect the rhizosphere's micropopulation (Bais et al. 2006; Kannan and Sureendar 2009; Dennis et al. 2010; Shi et al. 2011). Plant roots exude several chemicals which attract soil microorganisms to the rhizosphere, for example, flavonoids secreted by roots of

leguminous plants that attract *Rhizobia* to form symbiotic associations with leguminous plants and initiate nodulation for nitrogen fixation in the roots of these plants. Other root exudates such as carbohydrates and amino acids stimulate plant growth-promoting microorganisms (PGPM) by chemotaxis on root surfaces through certain mechanisms (Bais et al. 2006). These plant growth-promoting microorganisms including fungi, bacteria, etc. have manifold beneficial effects on plant health. Therefore, the qualitative and quantitative changes in the rhizosphere microflora besides the addition of organic matter or green manuring may be due to the alteration in the pattern of root exudates released by different plants. Root's exudates can attract beneficial organisms, but they can also be equally attractive to the pathogenic populations (Schroth and Hildebrand 1964), which may express virulence on limited numbers of host species.

11.4 Rhizomicrobiome and Wheat Health Dynamics

Rhizomicrobiome is involved in complex interactions leading significant impact on wheat crop health (Santana et al. 2020). By boosting food availability, creating plant hormones, enhancing tolerance to abiotic stresses, and responding to environmental changes, non-pathogenic rhizospheric microorganisms, such as helpful and mutualistic microbes, can stimulate plant growth (Yin et al. 2021). They may also act as a plant growth enhancer, soil bioremediator, plant-pathogen inhibitors, or disease incitant itself.

11.4.1 Rhizosphere Microflora as Potential Biological Control Agents

The function of rhizosphere microflora in promoting plant growth and biological control of plant diseases is well known (Jha and Jalali 2006; Kannan and Sureendar 2009). For the benefit of plant health, these rhizosphere bacteria have been used as biofertilizers and bioprotectants (Basnet et al. 2009) (Table 11.1). Ridiculous soilborne plant diseases and diseases which are seed-borne in nature are inhabited by beneficial rhizosphere microflora (Upadhyaya and Rai 1987). These microbes involved in the control of various soilborne and seed-borne phytopathogens are now popularly called biological control agents (BCA). For the effective control of the phytopathogens, these biological control agents adopt several mechanisms, viz., antibiosis; competition for growth factors or food, e.g., competition for iron; induced resistance; parasitism; production of extracellular enzymes, etc. (Whipps 2001).

Table 11.1 Some important rhizosphere microorganisms used as biocontrol agents against various phytopathogens of wheat

Beneficial rhizosphere microorganisms	Pathogens	Disease/ crop	References
Bacteria			
Bacillus spp.	*Rhizoctonia solani*, *Gaeumannomyces graminis* var. *tritici*	Take-all disease of wheat	Ryder et al. 1999
Burkholderia cepacia A3R	*Fusarium graminearum*	Wheat	Huang and Wong 1998
P. fluorescens Q8r1-96	*Gaeumannomyces graminis* var. *tritici*	Take-all disease of wheat	Raaijmakers and Weller 2001
Pseudomonas isolates WPS3 and WPS90	*Rhizoctonia solani*	Root rot disease in wheat	Dua and Sindhu 2012
Fungi			
Idriella bolleyi	*Bipolaris sorokiniana*	Spot blotch disease	Duczek 1997
Phialophora sp. 1-52	*Gaeumannomyces graminis* var. *tritici*	Take-all disease of wheat	Mathre et al. 1998
Pseudomonas chlororaphis MA342	*Tilletia caries*	Wheat	Johnsson et al. 1998
Trichoderma harzianum T-22	*Pyrenophora triticis-repentis*	Wheat	da Luz et al. 1998
Stenotrophomonas maltophilia, Bacillus cereus, Trichoderma harzianum	*Fusarium graminearum*	Wheat	Dal Bello et al. 2002
Sphaerodes mycoparasitica, Trichoderma harzianum	*Fusarium graminearum*	Wheat	Vujanovic and Goh 2012

11.4.2 Rhizosphere Microflora Is Used to Dissolve Several Plant Nutrients

The microorganisms in soil play an important role to make plant nutrients available to the plants (Table 11.2). They may convert an insoluble form of nutrients into soluble forms. The rhizosphere microflora function as biofertilizers in soils which include bacteria, fungi, blue-green algae, and mycorrhiza (Umamaheswari et al. 2001). Biofertilizers are famous for the advantages they provide to the soil and plant system, viz., plant nutrition and disease-pest resistance tolerance to crops against adverse climate and soil conditions (Fig. 11.1). These are very helpful to reduce increased salinity and stop the leaching of nutrients from the soil. Plant growth-promoting bacteria (PGPR), typically colonizing at the rhizosphere, are

Table 11.2 Rhizosphere microorganisms contributing to soil health and wheat plant health

Rhizosphere microorganisms	Contribution/role	References
Bacteria		
Arthrobacter, *bacillus*, *Massilia, Devosia, Oceanobacillus,* and *Lactococcus*	Important PGPR present in the wheat roots were higher at the jointing and ripening stages than at the tillering stage	Ofek et al. 2012; Upadhyay et al. 2012; Pontes et al. 2015; Vejan et al. 2016
Arthrobacter, bacillus, and *Devosia*	Increase the level of nitrogen (N) input by recruiting PGPR through secretion of organic acids	Kamilova et al. 2006; Rudrappa et al. 2008; Ling et al. 2011
Sphingomonas	Rhizospheric bacteria are mostly found in the later stages of wheat root maturation and also have a plant protective role against infestations	Kim et al. 1998; Khan et al. 2014; Araujo et al. 2019
Azospirillum brasilense Sp245, *Azospirillum brasilense* INTA Az-39, and *Azospirillum lipoferum*	Present around the roots and increased growth rate of wheat coleoptiles during drought conditions	Alvarez et al. 1996; Díaz-Zorita and Fernández-Canigia 2009; Arzanesh et al. 2011
Burkholderia phytofirmans	Increased grain yield, photosynthetic rate, water use efficiency, chlorophyll content of wheat during drought	Naveed et al. 2014
Bacillus sp.	Enhance indole-3-acetic acid, antioxidant defense system, SOD shoots and roots, shoot POD and CAT of wheat during heavy metal stress	Wang et al. 2013
Bacillus thuringiensis, Azotobacter chroococcum, Paenibacillus ehimensis, pseudomonas pseudoalcaligenes	Provide higher heavy metal resistance siderophore, indole acetic acid, HCN, P solubilization to wheat	Kumar et al. 2015b
Pseudomonas spp.	Provide IAA, P solubilization, rhamnolipids, and siderophores during cold stress	Mishra et al. 2009
Bacillus megaterium M_3, *Bacillus subtilis* OSU142, *Azospirillum brasilense* Sp245, *Raoultella terrigena*	Increase root and shoot dry weight, leaf total chlorophyll content, stomatal conductance, and leaf relative water content during cool weather	Tarun et al. 2012
Pseudomonas putida $AKMP_7$	Protect from heat stress	Ali et al. 2011
Pseudomonas putida, pseudomonas extremorientalis, pseudomonas chlororaphis and Pseudomonas aurantiaca	Wheat root tip colonizer, and promote salt tolerance	Egamberdieva and Kucharova 2009
Pseudomonas fluorescens 153, 169, *pseudomonas putida* 108	Strengthen grain yield and 1000 grain weight	Abbaspoor et al. 2009

(continued)

Table 11.2 (continued)

Rhizosphere microorganisms	Contribution/role	References
Bacillus pumilus, Pseudomonas mendocina, Arthrobacter sp. *Halomonas* sp., and *Nitrinicola lacisaponensis*	P solubilization, indole acetic acid (IAA), siderophore, ammonia, proline accumulation, salt tolerance, choline oxidase activity during salinity	Tiwari et al. 2011
Pseudomonas putida, Enterobacter cloacae, Serratia ficaria, and *P. fluorescens*	Increase germination rate percentage/index and improved nutrient status	Nadeem et al. 2013
Halobacillus sp. SL3 and *bacillus halodenitrificans* PU62	Boost root length, root elongation, and dry weight	Ramadoss et al. 2013
Enterobacter asburiae, Moraxella pluranimalium, pseudomonas stutzeri	Number of tillers, grain weight, growth, and yield buildup	Raheem and Ali 2015
Fungi		
AM fungi (*Rhizophagus irregularis*)	Maintain phosphorus level of soil and increase the yield of wheat	Landis et al. 2004; Assainar et al. 2018; Yuvaraj and Ramasamy 2020
Aspergillus, *Dipodascus*, and *Trichoderma*	Intensively colonize wheat roots as a protective layer	Nicolaisen et al. 2014; Bokati et al. 2016; Hertz et al. 2016; Barnett et al. 2017; Liu et al. 2018
Trichoderma harzianum	Significantly increased the wheat grown in terms of number of tillers, rootlets, number of grains per spike, and weight of 1000 seeds	Hyakumachi 1994; Sharma et al. 2012
T. longibrachiatum	Increase contents of chlorophyll, proline, soluble sugar, and protein in wheat seedlings under salt stress and enhance the relative levels of antioxidant gene expression in the stressed wheat plants	Zhang et al. 2016
T. koningii	Plant biomass, root-shoot growth	Hyakumachi 1994
Aspergillus niger	Support shoot and total plant length ratio	Gujar et al. 2013
Aspergillus flavus	Excellent growth-promoting endophytic fungi of wheat	Ripa et al. 2019
Penicillium sp.	Growth promotion in wheat also elevates phosphorus uptake	Wakelin et al. 2007
Phoma sp.	Increase wheat plant height, ear-head length and weight, seed number, and plant biomass at harvest	Shivanna et al. 1996

(continued)

Table 11.2 (continued)

Rhizosphere microorganisms	Contribution/role	References
Sphaerodes mycoparasitica	Improve wheat seed germination and seedling growth	Vujanovic and Goh 2012
Sterile red fungus	Increased fresh shoot and root weight of wheat along with root length and also provided significant protection to the hosts from infection by the take-all fungus	Dewan and Sivasithamparam 1988

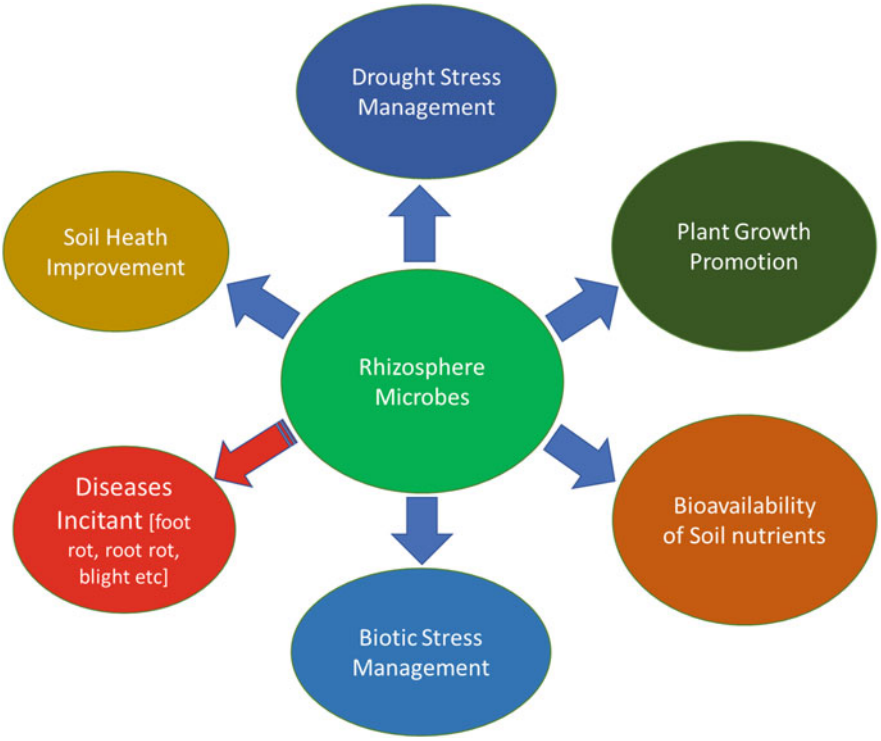

Fig. 11.1 Possible major contribution of rhizosphere inhabiting microbes to wheat crop health

known to increase the yield and help alleviate the effects of biotic or abiotic stresses (Backer et al. 2018).

The practice of efficient diverse plant growth-promoting rhizobacteria (PGPR) as biofertilizers and biological control agents is promising in reducing the use of chemical fertilizers, at the same time maintaining yields at commercially viable levels and/or maintaining grain protein content (Goswami et al. 2015; Dong et al. 2019; Adedeji et al. 2020; Anli et al. 2020; Atieno et al. 2020). As such, PGPR contribute to the improvement of both local and global environments, reducing

dependence on nonrenewable resources while still being economically competitive (both price and quality aspects) (Souza et al. 2015). Rhizosphere engineering using these PGPR has numerous applications, including eco-friendly sustainable agriculture development as well as crop fertilization. There is increased interest in stress-resilient PGPM and their application to induce stress (drought, salinity, and heat) tolerance mechanisms in plants as a result of the severe consequences of climate change on plants and rhizosphere biology (Hakim et al. 2021).

11.4.3 Rhizosphere Microflora as Plant Pathogens

Pathogenic microorganisms affecting plant health are a major and chronic threat to food production, food security, and ecosystem sustainability in a global scenario. The rhizosphere microflora plays an important role in plant disease development. All soil-borne phytopathogenic fungi, bacteria, etc. cause several plant diseases of economic importance in various crops (Table 11.3). These soil-borne phytopathogens are members of rhizosphere microflora. Like beneficial microorganisms, the soil-inhabiting pathogenic populations which may be already present in the rhizosphere can be attracted to their host plants through its root exudates (Schroth and Hildebrand 1964). These soil or rhizosphere inhabiting pathogenic populations may include phytopathogenic fungi, bacteria, viruses, nematodes, viroids, etc. These pathogens after successful establishment may express virulence on only a limited number of host species. This is a host-specific process. Numerous pathogenic organisms, including bacteria and fungus, coevolved with plants and are very host-specific (Raaijmakers et al. 2009). Plant disease is the exception rather than the rule in nature, though, as pathogens may not thrive in environments that are best for growing plants (Paulitz and Bélanger 2001).

11.5 Assertive Role of Beneficial Rhizosphere Microflora

An effective strategy to stop the rapid environmental deterioration while ensuring high agricultural productivity and better soil health is to promote sustainable agriculture with a gradual decrease in the use of synthetic agrochemicals and a more prominent utilization of biowaste-derived substances as well as the biological and genetic potential of crop plants and microorganisms (Basu et al. 2021). Microbial products can be used as nontoxic, environment friendly agents to promote plant development and manage disease. By using microbial formulations to fertilize crops, it is possible to boost the biological potential and fertility of soil while reducing the harmful impacts of agrochemicals (Raklami et al. 2019; Jabborova et al. 2020). Some advantageous roles of rhizosphere microflora are listed below:

Table 11.3 Rhizosphere microorganisms of wheat causing various soil-borne diseases

Phytopathogenic microbes	Name of the disease	Host crop	References
Fungal diseases			
Fusarium spp.	Wilt diseases	Various crops including wheat	Naguib 2018
Pythium spp. *rhizoctonia* spp.	Damping-off	Various crops including wheat	Sturrock et al. 2015; Lamichhane et al. 2017
Tilletia caries and *Tilletia foetida*	Common bunt	Wheat (*Triticum aestivum* L.)	Hoffmann and Waldher 1981
Tilletia indica	Karnal bunt	Wheat (*Triticum aestivum* L.)	Mitra 1931; Sharma et al. 2017
Urocystis agropyri	Flag smut	Wheat (*Triticum aestivum* L.)	Purdy 1965
Rhizoctonia solani	Seedling and root rots Sharp eyespot	Various crops, e.g., rice, maize, mung bean, cotton, crucifers, cucurbits (mainly cucumber), etc.	Sturrock et al. 2015
Gaeumannomyces graminis var. *tritici*	"Take-all" is the common name for a root, crown, and basal stem (foot) rot	Primarily affects wheat, but can also affect barley, oats, rye, as well as other grass crops and weeds	Wiese 1987; Hornby 1998; Freeman and Ward 2004
Pyrenophora tritici-repentis	Tan spot of wheat	Barley, wheat, and oats are common hosts	Saari 1998
Cephalosporium gramineum	*Cephalosporium* stripe	A vascular wilt-type disease of wheat and barley, which also affects other cereals and grasses	Nisikado et al. 1934; Bruehl 1956; Quincke and Peterson 2012; Quincke et al. 2014
Calonectria nivalis (Fusarium nivale)	Fusarium leaf blotch (snow mold)	The disease affects durum wheat and triticale more than bread wheat or rye	Rawlinson and Colhoun 2007
Microdochium [Fusarium] nivale	Pink snow mold	Occurring on wild grasses, lawns, and winter wheat	Murray et al. 1999, Ponomareva et al. 2021
Typhula idahoensis, *T. ishikariensis*, and *T. incarnata*	Speckled snow mold (*T. incarnata* is often found causing a root and crown rot of wheat and barley in the absence of snow cover)	Speckled snow mold on turf and crown and root rot of wheat and barley	Murray et al. 1999, Ponomareva et al. 2021
Myriosclerotinia borealis	Snow scald	The overall impact on winter wheat	Murray et al. 1999, Ponomareva et al. 2021

(continued)

Table 11.3 (continued)

Phytopathogenic microbes	Name of the disease	Host crop	References
Pythium iwayami and *P. okanoganense*	Snow rot	Overall impact on winter wheat	Murray et al. 1999, Ponomareva et al. 2021
Claviceps purpurea	Ergot	Ergot is found in all small grain cereal crops, especially if sterility occurs for some reason (e.g., frost)	Miedaner and Geiger 2015
Pseudocercosporella herpotrichoides (Syn. *Cercosporella herpotrichoides*)	Eyespot (Strawbreaker)	Wheat, triticale, rye, oats, and other related grasses can be affected by the disease, with wheat being the most susceptible	Fitt et al. 2007; Barton 2016
Bacterial diseases			
Xanthomonas campestris pv. *Translucens* syn. *X. translucens, X. translucens pv. undulosa, X. campestris* pv. *Undulosa*	Bacterial black chaff and bacterial stripe	Occurring worldwide on all small grain cereals and many types of grass including wheat	Duveiller 2008
Corynebacterium tritici	Bacterial spike blight (yellow ear rot)	Wheat is the only cultivated host, though some wild grasses are susceptible to attack	Duveiller et al. 2012
Viral diseases			
Soil-borne wheat mosaic virus (SBWMV)	Wheat mosaic disease transmitted by a fungal-like protist, *Polymyxa graminis*	Causes severe stunting and mosaic in susceptible wheat, barley, and rye cultivars	Littlefield et al. 1998; Hariri et al. 2001
Nematodes			
Heterodera avenae	Molya disease of wheat	Confined to the members of family Poaceae (=Graminae) such as wheat, barley, oats, rye, bajra, maize, and other various grasses	Sharma and Sharma 2000
Meloidogyne spp.	Formation of small knots or galls near the tips of the roots	Very wide host range, including all small grain cereals	de Brida et al. 2017

1. They increase the nutrient level in the soil or make them available to the plants, e.g., nitrogen, phosphorus, zinc, iron, etc.
2. Beneficial rhizosphere microflora produce plant growth-promoting compounds and hence they act as plant growth-promoting microorganisms (PGPM) for the plants.
3. Beneficial rhizosphere microflora reduce phytopathogenic microbes through several mechanisms.
4. The use and promotion of beneficial rhizosphere microflora as biofertilizers is environment-friendly, cost-effective, and helpful to replace agrochemicals to a certain extent.
5. Beneficial rhizosphere microflora may be useful in enhancing the soil's structure and water-holding ability.
6. Beneficial rhizosphere microflora increases seed germination and seed vigor.
7. They help improve soil fertility and fertilizer use efficiency and finally increase crop yield to an average of 15–20%.

11.6 Conclusion

The rhizosphere is an arena for different types of soil-dwelling microorganisms. These microorganisms may "cross-talk" or interact with the help of several chemical compounds and signals through various understood or even undiscovered mechanisms. This rhizosphere community of microbes has great importance for plant health. Their interaction with the plant may result in beneficial association, viz., PGPR, biological control agents, symbionts, antagonists, and phytopathogens, or sometimes as harmful association, e.g., development of catastrophic soil-borne diseases in crops. Therefore, identification of rhizosphere microflora and determination of their role in plant's health are the need of time. The use of the beneficial rhizosphere microflora to enhance crop production and productivity may be promoted. Also, there is a need to carry out more investigation on various research aspects of this rhizosphere microflora in the interest of the farmers of the country.

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Chapter 12
Exploring the Potential of Secondary Metabolites from Indigenous *Trichoderma* spp. for Their Plant Growth Promotion and Disease Suppression Ability in Pulses

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Abstract Pulses are an important part of the human diet; it has all the nutritional elements required for the body. Pulses contain various varieties like beans, lentils, peas, green gram, horse gram, and chickpeas. Pulses are rich in protein and are low in fats. This reduces the risk of cardiovascular diseases. The presence of phenols, flavonoids, saponins, oxalates, and enzyme inhibitors is the added health benefit for humans. Bioactive metabolite produced by *Trichoderma* species plays an important role in interaction with plants and pathogens. These bioactive metabolites have antibiotic properties, which inhibit or kill other organisms. These bioactive metabolites are used for crop protection and as biofertilizers. These bioactive metabolites are also able to induce systemic disease resistance in plants. *Trichoderma* is well known for its secondary metabolite production ability. The widespread use of secondary metabolites for the control of plant pathogens, plant growth promotion, and induction of host resistance may become popular in the coming years under IPM strategies.

Keywords *Trichoderma* spp. · Secondary metabolites · Disease suppression · Phytopathogen · Daucanes · IPM strategies

12.1 Introduction

Biological control of plant disease provides an alternative to synthetic pesticides due to increasing public concern over environmental changes. Soil-borne fungi can survive in a highly competitive environment. Natural antagonism between fungi

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has been observed in every fungal ecosystem (Wicklow 1998; Ghisalberti 2002). *Trichoderma* species are free-living fungi; they easily colonize the root, soil, and foliar environment. Being invasive in nature they can easily work against fungal phytopathogens directly (mycoparasitism) or indirectly (competition for food and space, plant growth promotion, antibiosis, modification of environment). *Trichoderma* species secrete a wide range of lytic enzymes (chitinase, xylanase, cellulases, etc.) (Gloer 1997) and secondary metabolites. The antagonism behavior of *Trichoderma* toward other fungi is due to the combined action of lytic enzymes and secondary metabolite (Mishra et al. 2020a). Induction of systemic and local defense response in many agricultural crops (cotton, lettuce, ball pepper, and tobacco) by *Trichoderma* has been reported (Harman et al. 2004; Yedidia et al. 2003).

Pulses are cultivated all over the world and around 50% of pulse production occurs in Asia. Canada is the major producer and exporter of pulses all over the world (Hoover and Ratnayake 2002). The annual global pulse production is around 904 kg/ha. Pulse consumption is higher in Africa and lower in Europe (FAOSTAT 2011). In several countries of the world, pulses are the major component of the diet. In India, pulse consumption is highest followed by Kenya and Turkey (FAOSTAT 2011). Pulses are high in protein, dietary fibers, micronutrients, and various other substances. Due to their high nutritional content, pulses have been described as a future major source of nutritional and health benefits by the Indian Pulses and Grains Association (FAO/WHO 2009). Pulses are rich in nutrition as compared to vegetables and are a cheap source of proteins as compared to animal protein. For pulse, production water is not required in higher amounts. So, the cultivation of pulses can be done in rain-deprived areas. Pulses are commonly known as "poor man's meat" (Iriti and Varoni 2017). The combination of essential amino acids with high protein content and fiber makes it useful for consumption. Pulse production is influenced by various abiotic and biotic factors. Among biotic factors, phytopathogens are the main cause of yield loss.

Trichoderma species are the most common and popular bioagents. It is commonly used as a biofertilizer for promoting plant health and antagonizing soil-borne pathogens (Mishra et al. 2020b). They can be applied directly to the plants in the form of talc-based formulation, or their secondary metabolites can be packed into a liquid form and applied to the stem of plants (Harman et al. 2004). *Trichoderma* applications made plants more resistant and increase their productivity for a safe agroecosystem (Vinale et al. 2008; Howell 2003).

12.2 Diversity of Different *Trichoderma* Isolates

Trichoderma spp. like *T. ressei* secrete cellulase enzymes which are used for the recycling of cellulosic wastes. Many *Trichoderma* strains are commercially used to control plant diseases like *Fusarium* wilt disease in pulses, *Sclerotium rolfsii* in tobacco and bean, *Botrytis cinerea* in apple, etc. (Cutler et al. 1999). Some

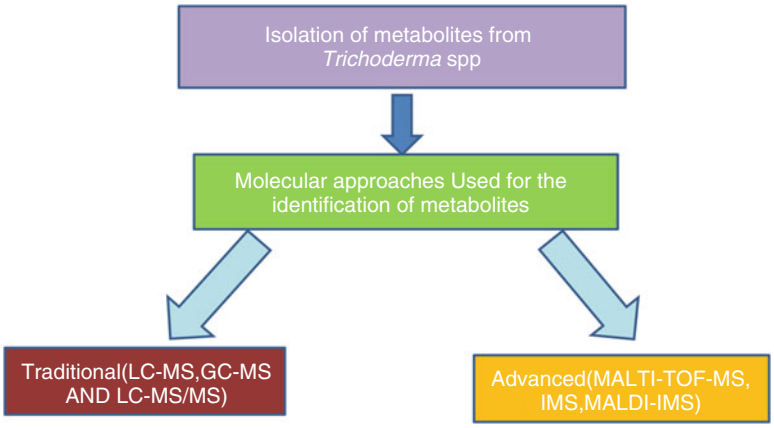

Fig. 12.1 Approaches used for the identification of metabolites from *Trichoderma* spp

Table 12.1 Some common *Trichoderma* metabolites with their physiological activities

Metabolite name	Function
Trichodermin, trichodermol, sesquiterpenoids	Growth inhibitors
Trichoviridin and isocyanides	Inhibited growth of *Micrococcus luteus*
1,3,6,8-Tetrahydroxyanthraquinone and 1-acetyl-2,4,5,7-tetrahydroxy-9,10-anthracenedione anthroquinone	Pigments
T-2 toxin	Toxin
Lignoren sesquiterpenoid, lipopeptaibol trichogin GA IV, 2′,4-′-dihydroxy-3′-methoxymethyl-5′-methylacetophenone and 2′,4-′-dihydroxy-3′,5′-dimethylacetophenone (clavatol) octaketide-derived acetal diol	Antifungal
Trichodermin	Antibiotic
Demethylsorbicillin and oxosorbicillinol, bisorbicillinoid	Free radical scavenging activity
Crude extracts, harzianopyridone	Antifungal, antimicrobial

commercial formulations are available in the market and are used for the control of several soil-borne plant diseases (Cardoza et al. 2005) (Table 12.2). *Trichoderma* metabolites can be classified into two categories: volatile and non-volatile. Volatile metabolites are low molecular weight compounds; these include simple aromatic compounds, polyketides, butenolides, volatile terpenes, and isocyanate metabolites. These are relatively non-polar substances with high vapor pressure. Non-polar metabolites are high molecular weight polar metabolites. They induce phytopathogenic action through direct interaction with a pathogen (Fig. 12.1). Here we will describe the different types of metabolites produced by *Trichoderma* species and their biological role (Table 12.1).

12.3 Metabolites Present in *Trichoderma* spp. Are Associated with Crop Plants

12.3.1 Anthraquinones

Metabolites of this class are the most common metabolites of *Trichoderma*. Slater et al. (1967) reported chrysophanol, pachybasin, and emodin from *T. viride*. Similar compounds were observed by Donnelly and Sheridan (1986) in *T. polysporum* grown with *Basidiomycetes* fungi. Extraction of chrysophanol and emodin was done from *T. aureoviride* in 1990. Combined cultures of *Trichoderma* and *Fusarium solani* produces trichodermaol. Anthraquinones are majorly involved in the pigmentation's acetyl and O-methyl derivatives of pachybasin, chrysophanol, and emodin and has been found to show a decrease in the mycelial growth of strains of *F. annosus*. Emodin possesses monoamine and tyrosine kinase inhibition activity. Trichodermaol exhibits antibacterial activity, and 50 μg/mL conc. of trichodermaol is found to be toxic to *Bacillus subtilis* and *Streptococcus aureus* (Adachi et al. 1983). Chrysophanol has been found to possess antifungal activity against *Candida albicans* and *Aspergillus fumigatus* at 25–250 μg/mL concentration (Agarwal et al. 2000).

12.3.2 Daucanes

This belongs to the class of sesquiterpenes and is also known as carotenes. During a long analysis of secondary metabolites produced by *Trichoderma* species, *T. virens* were found to produce a novel carotene-type metabolite having antifungal activity against yeast and dermatophytes (Watanabe et al. 1990). An oleic acid ester L-735,334 was isolated from the *T. virens*. These compounds were found to exhibit the growth of etiolated wheat coleoptiles (Macias et al. 2000).

12.3.3 Simple Pyrones

The pyrone 6-pentyl-2H-pyran-2-one is the representative metabolite of this series. This compound is responsible for the aromatic fragrance associated with the fungus. This compound was first identified by Collins and Halim in 1972. This compound has been found to be present in *T. viride, T. harzianum* (Claydon et al. 1987), and *T. koningii* (Simon et al. 1988). This metabolite is found to exhibit antagonism against *Rhizoctonia solani* and *Fusarium oxysporum* f. sp. *lycopersici* (Scarselletti and Faull 1994). This compound has been found to significantly reduce the growth of *B. cinerea*. From *T. harzianum,* four analogues of pyrone have been isolated exhibiting phytopathogenic action: *Penicillium* spp., *Aspergillus fumigatus*,

Candida albicans, and *Cryptococcus neoformans* (Claydon et al. 1987; Parker et al. 1997). In 1995 Hill et al. patented the hydro derivatives massoilactone and delta-decanolactone for their ability to control *Botrytis* or *Phytophthora*. These compounds inhibit the growth of *Aspergillus niger*, *Candida albicans*, and *Staphylococcus aureus* (Kishimoto et al. 2005). From *T. viride* viridepyronone has been isolated which has been found to show antagonistic activity in *Sclerotium rolfsii* at 196 μg/mL of concentration (Evidente et al. 2003).

12.3.4 Koninginins

This belongs to pyranes; they are found to be present in some species of *Trichoderma*. The culture broth of *Trichoderma koningii* showed the presence of koninginins (Cutler et al. 1989, 1991a). Koninginin has been found to show an inhibitory effect on the growth of *Rhizoctonia solani, Phytophthora cinnamomi, Pythium middletonii, Fusarium oxysporum*, and *Bipolaris sorokiniana.*

12.3.5 Trichodermides

Trichodermides have been isolated from the cultures of *T. virens.* These trichodermides showed inhibitory action toward human colon carcinoma, and the inhibitor concentration was 0.32 μg/mL and it was found to have a low cytotoxic effect against P388, A-549, and HL-60 cancer lines (Garo et al. 2003; Liu et al. 2005).

12.3.6 Viridans

These compounds possess an unusual furan ring which is fused with c-4 and c-6 carbons of the steroid framework (Hanson 1995). These were first described in 1945 (Brian and McGowan 1945). *T. koningii*, *T.* virens, and *T. viride* have been found to possess this metabolite. *Trichoderma* viridans have been found to possess the inhibitory action against the spore germination of *Botrytis allii*, *Colletotrichum lini*, and *Fusarium caeruleum* (MIC of 0.003–0.006 lg/mL), *Penicillium expansum*, *Aspergillus niger*, and *Stachybotrys atra* (6 lg/mL) (Brian and McGowan 1945; Ghisalberti 2002). The viridiol obtained from the *T. viride* and *Gliocladium* species has been found to possess phytotoxic and antifungal properties.

12.3.7 Viridiofungins

The structural element of this metabolite is citric acid. *T. viride* produces these metabolites from solid-state fermentation (Harris et al. 1993; Mandala et al. 1997). Viridiofungins are antifungal compounds and have been found to possess inhibitory action toward *Aspergillus* species (Harris et al. 1993).

12.4 Nitrogen Heterocyclic Compounds

Harzianopyridone is the chief representative of this class of metabolite and has been found to show antifungal activity against *Rhizoctonia solani* (Dickinson et al. 1989), *Gaeumannomyces graminis* var. *tritici*, and *Pythium ultimum* (Vinale et al. 2006). Harzianic acid also belongs to this category and was obtained from *T. harzianum* (Sawa et al. 1994).

12.4.1 Derivatives of Trichodenones and Cyclopentenone

5-Hydroxy-3-methoxy-5-vinylcyclopent-2-en-1-one was isolated from the cultures of *T. album* in 1977 (Strunz et al. 1977).

Harziphilone and fleephilone were isolated from the culture filtrate of *T. harzianum, Rhizoctonia solani, Pythium ultimum, and Gaeumannomyces graminis* var. *tritici* (Vinale et al. 2006).

12.4.2 Harzialactones and Derivatives

From *T. harzianum* harzialactones and their derivatives have been isolated. Harzianoilde is a secondary metabolite with butenolide ring that has been identified. These compounds have been found to show inhibitory action toward *Gaeumannomyces graminis* var. *tritici*, *Rhizoctonia solani*, and *Pythium ultimum* (Vinale et al. 2006). Vertinolide is a different series of metabolites isolated from the *T. longibrachiatum*. Sparapano and Evident (1995) reported the biological activity of these compounds.

12.4.3 Trichothecenes

Trichoderma genus is the predominant producer of these metabolites (Grove 1988, 1993, 1996). Trichothecenes can be divided into four categories. Type A has a

functional group other than keto at C-8 position, type B has a keto group at C-8 position, and type C and D have a second epoxide ring at c-7,8 or C-9,10 positions and a macrocylic ring was present between C-4 and C-5 with ester linkages. Trichodermin belongs to the category of trichothecenes and has been isolated from *T. viride*.

12.4.4 Isocyano Metabolites

These metabolites have a characteristic five-membered ring. Forty years ago the first report on cyclopentene in *Trichoderma* was published (Pyke and Dietz 1966; Meyer 1966). Isonitrile trichoviridin was first isolated by Tamura et al. form *T. koningii*.

12.4.5 Setin-like Metabolites

Various species of *Trichoderma* secrete setin-like metabolites which are phytopathogenic against *Fusarium* species.

12.4.6 Bisorbicillinoids

Bisorbicillinoids are a family of natural products which have many biological activities. Trichodermerol has been isolated from *T. longibrachiatum*.

12.4.7 Diketopiperazines

Trichoderma harzianum, *T. hamatum*, and *T. koningii* have been found to produce diketopiperazines. Gliotoxin was the first member of this category to be identified. There are various species of *Trichoderma* like *T. harzianum, T. hamatum*, *T. virens,* and *T. koningii* which produce this compound. These metabolites have been found to play an inhibitory effect against *Rhizoctonia solani* and *Pythium ultimum* (Howell and Stipanovic 1983).

12.4.8 Ergosterol Derivatives

In *Trichoderma* sterol production was first detected by Kamal et al. (1971) from the fermentation broth of *T. pseudokoningii*. Ergosterol is the most common sterol, and in 1975 this has been isolated from the *T. hamatum* (Hussain et al. 1975). Ergokinin,

a class of sterol, has been isolated from *T. koningii*. Ergokinin has been patented for its use in the inhibition of yeast and fungal mycelia (Reichenbach et al. 1990). Ergokinin is effective against *Candida* and *Aspergillus* but is ineffective against *Cryptococcus, Fusarium, and Saccharomyces* (Vicente et al. 2001).

12.4.9 Peptabiols

This is a large family of natural products. *Trichoderma* species are the main producers of this metabolite class. From *T. viride*, first compound, named alamethicin, of this class was isolated. The use of alamethicin, produced by *Trichoderma viride*, induces a defense response in *Phaseolus lunatus* and *Arabidopsis thaliana.*

12.4.10 Cyclonerodiol Derivatives

Cyclonerodiol was first reported from *T. koningii* (Cutler et al. 1991b; Huang et al. 1995) and from *T. harzianum* (Ghisalberti and Rowland 1993). Metabolites of this class have been found to have antimicrobial activity against various pathogens.

12.4.11 Statins

It is a diverse group of metabolites which have the ability to inhibit HMG-CoA reductase activity. Compactin belongs to this class and has been isolated from *T. longibrachiatum* and *T. pseudokoningii* (Fig. 12.2).

12.5 Secondary Metabolites Present in *Trichoderma* Isolates Associated with Pulse Rhizosphere

ICAR-IIPR 160 isolates of *Trichoderma* are present which have been characterized through ITS and TEF (Fig. 12.3). The mycoparasitic activity of all the isolates has been checked through dual culture. To check the production of secondary metabolites, inverse plate technique was performed (Fig. 12.4). Out of 160 isolates metabolite profiling of five isolates was done (Table 12.2), and it was observed that metabolites related to phytopathogenic activity and plant growth promotion activity were present in all the isolates (Fig. 12.5).

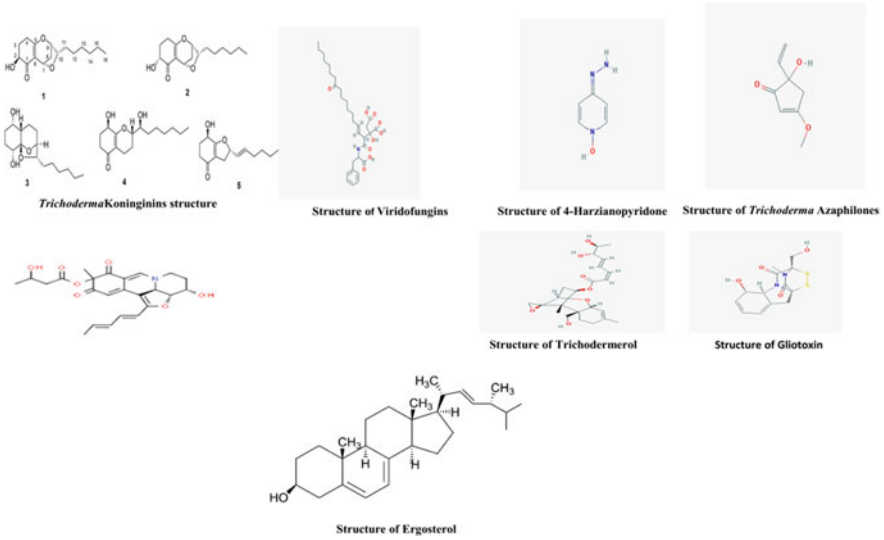

Fig. 12.2 Structure of secondary metabolites present in *Trichoderma*

Fig. 12.3 Diversity of *Trichoderma* isolates from pulses rhizosphere

12.5.1 Lytic Enzyme

Apart from secondary metabolites, *Trichoderma* species can produce lytic enzymes (Fig. 12.5).

Fig. 12.4 Inhibition of phytopathogen mycelial growth by the secondary metabolites produced by *Trichoderma* spp. (**a**) Inverse plate technique and (**b**) binary culture technique

12.5.2 Proteases

Trichoderma spp. are well-known producers of proteases. Proteolytic activity of *Trichoderma viride* is aimed to be involved in the biocontrol activity against *Sclerotium rolfsii*. *T. harzianum* produces serine proteases which are involved in the mycoparasitism against plant pathogens (Geremia et al. 1993). Proteases are reported to degrade cell walls, membranes, and proteins released by the lysis of pathogen (Goldman et al. 1994).

12.5.3 B1,3-Glucanases

β1,3-glucanases lyse the host cell wall and lead to the leakage of protoplasmic contents (Cherif and Benhamou 1990; Elad et al. 1983; Tronsmo et al. 1993). *T. harzianum* has been reported to produce N-acetylglucosaminidase, endochitinase, chitobiosidase, and endo β-1,3-glucanases which play a significant role against *R. solani*.

12.5.4 Chitinase

Chitinases are the linear polymer of β-1,4-N-acetyl glucosamine, the second most abundant polysaccharide present in nature (Deshpande 1986; Nicol 1991). The chitinolytic enzymes of *Trichoderma* are very effective against pathogens (Harman et al. 1993).

Table 12.2 Metabolite profiling of potential ICAR-IIPR *Trichoderma* isolates

A. Trichoderma asperellum (IIPRTas-1)		
Sl. no.	Name of metabolite	Chemical class of metabolite
1	1-Decanol, 2-hexyl-	Saturated alcohol
2	2-Ethyl-1-dodecanol	Antimicrobial, anti-inflammatory, anti-cancer, diuretic, hepatoprotective antiasthma, steroid
3	Dichloroacetic acid, heptadecyl ester	Phenolic compound Antimicrobial, anti-inflammatory, antioxidant
4	Silane, trichlorodocosyl-	Fatty alcohol
5	Dichloroacetic acid, tetradecyl ester	Alcohol
6	Heptafluorobutyric acid, *n*-octadecyl ester	
7	Dibutyl phthalate	Esters
8	Phthalic acid, isobutyl 2-pentyl ester	Esters
9	Phthalic acid, isobutyl 4-octyl ester	Esters
10	Phthalic acid, butyl 2-pentyl ester	Esters
11	Phthalic acid, butyl hexyl ester	Esters
12	Phthalic acid, isobutyl octyl ester	Esters
13	Phthalic acid, butyl dodecyl ester	Ester
14	Phthalic acid, butyl nonyl ester	Ester
15	Phthalic acid, decyl isobutyl ester	Ester
16	Phthalic acid, butyl tridecyl ester	
17	Phthalic acid, isobutyl pentadecyl ester	Monoacetoxyscirpenol, mycotoxin
18	Phthalic acid, butyl tetradecyl ester	Retinoid derivative
19	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	Ester
20	Dibutyl phthalate	Ester
21	Phthalic acid, butyl 2-pentyl ester	Diterpenes
22	Phthalic acid, isobutyl 2-pentyl ester	Sesquiterpenes
23	Phthalic acid, butyl hexyl ester	
24	Phthalic acid, 6-ethyl-3-octyl butyl ester	Tovena lactone
25	Phthalic acid, butyl tetradecyl ester	Diterpenes
26	Phthalic acid, butyl undecyl ester	Alcohol
27	Phthalic acid, 2-cyclohexylethyl isobutyl ester	Acid
28	Phthalic acid, isobutyl octadecyl ester	Ester
29	Phthalic acid, isobutyl pentadecyl ester	Ester
30	Didodecyl phthalate	Glycosides
31	1-Hexadecanol, 2-methyl-	Antifungal aldehyde
32	1-Decanol, 2-hexyl-	Enhanced biocontrol activity
33	Oxalic acid, isobutyl hexadecyl ester	Antimicrobial activity ester
34	Ethanol, 2-(octadecyloxy)-	
35	2-Ethyl-1-dodecanol	Ester
36	Dichloroacetic acid, heptadecyl ester	
37	Ethanol, 2-(octadecyloxy)-	

(continued)

Table 12.2 (continued)

A. Trichoderma asperellum (IIPRTas-1)		
Sl. no.	Name of metabolite	Chemical class of metabolite
38	Eicosane, 9-cyclohexyl-	
39	17-Pentatriacontene	
40	1-Hexadecanol, 2-methyl-	Alcohol
41	Hexadecane, 1,1-bis(dodecyloxy)-	
42	Octatriacontyl pentafluoropropionate	
43	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	
44	Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester	Ester
45	1,2-Benzenedicarboxylic acid, diisooctyl ester	
46	Bis(2-ethylhexyl) phthalate	
47	Phthalic acid, dodecyl 2-ethylhexyl ester	
T. asperellum (IIPRTh-31)		
Sl. no.	Name of metabolite	Chemical class of metabolite
1	2,2,6-Trimethyl-1-(3-methylbuta-1,3-dienyl)-7-oxabicyclo[4.1.0]heptan-3-ol	Saturated alcohol
2	Cholestan-3-ol, 2-methylene-, (3β,5α)-	Antimicrobial, anti-inflammatory, anti-cancer, diuretic, hepatoprotective, antiasthma, steroid
3	2-Hydroxy-2,4,4-trimethyl-3-(3-methylbuta-1,3-dienyl)cyclohexanone	Phenolic compound Antimicrobial, anti-inflammatory, antioxidant
4	1-Heptatriacotanol	Fatty alcohol
5	2-Methyl-4-(2,6,6-trimethylcyclohex-1-enyl)but-2-en-1-ol	Alcohol
6	Methyl 4,7,10,13-hexadecatetraenoate	
7	Methyl 5,7-hexadecadiynoate	Esters
8	2,5-Octadecadiynoic acid, methyl ester	Esters
9	Acetic acid, 8,8-dimethyl-2-oxo-3,3a,4,5,6,7,8,8b-octahydro-2H-indeno [1,2-b]furan-5-yl ester	Esters
10	Methyl 3,5-tetradecadiynoate	Ester
11	4,7-Octadecadienoic acid, methyl ester	Esters
12	6,9-Octadecadienoic acid, methyl ester	Esters
13	Murolan-3,9(11)-diene-10-peroxy	Ester
14	5-Benzofuranacetic acid, 6-ethenyl-2,4,5,6,7,7a-hexahydro-3,6-dimethyl-α-methylene-2-oxo-, methyl ester	Ester
15	Ledene oxide-(II)	Ester
16	6-(1-Hydroxymethylvinyl)-4,8a-dimethyl-3,5,6,7,8,8a-hexahydro-1H-naphthalen-2-one	
17	Desacetylanguidine	Monoacetoxyscirpenol, mycotoxin

(continued)

Table 12.2 (continued)

T. asperellum (IIPRTh-31)		
Sl. no.	Name of metabolite	Chemical class of metabolite
18	Fenretinide	Retinoid derivative
19	Murolan-3,9(11)-diene-10-peroxy	Ester
20	5-Benzofuranacetic acid, 6-ethenyl-2,4,5,6,7,7a-hexahydro-3,6-dimethyl-α-methylene-2-oxo-, methyl ester	Ester
21	Phorbol	Diterpenes
22	Ledene oxide-(II)	Sesquiterpenes
23	Tricyclo[6.3.0.0(5,7)]undecane, 1,8-epoxy-2,6,6,9-tetramethyl-	
24	Resibufogenin	Tovena lactone
25	Phorbol	Diterpenes
26	5-(7a-Isopropenyl-4,5-dimethyl-octahydroinden-4-yl)-3-methyl-penta-2,4-dien-1-ol	Alcohol
27	2-Butenoic acid, 2-methyl-, dodecahydro-8-hydroxy-8a-methyl-3,5-bis(methylene)-2-oxonaphtho[2,3-b]furan-	Acid
28	Androstan-17-ol, 2,3-epoxy-, (2α,3α,5α,17β)-	Ester
29	5H-Cyclopropa[3,4]benz[1,2-e]azulen-5-one, 9-(acetyloxy)-3-[(acetyloxy)methyl]-1,1a,1b,4,4a,7a,7b,8,9,9a-	Ester
30	Resibufogenin	Glycosides
31	Benzaldehyde, 4-nitro-	Antifungal aldehyde
32	2H-Pyran-2-one	Enhanced biocontrol activity
33	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	Antimicrobial activity ester
Trichoderma harzianum (IIPTh-10)		
Sl. no.	Metabolite identified	Chemical class of metabolite
1	1,3a-Ethano-3aH-indene	Alcohol
2	Oxacyclotetradeca-4,11-diyne	
3	Cyclopenta[1,3]cyclopropa[1,2]cyclohepten-3(3aH)-one	Esters
4	Aromadendrene	Esters
5	Seychellene	Esters
6	Tricyclo[6.3.3.0]tetradec-4-ene,10,13-dioxo-	Esters
7	1-Decanol, 2-hexyl-	Esters
8	Silane, trichlorodocosyl-	Esters
9	2-Hexyl-1-octanol	Ester
10	1-octanol, 2-butyl-	Ester
11	2-Ethyl-1-dodecanol	Ester
12	1-Dodecanol, 2-methyl-, (S)-	

(continued)

Table 12.2 (continued)

Trichoderma harzianum (IIPTh-10)		
Sl. no.	Metabolite identified	Chemical class of metabolite
13	Silane, trichlorodocosyl-	Monoacetoxyscirpenol, mycotoxin
14	1-Decanol, 2-hexyl-	Retinoid derivative
15	2-Ethyl-1-dodecanol	Ester
16	2-Hexyl-1-octanol	Ester
17	Dichloroacetic acid, tetradecyl ester	Diterpenes
18	1-Dodecanol, 2-methyl-, (S)-	Sesquiterpenes
19	Phthalic acid, 2,7-dimethyloct-7-en-5-yn-4-yl isobutyl ester	
20	Dibutyl phthalate	Tovena lactone
21	Phthalic acid, isobutyl non-5-yn-3-yl ester	Diterpenes
22	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	Alcohol
23	Phthalic acid, isobutyl 2-pentyl ester	Acid
24	Phthalic acid, butyl hex-2-yn-4-yl ester	Ester
25	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	Ester
26	Phthalic acid, 6-ethyl-3-octyl butyl ester	Glycosides
27	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	Antifungal aldehyde
28	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester	Enhanced biocontrol activity
29	Phthalic acid, butyl nonyl ester	Antimicrobial activity ester
30	1,2-Benzenedicarboxylic acid, butyl decyl ester	
31	Dibutyl phthalate	
32	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	Ester
33	1,2-Benzenedicarboxylic acid, butyl 2-ethylhexyl ester	Ester
34	1,2-Benzenedicarboxylic acid, butyl octyl ester	Ester
35	1,2-Benzenedicarboxylic acid, bis (2-methylpropyl) ester	Ester
36	Phthalic acid, butyl 2-pentyl ester	Ester
37	1,2-Benzenedicarboxylic acid, ditridecyl ester	Ester
38	1-Decanol, 2-hexyl-	
39	Silane, trichlorodocosyl-	
40	Chloroacetic acid, 4-hexadecyl ester	Ester
41	2-Ethyl-1-dodecanol	
42	Dichloroacetic acid, tetradecyl ester	
43	1-Hexadecanol, 2-methyl-	
44	Tert-Hexadecanethiol	Alcohol

(continued)

Table 12.2 (continued)

Trichoderma harzianum (IIPTh-10)		
Sl. no.	Metabolite identified	Chemical class of metabolite
45	1-Chloroeicosane	
46	Ethanol, 2-(octadecyloxy)-	Alcohol
47	17-Pentatriacontene	
48	1-Octadecanesulphonyl chloride	
49	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	
50	1,2-Benzenedicarboxylic acid, diisooctyl ester	
51	Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester	Ester
52	Bis(2-ethylhexyl) phthalate	
53	1,2-Benzenedicarboxylic acid, isodecyl octyl ester	
54	Phthalic acid, 2-ethylhexyl isohexyl ester	Ester
55	Phorbol	
56	Androstan-3-one, 17-hydroxy-1,17-dimethyl-, (1α,5α,17β)-	
57	Olean-12-ene-3,16,21,22,23,28-hexol, (3β,4α,16α,21β,22α)-	
58	5H-Cyclopropa[3,4]benz[1,2-e]azulen-5-one, 9a-(acetyloxy)-1,1a,1b,4,4a,7a,7b,8,9,9a-decahydro-4a,7b,9	
59	Androstane-3,17-diol, 17-methyl-, (3α,5α,17β)-	
60	Pregnane-3,11,17,20,21-pentol, cyclic 17,21-[(1,1-dimethylethyl)boronate], (3β,5α,11β,20R)-	
Trichoderma harzianum (IIPRTh-3)		
Sl. no.	Metabolite identified	Chemical class of metabolite
1	N-(4,6-Dimethyl-2-pyrimidinyl)-4-(4-nitrobenzylideneamino) benzenesulfonamide	
2	Benzaldehyde, 4-nitro-	Antifungal
3	Benzaldehyde, 3-nitro-	
4	Oxaziridine, 3-(4-nitrophenyl)-2-propyl-	Peroxidase reaction
5	2-Isobutyl-5-(2-nitro-phenyl)-[1,3,4] oxadiazole	
6	β-Chloro-2-nitroxy-3,5-O-di-p-nitrobenzoyl-d-arabinose	
7	4-Bromobutanoic acid, tetradecyl ester	Acid
8	1-Methyl-8-propyl-3,6-diazahomoadamantan-9-ol	
9	1,8-Diethyl-3,6-diazahomoadamantan-9-ol	
10	Spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-	

(continued)

Table 12.2 (continued)

Trichoderma harzianum (IIPRTh-3)		
Sl. no.	Metabolite identified	Chemical class of metabolite
11	1-Hydroxycyclododecanecarbonitrile	
12	4-Bromobutanoic acid, hexadecyl ester	
13	Pentanoic acid, 3-(3-hydroxy-5,5-dimethyl-1-oxo-2-cyclohexenyl)-4-oxo-	
14	5-Methyl-7-propyl-1,3-diazaadamantan-6-one	
15	Pyridine-3-carbonitrile, 2-[2-(3,4-dihydroxyphenyl)-2-oxoethylthio]-4-methoxymethyl-6-methyl-	
16	Diallyl(2,2,4a,7,7-pentamethyl-1,2,3,4,4a,5,6,7-octahydro[1,8] naphthyridin-1-yl)phosphate	
17	1,3-Dimethoxy-5-(1-methyl-heptyl)-benzene	
18	Spiro[4.5]decan-7-one, 1,8-dimethyl-8,9-epoxy-4-isopropyl-	
19	Propiolic acid, 3-(1-hydroxy-2-isopropyl-5-methylcyclohexyl)-, ethyl ester	Acid
20	2-Hydroxy-4-methoxy-7-methyl-7,8,9,10,11,12,13,14-octahydro-6-oxabenzocyclododecen-5-one	
21	Propiolic acid, 3-(1-hydroxy-2-isopropyl-5-methylcyclohexyl)-	
22	Dihydroxanthin	
23	2,15-Heptadecadiene, 9-(ethoxymethyl)-	
24	6-[4-(Tetrahydro-pyran-2-yloxy)-but-1-ynyl]-1,4-dioxa-spiro[4.5]decan-6-ol	
25	Cyclopropaneoctanoic acid, 2-[(2-pentylcyclopropyl)methyl]-, methyl ester, trans,trans-	
26	Cyclopropanepropionic acid, 2-[(2-decylcyclopropyl)methyl]-, methyl ester	
27	Phthalic acid, butyl undecyl ester	Ester
28	1-Methyl-8-propyl-3,6-diazahomoadamantan-9-ol	
29	Phthalic acid, butyl tetradecyl ester	Ester
30	Phthalic acid, 2-cyclohexylethyl isobutyl ester	Ester
31	Octadecanoic acid	
32	7-methyl-Z-tetradecen-1-ol acetate	
33	9-Hexadecenoic acid	
34	Octadecanoic acid, 2-(2-hydroxyethoxy) ethyl ester	

(continued)

Table 12.2 (continued)

Trichoderma harzianum (IIPRTh-3)		
Sl. no.	Metabolite identified	Chemical class of metabolite
35	Hexadecane, 1,1-bis(dodecyloxy)-	Ester
36	Estra-1,3,5(10)-trien-17β-ol	
37	17-Pentatriacontene	
38	Z-5-Methyl-6-heneicosen-11-one	
39	Fumaric acid, 10-chlorodecyl decyl ester	
40	2-(4-Nitrobutyryl)cyclooctanone	
41	Oxiraneoctanoic acid, 3-octyl-, methyl ester, cis-	
42	2-Heptadecanol, acetate	
43	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	
44	1,2-Benzenedicarboxylic acid, diisooctyl ester	Enhanced biocontrol activity
45	Bis(2-ethylhexyl) phthalate	
46	Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester	
47	Phthalic acid, 2-ethylhexyl isohexyl ester	
48	1,2-Benzenedicarboxylic acid, isodecyl octyl ester	
49	Bis(2-ethylhexyl) phthalate	
50	Phthalic acid	
51	1,2-Benzenedicarboxylic acid	
Trichoderma longibrachiatum (IIPRTg-3)		
Sl. no.	Metabolite identified	Chemical class of metabolite
1	Phenylethyl alcohol	
2	Hydrazine	
3	Toluene	
4	Cyclobutene	
5	1,3,5-Cycloheptatriene	
6	Benzeneethanol	
7	2-Hexanone	
8	Thiophene	
9	2-[2-(5-Norbornenyl)oxy]-tetrahydropyran	
10	2(3H)-Furanone	
11	Tetrahydrofuran-2-one, 5-[1-hydroxyhexyl]-	
12	Thiophene, 2,3-dihydro-	
13	N-(4,6-Dimethyl-2-pyrimidinyl)-4-(4-nitrobenzylideneamino) benzenesulfonamide	
14	Benzaldehyde, 4-nitro-	
15	Benzaldehyde, 3-nitro-	
16	β-Chloro-2-nitroxy-3,5-O-di-p-nitrobenzoyl-d-arabinose	

(continued)

Table 12.2 (continued)

Trichoderma longibrachiatum (IIPRTg-3)		
Sl. no.	Metabolite identified	Chemical class of metabolite
17	n-butyl 4-nitrobenzoate	
18	Methanediol, (4-nitrophenyl)-, diacetate	
19	Benzeneethanol, 4-hydroxy-	Aldehyde
20	Hydrazine, 1-(3-hydroxybenzyl)-	
21	Benzeneethanol, 2-hydroxy-	
22	DL-Tyrosine	
23	1,4-Benzenedimethanol	Ethanol
24	Phenol, 4-(methoxymethyl)-	
25	Cholestan-3-ol, 2-methylene-,	
26	Limonen-6-ol, pivalate	
27	Aspidospermidin-17-ol, 1-acetyl-19,21-epoxy-15,16-dimethoxy-	
28	2-Dodecen-1-yl(−)succinic anhydride	
29	(2,2,6-Trimethyl-bicyclo[4.1.0]hept-1-yl)-methanol	
30	1,2-15,16-Diepoxyhexadecane	
31	2-Furanmethanol	
32	2H-Pyran-2-one	Enhanced biocontrol activity
33	Cyclopentanemethanol	
34	Acetic acid	Acid
35	2-butanol, 4-	
36	2-Hydroxy-2,4,4-trimethyl-3-(3-methylbuta-1,3-dienyl)cyclohexanone	
37	Neoisolongifolane,	
38	3-(1,5-dimethyl-hex-4-enyl)-2,2-dimethyl-cyclopent-3-enol	
39	9-Undecenal, 2,10-dimethyl-	
40	(−)-Globulol	
41	Epiglobulol	
42	2s,6s-2,6,8,8-Tetramethyltricyclo[5.2.2.0(1,6)]undecan-2-ol	
43	Dichloroacetic acid, tetradecyl ester	
44	Ethanol, 2-(tetradecyloxy)-	
45	1-Decanol, 2-hexyl-	
46	2-Ethyl-1-dodecanol	
47	Dichloroacetic acid, heptadecyl ester	
48	Dichloroacetic acid, tridecyl ester	
49	Phthalic acid, butyl undecyl ester	
50	Phthalic acid, butyl tetradecyl ester	
51	Phthalic acid, 2-cyclohexylethyl isobutyl ester	Ester
52	Phthalic acid, isobutyl octadecyl ester	Ester

(continued)

Table 12.2 (continued)

Trichoderma longibrachiatum (IIPRTg-3)		
Sl. no.	Metabolite identified	Chemical class of metabolite
53	Phthalic acid, isobutyl undecyl ester	Ester
54	Phthalic acid, isobutyl pentadecyl ester	Ester
55	Octadecanoic acid	Acid
56	Octadecanoic acid, 2-(2-hydroxyethoxy) ethyl ester	Ester
57	L-ascorbic acid, 6-octadecanoate	
58	Distearyl sulfide	
59	Ethanol, 2-(octadecyloxy)-	
60	Tetracosanoic acid	
61	Carbonic acid, isobutyl octadecyl ester	Ester
62	Carbonic acid, heptadecyl isobutyl ester	Ester
63	Dichloroacetic acid, heptadecyl ester	Ester
64	2-Heptadecanol	
65	1-Hexadecanol, 2-methyl-	
66	Hexatriacontyl pentafluoropropionate	
67	1,2-Benzenedicarboxylic acid, mono (2-ethylhexyl) ester	
68	1,2-Benzenedicarboxylic acid, diisooctyl ester	Ester
69	Bis(2-ethylhexyl) phthalate	
70	Phthalic acid, 6-ethyloct-3-yl 2-ethylhexyl ester	
71	Phthalic acid, 2-ethylhexyl isohexyl ester	Ester
72	1,2-Benzenedicarboxylic acid, isodecyl octyl ester	Ester

12.6 Future Line of Research

Isolation of metabolic compounds which affect plant metabolism may help in dealing with the problems related to living microorganisms. The use of *Trichoderma* metabolites for inhibiting pathogen growth and inducing plant growth is a topic of research at present. These metabolites can be produced on a large scale and can be separated from the fungal biomass and formulated for foliar spray.

12.7 Conclusion

Due to the growing interest in the biological control properties of *Trichoderma*, several bioactive metabolites have been isolated and identified which inhibit the growth of phytopathogens. *Trichoderma* species produce different volatile and

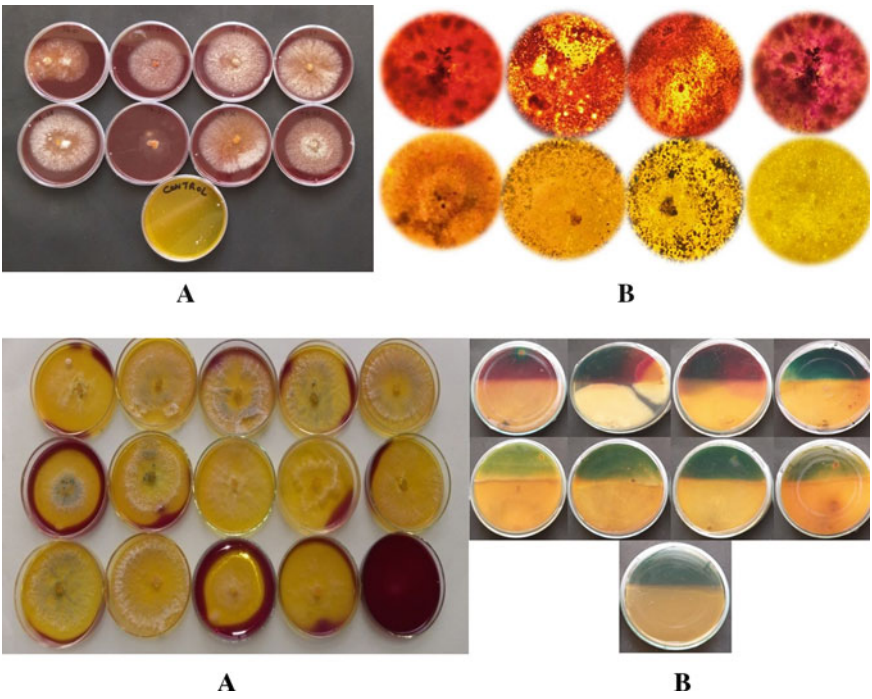

Fig. 12.5 (I): Chitinase and lipase enzyme production by *Trichoderma* species on their specific medium. (II): (**a**) Phosphate and (**b**) siderophore enzyme production by *Trichoderma*

non-volatile metabolites which inhibit the growth of phytopathogens. Among *Trichoderma* gliovirin, gliotoxin, viridin, pyrones, peptabiols, and others are the main metabolites that are extensively secreted by *Trichoderma* (Vey et al. 2001). Apart from secondary metabolites, *Trichoderma* species also secrete ethylene oxide, hydrogen cyanide, alcohol, aldehyde, and ketones which also play an important role in biocontrol activity. Peptabiols are synthesized non-ribosomally and are antimicrobial peptides that have antifungal and antibacterial properties and play an important role in antagonism (Landreau et al. 2002). At the present time around 309 metabolites have been sequenced out of which 180 are from *Trichoderma*. Further research is needed to study the toxicity and mechanisms of action. *Trichoderma* species are well-known biocontrol agents that are used efficiently for crop production. *Trichoderma* species are well known for their capacity to generate antibiotic substances which inhibit phytopathogens. In addition, *Trichoderma* species secrete several plant growth-promoting metabolites which significantly increase the plant growth and impart tolerance to abiotic stress. The ecological influence of *Trichoderma* metabolites should be studied for managing the secure and sustainable use of *Trichoderma* metabolites. For the green era of economy, use of *Trichoderma* species should be promoted so that we can save the environment and human health from the harmful effects of pesticides.

Metabolomics and expressomics are the techniques that should be used for the identification of molecular bioactive which are involved in the interaction among plants, microbes, and pathogens. Novel techniques developed should be used to identify *Trichoderma* strains that can produce beneficial metabolites (antibiotics, plant growth promoters, or inducers) (Mukherjee et al. 2012). The application of secondary metabolites to promote crop yield is an innovative and interesting approach, but further studies are needed to study the fate of metabolites applied.

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Chapter 13
Uncultivable Soil Microbes Contributing to Sustainable Agriculture

Manish Kumar, Neha Sharma, Raghvendra Saxena, and R. S. Tomar

Abstract The science of microbial genomics and metagenomics is necessary to understand the complete microbial composition in soil, water, and other environmental samples. There is an assemblage of hidden microbes that cannot be cultured or isolated in the laboratory. Moreover, hidden microbes are unculturabe due to its unkown media requirement for researchers. The metagenomics pave the way to identify uncultivable microbes present in the environmental samples. Of late sophisticated research is going on to rediscover this hidden unculturable microbial communities based on several recent molecular techniques and next-generation sequencing technology. The whole genome sequencing is also utilized to get the complete genome sequences of isolates, whereas this technique can be applied to obtain all conserved DNA sequences of complex microbial communities to fulfill the need of metagenomic study. Each individual microbial community can be identified and obtained by clone library preparation and DGGE (denaturing gradient gel electrophoresis) along with conventional or next-generation DNA sequencing. This review illustrates the application and exploitation of recent and conventional techniques to identify uncultivable microbial communities from soil ecosystem.

Keywords Metagenomics · Uncultivable · DGGE · Whole genome

13.1 Introduction

A number of soil microbes are reported to make agriculture sustainable and mostly culturable microbes, viz., nitrogen-fixing microbes, methylotrophic bacteria, ammonia-oxidizing bacteria, and PGPRs that can be isolated on the media plates are applied to the field. Soil ecosystem has the capacity to self-produce the necessary resources for the development of living microorganisms (Furtak and Gajda 2018; Russel 2005). Soil qualities are frequently defined as the balance between high

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activity and high microbiological biodiversity (Li et al. 2013). The presence of microorganisms in soil depends on their chemical composition, pH, moisture, and structure. Soil microorganisms like bacteria and fungi are responsible for biomass decomposition and biodegradation of impurities and help in maintenance of soil structure. Soil helps in biogenic component circulation which makes nutrients available to plants (Furtak and Gajda 2018). This makes a living environment with over 30% of the species existing on Earth. Hence, soil microorganisms are called as "biological engine of the earth" (Haygarth and Ritz 2009). Soil quality plays a crucial role in protection and preservation of the environment and biodiversity (Girvan et al. 2003; Lemaire et al. 2014).

The biological activity of the soil depends on the right number and species composition of microorganisms and their enzymatic activity. Soil microorganisms mediate 80–90% of all processes occurring within the soil (Nannipieri and Badalucco 2003). They are involved in many biogeochemical processes and responsible for mineralization of organic matter, nucleic acids, and proteins synthesis as well as transformation of various phosphorus forms. Rhizosphere microorganisms enhance plant health and may protect against pathogens. (Abigail et al. 2005; Nannipieri et al. 2017). They generate favorable conditions for germination of seeds and growth of the basis system of cultivated plants, which is extremely important for a high yield (Girvan et al. 2003).

Plants emit a large number of varied chemical compounds into the soil, which shape the composition of microorganisms within the environment. Microorganisms use these root secretions as a source of food. The rhizosphere is a habitat mainly for bacteria and mycorrhizal fungi. Some microorganisms may produce antibiotics that block harmful microorganisms. Additionally, soil microorganisms also can improve the condition of plants by releasing growth regulators (e.g., auxin, ethylene, and cytokine) and making available some nutrients (e.g., phosphorus). Polymer-producing microorganisms can advance the soil structure. Among the many soil microorganisms, it's worth mentioning the bacteria of the genus *Pseudomonas* sp., bacteria that inhabit the root zone of plants (Ramos 2011). Bacteria of this type produce various biologically active compounds like antibiotics, lytic enzymes, ethylene, auxin, and gibberellin. In addition, *Pseudomonas* competes for nutrients with pathogenic microorganisms, e.g., for iron by creating siderophores. The bacteria binding atmospheric nitrogen are also important for the cultivation of plants such as *Azotobacter, Clostridium, Rhizobium, and Bradyrhizobium* (Gałązka et al. 2015).

Microbial communities play a significant role in nutrient recycling by mineralization and organic material decomposition. They are released into the soil as nutrients which are essential for proper development of plant and influence root physiology. These communities can influence nutrient accessibility by different chemical reactions like solubilization, chelation, and oxidation/reduction processes. It has been suggested by many studies that microbial populations are important components for agro-environmental problems because microbial communities have the capacity to promote plant growth (Compant et al. 2010; Lugtenberg and Kamilova 2009), increase nutrient availability and absorbance (Adesemoye and

Kloepper 2009; Yang et al. 2009; Berendsen et al. 2012), and improve plant health (Berendsen et al. 2012).

The new gene pools introduce biodiversity or identification of the variability of microbial community which is the basis for biotechnological application. Diversity of microorganisms without the utilization of various ways to look at microorganisms and identify the differences between them isn't possible. Identification of differences between the morphology, physiology, and phylogenetics of microorganisms is possible only with the support of different methods and powerful tools. These methods are often divided into two parts, culture-dependent and culture-independent methods (Reddy et al. 2016).

13.2 Microbes Isolated Through Culture-Dependent Approach

Cultivation-dependent studies also are referred to as traditional or culture-dependent method, which is employed to study microbial diversity (Muthukumar et al. 2003; Thomassin-Lacroix et al. 2001). Culture-dependent methods depend on different morphological, cellular, metabolic, and physiologic characters of microorganism. These studies include isolation and cultivation of microorganisms from collected sample(s) by using different types of solid media, most probable number (MPN)-type liquid media, and Biolog plates to review substrate utilization profile and identification of isolates. Enrichment, isolation, and characterization of bacterial and archaeal species having ability to perform their metabolic processes. Culture-dependent characterization of microbial communities depends on several factors that limit its microbial activities in several ecosystems to completely explain community ecosystem. Microorganisms that are easy to get in pure cultures aren't necessarily abundant and/or metabolically active in place; therefore isolation of one microorganism which mediates a selected metabolism generally doesn't represent the whole community (Varjani and Upasani 2013, 2017a, b; Varjani 2017; Lazar et al. 2015; Rappé and Giovannoni 2003).

Use of cultivation-dependent methods confirmed the presence of the aforementioned groups of bacteria and led to the invention of the many new species of bacteria in several environment conditions (Reddy et al. 2016). Endophytic and phyllosphere bacteria have typically been characterized and enumerated using traditional culture-based approaches, although such methods are highly dependent on the medium used for isolation and therefore the incubation conditions (Kobayashi et al. 2000).

Culture-based approaches, while extremely useful for understanding the physiological potential of isolated organisms, don't necessarily provide comprehensive information on the composition of microbial communities. Due to this documented disparity between cultivatable and in place diversity, it's often difficult to assess the importance of cultured members in resident microbial communities (Orphan et al. 2000).

Recent studies have pointed out that use of culture-independent molecular methods to explain cultivated microorganisms from different environmental conditions often may represent very minor components of the microbial community.

13.3 Microbes Not Isolated But Identified Through Culture-Independent Approach

In recent years, culture-independent methods are utilized in preference to traditional isolation techniques for microbial community analysis (Ellis et al. 2003). The use of culture-independent approaches will remove the preconception imposed by isolation of bacteria on laboratory media but, equally, will fail to monitor differences in cellular activity (Lebaron et al. 2001). Application of the molecular methods, like nucleic acid probes, cloning and sequencing of 16S rRNA, gene amplification, and DNA microarrays to review microbial ecology, would help us to comprise important set of knowledge regarding microbial ecosystem (Varjani et al. 2018).

Taxa identified by culture-independent approach included widely known plant pathogens or symbionts (e.g., species of *Pseudomonas, Ralstonia, Stenotrophomonas, Erwinia, Xanthomonas, Janthinobacterium, Massilia, Chryseobacterium*), but also some genera that contain species that are potential human pathogens (e.g., *Pseudomonas, Serratia, Providencia, Enterobacter, Morganella, Bacillus, Streptococcus, Staphylococcus*) (Jackson et al. 2013). Culture-independent methods are receiving particular attention because it's commonly held that only a little proportion of bacteria present in any environment will form colonies on general laboratory media. Although methods and media that improve on the proportion of bacteria which will be cultured from environmental samples are now coming to light (Janssen et al. 2002; Sait et al. 2002; Zengler et al. 2002).

13.4 Microbe Sequence Information Through Culture-Independent Approach

Culture-independent 16S rRNA-based methods can detect unculturable bacterial colony, also those bacteria that are in low abundance or grow slowly and, therefore, unidentified by traditional culture-based protocols. Next-generation pyrosequencing of 16S rRNA genes provides a high resolution approach to evaluate microbial communities (Redford et al. 2010; Leff and Fierer 2013). The utilization of pyrosequencing also allowed for the identification of various low abundance bacteria that might not be identified otherwise by culture-dependent methods (Jackson et al. 2013).

Cultivation-independent approaches for community analysis began with examination of metabolically active microorganisms using fluorescence in situ hybridization (FISH), bulk analysis of total protein binding, and phospholipid carboxylic acid analysis (Pelz et al. 2001). Nowadays, PCR-based approaches are applied in research to review specific microorganisms or groups of microorganisms and specific genes to judge overall community profiles. Methods to evaluate community profiles include denaturing gradient gel electrophoresis (DGGE), reverse sample genome probing, ribosomal intergenic spacer analysis, restriction fragment length polymorphisms (RFLP), single-strand conformation polymorphism, terminal-RFLP (T-RFLP), internal transcribed spacer-restriction fragment length polymorphism, random amplified polymorphic DNA, amplified ribosomal DNA restriction analysis, and real-time PCR (RT-PCR)/quantitative PCR (qPCR) (Varjani and Upasani 2013, 2017a, b; Varjani 2017; Lazar et al. 2015; Rappé and Giovannoni 2003).

Culture-independent methods may detect species that are missed by plating, as long as the amplification efficiency is high enough. However, they're typically dependent on PCR and other molecular biological techniques. Several potential biases are shown or are conceivable for the specified extraction of community DNA, the PCR step, and other enzymatic reactions (Wintzingerode et al. 1997). Also, cloning into vectors or separation of 16S ribosomal DNA (rDNA) by denaturing or gradient electrophoresis has its own potential shortcomings regarding accurate separation of taxa (Muyzer and Smalla 1998) (Table 13.1).

13.5 Methods to Identify Uncultivable Soil Microbes (Metagenomic Approach)

Previous findings have elaborated the characteristics and characterization of microbes based on isolates obtained on the known media plates or broths from various environmental samples. The uncultivable soil microbial communities exceed the number of cultured prokaryotes. The isolated culturable microbial entities are assumed to be 1% that can be identified by taking out the genomic DNA and PCR amplification of ribosomal genes or other housekeeping genes. And the rest of the 99% microbes are assumed to be unculturable for which no definite and known media composition is known. There are a number of techniques for the identification of uncultivable microbes from soil ecosystem. This includes techniques like PCR (polymerase chain reaction), DGGE (denaturing gradient gel electrophoresis), cloning, DNA sequencing of conserved genes, and whole genome sequencing along with computational tools. The uncultivable microbes are identified on the basis of their DNA sequences obtained after DNA sequencing with the help of homology similarity search on NCBI BLAST (Basic Local Alignment Search Tool). The similarity percentage of each gene sequences obtained after metagenomics analysis provides a close match with the already available identified data in the public database. The soil

Table 13.1 List of uncultivable soil microbes identified on the basis of culture-independent techniques

S. No.	Environment sources	Name of uncultured and identified microbes by metagenomics	Methods of identification	Classification	References
1	Soil ecosystem	*Arthrobacter*	16S rRNA genes sequencing with denaturing gradient gel electrophoresis (DGGE)	*Actinobacteria*	He et al. (2008, 2013)
		Proteobacteria		*Acidithiobacillia*	
2	Heavy metal contaminated soil ecosystem	*Bacteroidetes*	16S rRNA genes sequencing with denaturing gradient gel electrophoresis (DGGE)	*Bacteroidia*	Ellis et al. (2003)
		Firmicutes		*Bacilli* and *Clostridia*	
		Deinococcus-Thermus		*Deinococci*	
		Chloroflexi		*Chloroflexia*	
3.	Hydrocarbon contaminated soils	*Proteobacteria*	(High-throughput sequencing) 454-pyrosequencing	Acidithiobacillia	Stefani et al. (2015)
		Rhodocyclales		*Betaproteobacteria*	
		Burkholderiales		*Betaproteobacteria*	
		Actinomycetales		*Actinobacteria*	
		Rhizobiales		*Alphaproteobacteria* *Gammaproteobacteria*	
		Xanthomonadales			
		Sphingomonadales		*Alphaproteobacteria*	
4.	Petroleum reservoirs	*Thermococcales*	RFLP	*Thermococci*	Orphan et al. (2000)
		Thermotogales		*Thermotogae*	
		Methanobacteriales		*Methanobacteria*	
		Methanococcales		*Methanococci*	
5.	Leafy vegetable ecosystem	*Proteobacteria*	Pyrosequencing	Acidithiobacillia	He et al. (2013)
		Bacteroidetes		Bacteroidia	
6.	Pea plants	Fungal species	Pyrosequencing	Phylum *Ascomycota* and *Basidiomycota*	Xu et al. (2012)
7	Riverine wetland soil	*Proteobacteria* (22.7–59.2% across all samples), *Acidobacteria* (4.5–28.5%), *Bacteroidetes* (5.0–14.3%), Verrucomicrobia (2.2–7.5%), *Nitrospirae*	Illumina sequencing (high-throughput sequencing of 16S rRNA gene)	Bacterial species from a number of phylum	Ligi et al. (2014)

		(0.3–7.4%), *Actinobacteria* (2.0–27.8%), *Chloroflexi* (1.4–5.0%), *Gemmatimonadetes* (0.5–7.8%), *Planctomycetes* (1.0–3.5%), WS3 (0.5–2.6%), *Firmicutes* (0.1–4.0%), and *Elusimicrobia* (0.1–0.3%)			
8	Forest and grassland soil	Dominant taxonomic group such as *Acidobacteria*, *Alphaproteobacteria*, *Actinobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Gammaproteobacteria*, and *Firmicutes*	Pyrosequencing	A number of taxonomic groups were identified	Nacke et al. (2011)
9	Rhizosphere soil of sugarcane	*Bradyrhizobium, Streptomyces, Sphingomonas, Burkholderia*, and *mycobacterium*	Illumina Miseq high-throughput sequencing	Nitrogen-fixing bacteria, *Actinobacteria*, *Proteobacteria*	Pang et al. (2021)

metagenomics comprises the following tools for the identification of uncultivable soil microbial communities:

13.5.1 Characterization of Soil Bacterial Communities by DGGE and Clone Library Preparation

The total DNA extracted from any environmental sample like soil is called as metagenomics DNA. This contains a mixture of culturable and unculturable microbes in the sample DNA. This is very important and crucial for separating the individual DNA fragments for their identification. This is facilitated by one of the techniques like DGGE (denaturing gradient gel electrophoresis).

For the assessment of soil bacterial diversity, first of all, gene is selected for the identification. Either ribosomal or housekeeping genes can be chosen for the PCR amplification by using forward and reverse primers followed by denaturing gradient gel electrophoresis. DGGE is a popular and strong molecular technique which separates the DNA fragments of similar length but different in nucleotide sequences.

In the metagenomics study, first step is to isolate total DNA from environmental sample and further subject it to PCR. To carry out PCR, conserved gene primers of 16S rRNA gene are required for the identification of bacterial communities present in soil. The amplified gene products of ribosomal genes constitute amplified gene product of a mixture of bacterial cells present in the soil. When amplified product is run in gel electrophoresis, it shows a single band. To separate all amplicons of individual bacterial cells, DGGE is used as a separation technique. This technique was very popular in metagenomics, and Muyzer et al. (1993) introduced it in microbial ecology.

In one of the investigations, methylotrophic bacterial communities were obtained and analyzed through culture-independent approach. In the study soil DNA was extracted with the help of UltraClean Soil DNA kit (Mo Bio Laboratories Inc.). Manual methods are also applied, but humic acid may present in soil that interferes with the Taq polymerase activity during PCR. The phylogenetic relatedness has been observed on the basis of both metagenomic and genomic sequences.

In a study, unculturable bacterial cells were obtained in the form of high molecular weight DNA after DGGE followed by clone library preparation and DNA sequencing between two grassland soil samples (Brons and van Elsas 2008). The DGGE bands obtained in the study merely represent an individual bacterial strain or represent a dominant microbial community present in the sample. Sekiguchi et al. (2001) explained that a single DGGE band in the DGGE band profile does not *always* represent a pure and single bacterial strain.

Soil metagenomics was studied in a Flevo silt loam (FSL) soil micro plot to observe the total bacterial communities (Gelsomino et al. 1999). The study included the total soil DNA extraction followed by DNA fingerprinting. The variable region

of 16S rRNA was amplified from soil DNA and subjected to DGGE for the separation of mixture of amplicons. The amplicons separated represent the dominant bacterial community. Further this was confirmed as *Arthrobacter* sp. and *Enterobacter cloacae* after DNA sequencing (Gelsomino et al. 1999).

13.5.2 Characterization of Soil Fungal Communities by DGGE and Clone Library Preparation

The fungal community profiling present in soil was done through culture-independent approach in a study done by Van Elsas et al. (2000). Investigation was based on nested PCR from environmental DNA followed by DGGE, clone library preparation, and DNA sequencing. DGGE separated the mixtures of mixed amplified template of ribosomal gene fragments into clear distinct banding patterns. The assessment of fungal species persistence in soil was done using *T. harzianum* spores and *A. oligospora* hyphal fragments added to microcosms. Cloning of selected bands obtained in DGGE gel profile and their DNS sequence analysis revealed with the 18S rDNA sequences of the closely related species of *Nectria haematococca*, *N. ochroleuca,* and *Fusarium solani*. Moreover, fungal isolates were obtained through culture-dependent approach on PDA plates and identified as *Trichoderma* sp., whereas some on Comada agar as *Cylindrocarpon* group (anamorph of *Nectria* spp.) (Van Elsas et al. 2000).

In a soil sample collected from Yellow Sea of Korea, fungal community structure was determined through metagenomics using pyrosequencing. The amplicons of 18S rRNA gene were subjected to high-throughput sequencing. More than 10,000 reads were obtained, and assembly was done to get a total of 372 distinct fungal taxa. The fungal phylotypes were detected with % homology of 95–99% and mostly fungal phylotypes with 99% similarity were obtained. The BLAST search revealed predominant species of *Ascomycota* described as plant parasites, saprophytes, lichen-forming fungi, and wood decomposers. This study revealed that the utilization of high-throughput pyrosequencing provides the actual abundance of fungal communities in soil (Lim et al. 2010). Moreover, a number of microbial groups are identified through culture-independent approaches from various environmental samples (Fig. 13.1).

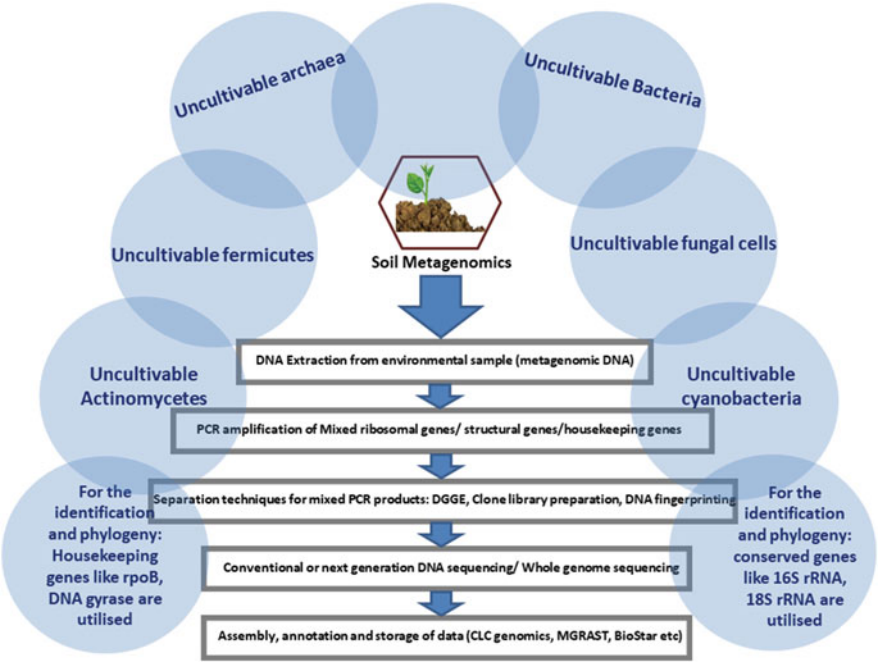

Fig. 13.1 The outline of metagenomic study and identification flow diagram for uncultivable microbial communities

13.6 Uncultivable Microbes Making Agriculture Sustainable

Organic phosphates in the form of phytic acid are a major requirement for plant growth and flowering in many soils. In one of the investigations, an experiment was conducted with *Lotus japonicus* by taking experimental field soil to observe the usage of phytic acid applied to the soil for plant growth. Further soil microbial communities in rhizosphere were analyzed using molecular ecological approaches. Microbial community composition involved in the soil phytic acid utilization was analyzed through culture-independent approach along with molecular fingerprinting. Metagenomic study revealed the variation in the abundance of plant growth-promoting bacterial cells of classes *Betaproteobacteria*, *Dehalococcoidetes*, *Methanobacteria*, *Chlorobi*, and *Bacteroidetes*. The metagenomic study, therefore, provided a better understanding of rhizospheric microbes involved in phytic acid utilization in the soil of *L. japonicus* (Unno and Shinano 2013).

Microbial formulations applied to agriculture fields have a great potential to enhance plant growth that leads agriculture toward sustainability. In this context, metagenomics or culture-independent investigations reveal hidden, unknown, and unidentified microbial strains interacting with plants. The metabolites and chemicals

released by hidden plant growth-promoting microbial communities in the rhizospheric and mycorrhizospheric soil provide a clue of community structure profiling, phylogenetic position, and horizontal gene transfer of microbial strains (Kaushik et al. 2020; Schlaeppi and Bulgarelli 2015; Bulgarelli et al. 2013).

Agriculture practices, change in land use, and agriculture management impacted the soil microbiome to a greater extent. The large untapped microbial assemblages present in soil can be assessed by metagenomics for identification of novel genes, biomolecules, and metabolites. The identification of novel genes from genetic reservoir in the soil ecosystem through metagenomics allows us to rethink about application of microbial formulations with uncultivable soil microbes to agriculture fields. The development of biopesticides can be understood well with the identification of uncultivable microbial communities along with cultivables present in the soil and their probable interaction with several impacts (Gupta et al. 2018).

The dynamics of microbial community structure and composition was determined by Illumina Miseq high-throughput sequencing in the rhizospheric soil of sugarcane (Pang et al. 2021). In this investigation, the effect of continuous cropping of sugarcane on the soil microbiome was studied extensively along with functionality.A change in the soil physiochemical properties and natural soil biodiversity of fungi and bacteria was found due to continuous cropping of sugarcane and, therefore, results in the reduction of crop productivity. Microbial community structure was obtained through next-generation sequencing technology from rhizospheric soil sample of sugarcane with and without continuous cropping years.

Interestingly, bacterial communities associated with sulfur and nitrogen cycling in the rhizosphere soil were diminished in the soil sample of sugarcane with continuous cropping years with the reduction in soil sulfur and nitrogen content. Furthermore, this leads to reduction in crop yield. The metagenomic approach reveals the actual soil microbiome information that may help to understand the underlying mechanism of changes in the community structure and helps to achieve sustainable development of crops (Pang et al. 2021).

13.7 Conclusion

To have insights of microbial communities present in soil microbiota and their interaction with plants can be best studied using soil metagenomics that facilitates the designing of crop systems. The culture-independent study of soil supplemented with organic manures and biofertilizers would be very helpful in the development and formulation of biofertilizers and fertilization strategies. This will lead to less dependence on the application of inorganic fertilizers in the field. Therefore, the plant growth-promotoing culturable and unculturable soil microbes are contributing a lot toward sustainable yield in the agriculture fields. Metagenomics enhance the understanding of beneficial microbial community abundance present in the soil along with fundamental and basics of soil community structure. Metagenomics raise a question and a challenge to researchers to find out these unculturable

beneficial groups of microbes on suitable culture plates also by finding a new media composition for them. Culture-independent approach opens the hidden microbial entities that cannot be isolated, but contribute precisely, abundantly in plant growth promotion and in making agriculture more sustainable.

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Chapter 14
Rhizosphere Microbiome: Significance in Sustainable Crop Protection

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Abstract Rhizosphere is a niche of rich microbial diversity than ever thought as revealed by recent metagenomic studies. The functional roles of rhizosphere microbiome decide the plant phenotype in terms of health and disease. Bacteria dominate the plant rhizosphere as a single most abundant domain followed by fungi, nematodes, protozoa, oomycetes, algae, archaea, arthropods and phages. The beneficial microflora associated with the rhizosphere of healthy plants can be exploited for development of bio-stimulants and microbial pesticides, a current ray of hope to mitigate the losses due to climate impact and plant diseases. Plant disease management by agrochemicals is the major phenomenon accepted and practised as well, but agrochemicals have been criticized from decades for their chemical footprint in the environment. This has further paved ways to different problems like emergence of new diseases, soil health deterioration, chemical residue issues etc. Unexploited potential microbial species can be used for designing new methods for sustainable crop protection. This chapter deals with (1) rhizosphere microbiome composition of few important crops and (2) the beneficial roles of rhizosphere microbiota for plant growth and strategies to deal with plant diseases.

Keywords Rhizosphere microbiome · Crop protection · Rhizosphere microbiome · Bio-stimulants · Microbial pesticides

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14.1 Introduction

Survival is the basic instinct of life, and food has become the major driving force for the entire human kind for survival. The basic motive of all developmental programmes set by different countries of the world is to provide food for its people. The growing population everyday has challenged food production to meet the demand. Stable as well as staple food production is very important for every country to maintain food security. Wheat, maize and rice take the major share in world food consumption. Recently, the report of Fernandez and Orth (2018) has clearly stated that there should be an increase of 25% in rice production to meet the global demand by 2030. With this huge demand in place, food production should see a huge hike in spite of constraints like biotic and abiotic stresses which incur huge losses to food production. Tackling these issues with ensured food production, in other words sustainable crop production with the help of sustainable crop protection is the need of the hour.

14.1.1 Focus on 'Microbiome to Medicine' for Sustainable Crop Protection

The recent report of microbiome playing important roles in human health and disease by Cho and Blaser (2012) has knocked the door of exploration of microbiome for plant health management. Ezenwa et al. (2012) opined that microbiome associated with any organism is responsible for shaping its behaviour. Young (2017) has reported that disease results from loss of beneficial functions of microbiome. The revelation of importance of human microbiome in human health and disease management has led to the exploration of plant-associated microbiome for plant health management. Plant-associated microbiome also referred as phytobiome includes microbiome of rhizosphere and phyllosphere. Rhizosphere is the area around the roots, while phyllosphere is the area around the aboveground parts of plants. The extreme diversity and variability of these plant niches provide opportunities to explore beneficial microbes which interact and help the plants to alleviate both biotic and abiotic stress. 'Microbiome to Medicine' is an important research area even in plant science where the biological control of insect-pest and diseases is possible effectively without harmful effects on environment. Hence for sustainable crop protection, biological control measures must be employed to mitigate losses.

14.1.2 Rhizosphere and Its Relevance in Plant Disease Management (PDM)

Soil has occupied a significant importance in plants health and growth since plants are anchored tightly by it. Soil also referred to as 'the storehouse of nutrients' is very important throughout the plant life in its establishment, growth and development. Roots are the plant parts which are anchored by soil in majority of the crops with a few exception of plants where fruits are obtained underground. Rhizosphere was studied much with reference to plant pathogens while ignored the vast wealth of diverse organisms positively influencing plant health. The trench warfare between plant and soil-borne pathogens ignored the third-party contribution, i.e. rhizosphere microbiome. Plants are always found in association with the soil microorganisms. The greatest biological diversity known so far is reported to be found in the soil microbial life. Microbial cells up to 10^{11} per gram root and more than 30,000 prokaryotic species are represented in the rhizosphere. The contribution of genome by the soil microbial life is much larger than that of the plant. This is also referred to as the plant's second genome (Berendsen et al. 2012). Much analogy has been reported in the functions of gut microbiome and rhizosphere microbiome. Rhizosphere is a reservoir of microbial life which includes symbionts, commensals, antagonists and pathogens. Initially, the loss of low molecular weight organic compounds during seed germination and seedling development process especially by roots is assumed to be the major force for microbiome development in the rhizosphere. This phenomenon of rhizosphere microbial life selection and adoption by the root exudates is also referred as *rhizosphere effect* (Whipps 2001). The rhizosphere effect can be exploited for biological control of plant diseases.

14.2 Rhizosphere Microbiome Composition

The composition of rhizosphere is complex with high diversity and low variability as compared with phyllosphere where we find microbes with low diversity and high variability (Lebeis 2015). Revealing the same requires advanced deep sequencing techniques from genomics. Many researchers have used shotgun metagenome sequencing with the help of next-generation sequencing technologies to unravel the microbial diversity of this important niche of most plant species. Irrespective of plant species, the rhizosphere species richness in disturbed and natural ecosystems highly varies, and in comparison with rhizoplane and endosphere, the species richness is very high (Edwards et al. 2015). The composition of rhizosphere microbiome depends on the succession activities and also changes with crop species, age, root exudates, available carbon sources, anthropogenic activities and also the climate. Most of the rhizosphere microbes are beneficial for plant growth and health while they influence the host plant physiology and innate immune systems. Some of the member species are pathogenic on plants, causing severe disease; they create

losses especially in nurseries and main field. Another group is either true or opportunistic human pathogens when they enter humans. These microbes can be largely placed in two groups called beneficial and deleterious. The well-studied beneficial microbes of rhizosphere are nitrogen-fixing bacteria, vascular arbuscular mycorrhizal fungi, plant growth-promoting rhizobacteria (PGPR), biocontrol microorganisms, myco-parasitic fungi and protozoa. Most of the deleterious microbes to plant growth and health belong to the category of pathogenic fungi, oomycetes, bacteria, virus and nematodes (Mendes et al. 2013).

To exploit native microbiome for crop protection activities, restoration of degraded lands, bioremediation, etc., we must know the existing species diversity and their functional role. The 'core species concept' which came out of this diversity studies is a best example to understand the co-evolution of plants and microbes (Toju et al. 2018), and the same can be exploited for designing the crop improvement programmes for quality traits like enhancement of aroma of Basmati cultivars of rice apart from plant growth and health. Some of the crop species widely grown as staple food such as rice, wheat, maize and also fruits like citrus were targeted for characterization of their rhizosphere microbial diversity and species richness, and the impact of various biotic and abiotic factors on shaping their root microbes is also elucidated. Mainly bacterial diversity was targeted in most of these studies through metagenomic approaches as culturable methods may reveal <5% total diversity. The advancement in genomic approaches still required to find the whole species diversity instead of targeting only two domains, i.e. *Archaea* and *Bacteria*. Some of the important studies on rhizobiome of plants are discussed below.

14.2.1 Rice

Studies on rice root-associated microbes revealed the alpha diversity of rhizosphere is high in comparison with rhizoplane and endosphere. The genera associated with rice rhizosphere mostly belong to the *Acidobacteria* followed by *Proteobacteria*, *Chloroflexi*, *Gemmatimonadetes*, *Planctomycetes*, *Verrucomicrobia*, *Nitrospirae*, *Armatimonadetes*, *Firmicutes*, *Bacteriodetes*, etc. (Arjun 2011; Edwards et al. 2015). Methanogenic genera like *Methanosarcina, Methanocella* and *Methanosaeta* are highly abundant in rice rhizosphere as core methane producers. However, the microbiome changes with geographical location, variety and cultivation practice. In case of organic cultivation of rice, *Alphaproteobacteria*, *Actinobacteria* and *Gemmatimonadetes* are found as prominent groups. Nitrogen-fixing cyanobacterial genus *Anabaena* and the alphaproteobacterial genera *Azospirillum* and *Rhodobacter* were also found enriched in organically cultivated samples. The genus *Streptomyces* which produces a variety of antimicrobial compounds was found to be more in organic rice fields, whereas eco-farmed samples were enriched in *Deltaproteobacteria*, *Chloroflexi* and *Spirochaetes* (Edwards et al. 2015).

14.2.2 Wheat

Wheat rhizosphere is mainly composed of nine bacterial phyla, viz. *Actinobacteria*, *Bacteroidetes*, *Fibrobacteres*, *Firmicutes*, *Gemmatimonadetes*, *Proteobacteria*, *Synergistetes*, *Tenericutes* and *Verrucomicrobia*. Indigenous populations of beneficial phenazine-producing (Phz+) *Pseudomonas* spp. were present in the rhizosphere of wheat, but the colonization by the very same genera became scanty in irrigated and high rain fall areas (Mavrodi et al. 2018). Wheat rhizosphere harbours disease-suppressive microbes. In Yin et al. (2013) study, *Chryseobacterium* and *Pedobacter* from wheat soils were found to produce antifungal compounds and antagonized the mycelial growth of *Rhizoctonia solani* AG-8 under in vitro conditions. Plant growth-modulating bacteria like *Promicromonospora* and *Sphingobacterium* which secrete gibberellins and modulate the ethylene-induced stress levels are also part of wheat rhizosphere microbiome (Kang et al. 2012). Interestingly, the selection of microbiome by plant genotype is evident from a recent study showing that tall varieties of wheat possess a differential rhizosphere microbiome abundance of *Actinobacteria*, *Bacteroidetes* and *Proteobacteria* compared with a higher differential abundance of *Verrucomicrobia*, *Planctomycetes* and *Acidobacteria* in semi-dwarf wheat cultivars (Kavamura et al. 2020). However, the variance in rhizosphere microbiome depends nearly on genotype (2%), soil (57%) and agricultural practices such as organic and conventional (10%). Despite these changes, it is observed that wheat possesses 177 taxa (2 archaea, 103 bacteria, 41 fungi and 31 protists) as its rhizosphere core microbiome (Simonin et al. 2020).

14.2.3 Maize

Bouffaud et al. (2012) have put forth an important hypothesis that crop evolution (maize diversification) has an impact on selection of their rhizosphere bacterial communities. They also found that rhizobacterial community composition of five maize inbred lines depended on their genetic group when grown in same soil. This study was supported when pyrosequencing of 27 modern maize inbred's rhizosphere soil bacterial 16S ribosomal DNA revealed significant heritable variation in total rhizosphere microbiota by Peiffer et al. (2013). These studies shown *Proteobacteria* as the most abundant phyla followed by *Actinobacteria*, *Acidobacteria*, *Bacteroidetes*, *Cyanobacteria*, *Chloroflexi*, *Archaea*, etc., along with many other unclassified bacteria and archaea. Mycorrhiza is another important member of the rhizosphere in the studies showing the mycorrhizal dependence (MD) on almost all land plants take part. Kapulnik and Kushnir's (1991) study showed *Triticum tauschii* (diploid wheat ancestor) had a higher MD compared to tetraploid or modern hexaploid wheat genotypes.

14.2.4 Pea

Pisum sativum is a widely grown legume known to harbour a distinct rhizosphere microbiome compared to cereals like wheat and oat (Turner et al. 2013). It was reported that 0.5% *Archaea*, 20.7% *Eukaryotes* and 73.7% *Bacteria* along with other cellular organisms are part of the pea rhizosphere. In general, the fungal diversity and abundance followed by nematodes; bacterivorous protozoa represent pea rhizobiome whereas oat is having more nematode abundance followed by fungi.

14.2.5 Citrus

Global citrus rhizosphere microbiome was characterized both by the amplicon and deep shotgun metagenomic sequencing by collecting samples from six continents covering distinct biogeographical regions (Xu et al. 2018). Predominant taxa found are *Proteobacteria*, *Actinobacteria*, *Acidobacteria* and *Bacteroidetes* which can be noted to find its similarity with cereal crops. However, this study remarkably identified the core citrus rhizosphere microbiome which comprises *Pseudomonas, Agrobacterium, Cupriavidus, Bradyrhizobium, Rhizobium, Mesorhizobium, Burkholderia, Cellvibrio, Sphingomonas, Variovorax* and *Paraburkholderia,* some of which are known as potential plant beneficial microbes.

14.2.6 Grape

The rhizosphere soil microbial functional diversity and its metabolic activity of grapevines were found to rise with the age of plants (Ji et al. 2019). In the same study they observed physiochemical indices of grape berry were also significantly influenced by its rhizosphere microbiome. *Proteobacteria*, *Firmicutes* and *Actinobacteria* are the main microbial phyla found in grapevine rhizosphere soils. Water stress acts as another factor to determine the diversity where the relative abundance of *Firmicutes* was significantly lower, and two main orders *Xanthomonadales* and *Clostridiales* also showed the same pattern (Zhang et al. 2019a, b).

14.2.7 Glacier Buttercup

Ranunculus glacialis is ubiquitously present in high alpine altitudinal gradient of southern European region. Praeg et al. (2019) have shown pH and temperature being the strongest influencing factors on rhizosphere microbial diversity of this plant. On

average, *Proteobacteria* (34%), *Actinobacteria* (15%), *Acidobacteria* (9%), *Planctomycetes* (8%), *Verrucomicrobia* (5%), *Chloroflexi* (3%) and *Bacteroidetes* (6%) were found in this Illumina MiSeq v2 platform-based amplicon sequencing study. *Nitrososphaera* sp. is the only species observed from *Archaea* (0.5%) in the case of fungi, while Basidiomycota and Ascomycota are found to be the most abundant with relative abundance 63% and 13%, respectively.

14.2.8 Others

Every single factor of modern cultivation bears direct or indirect effect which leads to the 'biased rhizosphere' and the advanced omics tools which help in finding the rhizosphere hotspots to design future biofertilizers, biocontrol agents and plant defence elicitors (Mohanram and Kumar 2019). Efforts are being made from every corner to characterize the diversity of rhizosphere soils of different crop species such as soybean (Sugiyama et al. 2014); sugarcane (Pisa et al. 2011); red clover (*Trifolium pratense*) (Hartman et al. 2017); cucumber (*Cucumis sativas* L.) (Zhang et al. 2018); lettuce (Schreiter et al. 2014); *Chrysanthemum* sp. L. (Chen et al. 2019); amazon forests (Goss-Souza et al. 2020); oak (Maghnia et al. 2019); solanaceous crops—potato, tomato, chilli and brinjal (Goswami et al. 2019); red kidney bean (*Phaseolus vulgaris)* (Suyal et al. 2015); *Arabidopsis* (Bodenhausen et al. 2013); etc.

14.3 Rhizosphere: The Destination in Search of Beneficial Microbes

The rhizosphere hotspots contribute in identification of beneficial microbial species such as plant growth-promoting rhizobacteria (PGPR), biocontrol agents (BCA), mycorrhizae, etc. (Table 14.1). Plants grown in diseas- suppressive soils can be a source for potential antagonistic species of certain economically important pathogens. Since plants have the ability to shape their own microbiome, manipulation of plant's holobiome with few beneficial species or consortia can be an option to devise sustainable protection measures. As discussed, rhizosphere microbial life influences plant growth and health in different ways and it helps in evolving strategies for PDM.

14.3.1 Disease-Suppressive Soils

Disease-suppressive soils are recognized for their low disease incidence in spite of the presence of virulent pathogen and a susceptible host. But a major group of

Table 14.1 List of beneficial microbial species from rhizosphere showing enhanced plant growth and defence activities

Source	Beneficial organism in rhizosphere	Defence mechanism	Target diseases/pathogens/crop	References
Suppressive soils	Fluorescent pseudomonads	Phenazine antibiotic production [2,4-diacetylphloroglucinol (DAPG)]	Various soil-borne plant pathogens (*Fusarium oxysporum, Macrophomina phaseolina, Helminthosporium tetramere, Alternaria tenuis*)	Ganeshan and Manoj Kumar (2005), Saber et al. (2015)
	Non-pathogenic *Fusarium* strains	Systemic acquired resistance (SAR), competition and parasitism	Fusarium wilt of tomato, egg-plant, *Gerbera*, watermelon, etc.	Mazzola (2002), Silva and Bettiol (2005), Kaur et al. (2011)
	Trichoderma harzianum	Competition, parasitism, fungistasis, hydrolytic enzymes, siderophores, antibiotics	Various foliar fungal plant pathogens (*Bipolaris oryzae, Rhizoctonia solani, Pythium ultimum, Alternaria solani*)	Mazzola (2002), Benítez et al. (2004), Abdel-Fattah et al. (2007), Mazrou et al. (2020)
Biocontrol agents	*Agrobacterium radiobacter* K84	Agrocin 84	Crown gall of apple disease (*Rhizobium rhizogenes*)	Whipps (2001)
	Bacillus subtilis	Production of chitinase, β-glucanases, mycolytic enzyme activity, biofilm formation	Soil-borne fungal and bacterial diseases of vegetables (*Colletotrichum* spp., *Rhizoctonia solani, Fusarium oxysporum* and *Rosellinia necatrix*)	Bais et al. (2004), Ashwini and Srividya (2014), Khedher et al. (2015)
	Fluorescent pseudomonads	Phytohormones, antibiotics (DAPG, pyoluteorin, pyrrolnitrin) and volatile compounds (hydrogen cyanide)	Various soil-borne plant pathogens	Höfte and Altier (2010), Saber et al. (2015)
	Pseudomonas aeruginosa	Antibiotics (phenazine-1-carboxylate (PCA) and phenazine-1-carboxamide (PCN))	Rice pathogens (*Rhizoctonia solani, Xanthomonas oryzae* pv. *oryzae*)	Shanmugaiah et al. (2010)

	Trichoderma harzianum	(a) Volatiles (6-*n*-pentenyl-2H-pyran-2 one and 6-*n*-pentenyl-2H-pyran-2-one) (b) Cell wall-degrading enzymes	Various foliar fungal plant pathogens (*Bipolaris oryzae, Rhizoctonia solani, Pythium ultimum, Alternaria solani*)	Jeyarajan and Nakkeeran (2000)
	Trichoderma sp.	(a) Peptaibols (trichovirins and harzianins) (b) Polyketides (c) Diketopiperazine-like compounds (gliotoxin)	Various fungal diseases of pulses, grapes, maize, cotton, onion, etc.	Whipps (2001), Hermosa et al. (2014)
	Phagotrophic protists	Predation and prey, enhance secondary metabolite gene function	Pathogen population dynamics to suppress the plant diseases	Xiong et al. (2020)
Plant growth-promoting rhizobacteria (PGPR)	*Bacillus* spp.	N fixation Siderophore production Exopolysaccharide (EPS) production	Tomato, rice, maize, *Arabidopsis*, wheat, soybean	Akinrinlola et al. (2018), Khan et al. (2020)
	Azospirillum brasilense	Phytothormone production and root hair induction	Wheat, maize	Vessey (2003)
	Fluorescent pseudomonads, *Bacillus polymyxa*	Nutrient solubilization, hormone modulation	Various economically important crop species	Höfte and Altier (2010)
	Cyanobacteria	Nitrogen fixation	Ubiquitous presence in saline rhizosphere soils	Vessey (2003), Issa et al. (2014)
	Azotobacter chroococcum, Bacillus circulans, Cladosporium herbarum, Pantoea spp.	P solubilization	Strawberry, rice, tomato, etc.	Vessey (2003), Liu (2019)
Phages	Lytic bacteriophages	Lysis of bacteria	Phytopathogenic bacteria (*Xanthomonas campestris* pv. *Campestris, Psedomonas* spp., *Xylella, Pectobacterium carotovorum* ssp. *Carotovorum, Ralstonia solanacearum*)	Jones et al. (2007), Fujiwara et al. (2011), Buttimer et al. (2017)

(continued)

Table 14.1 (continued)

Source	Beneficial organism in rhizosphere	Defence mechanism	Target diseases/pathogens/crop	References
Resistance modulators	*Pseudomonas* spp.	Triggers immune response by signalling, hormone network modulation, lipopolysaccharides (LPS)	Wheat, rice, tomato, soybean, etc.	Lebeis et al. (2015), Legein et al. (2020)
Gene source for transgenics	*Epichloë* species	Fhb7 gene for fungal disease resistance	Wheat, grasses	Wang et al. (2020)
	Bacillus thuringiensis	Bt toxin *Cry* protein genes for insect pest resistance	Cotton, brinjal, corn, tobacco	Sanahuja et al. (2011)

studies have indicated that the major contribution towards disease suppression is by the biotic element which is constituted by soil microbial life. Culture-dependent microbiome function analyses and metagenomic studies have resulted in identification of more than 33,000 bacterial and archaeal species in rhizosphere. More importantly, phylum like *Proteobacteria*, *Firmicutes* and *Actinobacteria* are found closely associated with disease suppression. *γ-Proteobacteria* members were able to produce non-ribosomal peptide synthetases which enabled them with disease-suppressive activity (Mendes et al. 2011). The beneficial microbial life of suppressive soils can be exploited for PDM.

Take-all decline is the best example for disease-suppressive soils. Take-all disease of wheat caused by *Gaeumannomyces graminis* var. *tritici* was controlled by repetitive mono-culturing of wheat where the population of beneficial fluorescent pseudomonads increased leading to disease decline. They are capable of producing antibiotic DAPG. The mechanisms of soil suppressiveness induced by soil microbial life are competition, antibiosis and induction of host resistance. Suppression of *Fusarium* wilts by non-pathogenic *Fusarium* spp. and fluorescent *Pseudomonas* spp. in soil is also reported by many groups (Mazzola 2002).

14.3.2 Biocontrol Agents (BCAs)

'Biological control of plant diseases, in its widest sense, is any means of controlling disease or reducing the amount or effect of pathogens that relies on biological mechanisms or organisms other than man' (Campbell 1989). Biocontrol agents are further broadly referred to as biopesticides, and there is a genuine growing interest for biocontrol agents to be commercialized in the recent past. BCAs are the preferred alternative to chemical management and non-availability of resistance varieties. BCAs are even accepted when there is no other option available for disease management (Fravel et al. 1990). It is one of the important methods which revolutionized the concept of organic farming. The quest for BCAs is gaining momentum since the whole world is preferring chemical residue-free food. So far biological control is assumed to be the best strategy to combat the diseases in an eco-friendly manner. Host resistance with resistance genes and agrochemicals are not sustainable management options for plant diseases. Emergence of new virulent races and chemical residue problems has questioned both the methods. Adoption of a better management tactic is very important, and biocontrol is one method giving a ray of hope in this direction. Rhizosphere is a rich resource of biocontrol agents. Majority of the BCAs registered globally are rhizosphere agents. In this quest, rhizosphere has emerged as a destiny to BCAs. The rich diversity of microbial life in rhizosphere has opened the vast route for selection of BCAs. For any organism to be commercialized as BCA, it should have certain qualities. These qualities are also considered to be the criteria for selection of BCA. The following are the criteria for selection of biocontrol agents according to Weller (2007):

- Grow rapidly in vitro and to be mass produced
- Rapidly utilize seed and root exudates
- Colonize and multiply in the rhizosphere and spermosphere environments and in the interior of the plant
- Produce a wide spectrum of bioactive metabolites (i.e. antibiotics, siderophores, volatiles, antimicrobial peptides and growth-promoting substances)
- Compete aggressively with other microorganisms
- Adapt to environmental stresses

To allay potential pathogens from causing severe yield loss, modern agriculture is majorly dependent on deployment of different agrochemicals and their formulations. The world agrochemical market is about $12 billion out of which only 1% is contributed by biological control agents (BCA) (Powell 1993). Breaking this dependency on agrochemicals is needed to assure long-term soil health and sustained production. The recent ban on certain food crops due to chemical residue at global market has made every country to make more stringent regulations on the use of agrochemicals and their formulations.

Majority of the BCAs registered so far are selected from rhizosphere. The most exploited BCAs like *Trichoderma* species, pseudomonads, *Agrobacterium* and *Streptomyces* are rhizosphere inhabitants. These BCAs have ability to produce antibiotics, volatiles, siderophore and other antimicrobial compounds. These substances further enable them to be the more potential antagonists against pathogens. Major antibiotics are phenazine, DAPG, pyrrolnitrin, pyoluteorin, etc. Strain identification is very important in case of selection of BCAs. Recent techniques of metagenomics have explored the rich diversity of microbiome wealth in rhizosphere which has paved way for further characterization of BCAs. Further transcriptomics and metabolomics has shed light on important antimicrobial gene clusters and metabolites which will help in better disease management. Identification of such strains and their characterization by culturing and molecular techniques will lead to development of potential BCAs. Development of consortium with naturally occurring organisms will further help for faster adaptation.

14.3.3 Plant Growth-Promoting Rhizobacteria (PGPR)

Plants are subjected to various stresses in its life cycle. It can be biotic or abiotic stresses. Stress amelioration is important to save plants. Certain organisms have the ability to influence plant beneficially in growth and development. These characters are also called as plant growth-promoting traits. Rhizosphere has enormous microbial support for plant growth. PGPRs are such organisms which are soil dwellers and contribute to plant health and growth. PGPRs can be employed as substitute to chemicals for plant development. Soil species like *Bacillus, Pseudomonas* and *Azospirillum* have contributed enormously towards plant growth. PGPRs are very helpful in this current scenario where chemical deployment has taken a back step.

PGPRs like BCA also possess different traits which help plants. PGPRs are reported to have both direct and indirect effects. Direct effects include characters which are influencing plant growth. Indirect effects include certain other characters which help plants to defend pests and pathogens. Direct effects include nutrient solubilization, fixation of nitrogen and siderophore production. Indirect effects include traits involved in biocontrol in a larger sense like biofilm formation and extracellular polysaccharide production. The most important mechanism or attribute of PGPRs is nutrient solubilization. The main character of PGPRs is the root colonization and inducing ISR. Only few organisms have been reported to have PGP traits from the vast diversity of rhizosphere. They have ability to combat abiotic stresses as well. Bacteria represent the majority of soil microbial life; majority of PGP organisms are identified to be bacteria (Tiwari et al. 2019).

According to Etesami and Maheshwari (2018), bacteria mainly PGPRs have the ability to increase plant growth and yield. PGPRs deploy various mechanisms to ensure better plant stand. Few of them are (1) bioremediation (by bacterial exopolysaccharides), (2) enzyme synthesis (1-aminocyclopropane-1-carboxylate deaminase), (3) biological nitrogen fixation, (4) production of siderophores, (5) the generation of phytohormones, (6) protection against pathogens (competition for nutrients and space with pathogens in soil, induction of ISR, production of antibiotics, siderophores, hydrolyzing enzymes, etc.), (7) nutrient solubilization and mineralization (especially phosphate) and (8) priming of plants for abiotic stress resistance. Rhizosphere can be the right place for harnessing such organisms and proving the potential of PGPRs in plant growth and development (Etesami and Maheshwari 2018). Pooling of such originally soil occurring organisms will help for proper maintenance of plants.

14.3.4 Mycorrhiza: An Important Member of Rhizosphere

Interactions between mycorrhizal fungi and plant pathogens have been studied since the 1970s, and majority of the studies have reported inhibitory effect on plant pathogens and reduction of disease severity, whereas some reports have put their effect as neutral (Bååth and Hayman 1984) or, even, enhancement in disease severity. Disease symptom reduction has been described for a large number of fungal pathogens such as *Fusarium*, *Rhizoctonia*, *Pythium*, *Thielaviopsis*, *Gaeumannomyces*, *Sclerotium*, *Phytophthora*, *Ganoderma*, *Verticillium*, *Macrophomina*, *Aphanomyces*, *Urocystis*, *Bipolaris*, *Microcyclus*, *Phoma*, *Cylindrocarpon* and *Olpidium* mycelium; for bacterial pathogens such as *Pseudomonas* and *Erwinia* (Garcia-Garrido and Ocampo 1989); and for nematodes such as *Radopholus*, *Pratylenchus*, *Heterodera*, *Rotylenchus*, *Aphelenchus*, *Tylenchorhynchus*, *Meloidogyne* and *Tylenchulus*. However, increased disease severity due to viral diseases has been reported due to vesicular-arbuscular mycorrhizal (VAM) fungi (WtRoot 1984). Different mechanisms could be accounted for the ability of mycorrhizal fungi to control plant diseases which include host nutrition

improvement, changes in the morphological attributes of host root, compensation for root damage, competition for host photosynthates and infection site, mycorrhizospheric changes in microbial communities, activation of defence mechanisms and antibiosis.

Mycorrhiza-induced suppression of soil-borne pathogens has been proven beyond doubt and has demonstrated potentiality in terms of controlling plant pathogens. Still, instances of successful practical applications are rare (Hooker et al. 1994; Linderman 1994) due to the sophisticated tripartite association (plant-soil-mycorrhiza) and the overarching effect of the existing environmental conditions. Usually, experiments have tested a single host genotype and a single mycorrhizal fungus, and hence, the mycorrhizal diversity for biocontrol of plant pathogens is not known. Certain other antagonists for soil-borne pathogens like *Trichoderma*, *Bacillus*, PGPR (plant growth-promoting rhizobacteria), *Gliocladium,* etc. work in tandem with the mycorrhizal fungi for biocontrol, and the role of mycorrhizal fungi, particularly in phytosanitary terms, can be transformed into a more effective one when integrated with other measures of plant protection. Hence, for the best exploitation of prophylactic activity of mycorrhizae, the optimum combinations of contributing factors must be explored with utmost importance and priority given to selection of efficient and appropriate mycorrhizal fungi.

14.3.5 Host Plant Resistance Modulators

Plants have same mechanism for identifying both pathogens and biocontrol agents. There are certain molecular or surface cues called PAMPs (pathogens) and MAMPs (any microorganism). Any microorganism which comes in contact with the plant has to face initial plant defence. The difference lies in amplitude of secondary defence activated against pathogens and BCAs. BCAs surpass this strong defence mechanism and signal the plant to develop induced systemic resistance. Defence signalling by jasmonic acid is the most accepted phenomenon of defence induction by BCAs and PGPRs. Several studies have investigated the effects of defence signalling on the commensal microflora. Successful colonization and minimization of immune response triggered by plant are important characters of BCAs. BCAs have the ability to minimize the strong defence response by reprogramming the plant's transcriptomics and proteomics. This occurs basically by controlling defence signalling network.

Along with defence signalling, BCAs and PGPRs aid in production of lipopolysaccharide and siderophore which are stimulants for defence signalling. The hormones involved in defence are also capable of selection of root microbiome. The induction of ISR by BCAs is well demonstrated in radish, carnation, cucumber and many more plants. In radish, ISR induction was attributed to the O-antigenic side chain of the lipopolysaccharide present on the outer membrane of *Pseudomonas* strains. Pathogens causing wilts, anthracnose and leaf spots are more efficiently controlled by BCAs through ISR. The role of rhizosphere microbiome in induction

of ISR was studied extensively and concluded that mutant microbiome impaired defence signalling (Weller 2007). There are differences in the root exudate profile of healthy and diseased plant, and infection by a pathogen or attack by an insect can cause significant changes in exudate profile in the rhizosphere. This change further plays a role in selection of rhizobiome (Berendsen et al. 2012).

14.3.6 Source of Antibiotics

The soil, in itself, consists of five major constituents, viz. water, organic matter, mineral matter, air and living organisms. The soil body's portion consisting of living organisms includes microorganisms as well as small animals, but,it is a widely accepted view that soil microorganisms mostly contribute to carbon dioxide and nutrient liberation for growth of plants. Even under the category of soil microorganisms, bacteria constitute as the most abundant group. Bacteria residing in the soil can have different shapes like rod-shaped (bacilli), spherical (cocci), spirilla (spirals), etc., but the numerical majority out of these different shapes are exhibited by rod-shaped bacteria. They are very widely distributed and one of the major groups of bacterial population in the soil (Bhagabati et al. 2004). The literal meaning of the term 'antibiotic' is 'against life'. However, in our everyday usage, the word describes those set of chemicals that can kill or inhibit bacteria. One of the most important secondary metabolites which are produced commercially by bacteria (or sometimes, synthetic means), to be exploited against a wide range of bacteria are antibiotics. A number of soil bacteria have the potential to produce antibiotics. For instance, a number of *Bacillus* species produce antibiotics like gramicidin, bacitracin and pumulin which show activity against Gram-positive bacteria, namely, *Streptomyces*, *Streptococcus*, *Staphylococcus* and *Corynebacterium*, while other bacterial genera produce antibiotics like tetracycline, gentamycin, chloramphenicol and vancomycin which show activity against Gram-negative bacteria.

Streptomyces is perhaps the largest genus, having around 150 member species. *Streptomyces* species are involved in production of antibiotics and are very important medically. Mostly, soil is the natural habitat of *Streptomyces* species, where they may contribute up to 20% of the population which is culturable. They are best known for the production of a vast number and diversity of antibiotics (Willey and Gaskell 2011). Waksman's discovery that *Streptomyces griseus* produces streptomycin was a contribution of immense significance to public health and science. The drug streptomycin was the first one able to effectively combat tuberculosis, and in 1952, Waksman was conferred with the Nobel prize. An enormous effort for searching and isolating new species of *Streptomyces* producing other compounds having important medicinal values was undertaken after the discovery of streptomycin. Over 10,000 bioactive compounds have been found to be produced by different *Streptomyces* species since then. Hundreds of these natural products are now used in industry and medicine. *Streptomyces* contribute to around two-thirds of the antimicrobial agents employed in veterinary and human medicine. Other

examples are erythromycin, amphotericin B, chloramphenicol, nystatin and tetracycline. Some species of *Streptomyces* even produce more than one antibiotic. *Bacillus* species produce different kinds of antibiotics such as bacitracin which exhibits a full range of antimicrobial activity and is produced by *Bacillus licheniformis*. An antibiotic called gramicidin is produced by *Bacillus brevis*. *B. thuringiensis* and *B. sphaericus* form a parasporal body next to their endospores during formation of spores which contain a toxic protein which dissolves in the caterpillars' alkaline gut and kills moth species by destruction of epithelium. *Lactobacillus lactis* produces an antibiotic called nisin which acts against many Gram-positive organisms such as *Clostridia*, anaerobic cocci and *Corynebacterium* (Waites et al. 2008).

14.4 Strategies of Crop Protection Derived from Rhizosphere Microbiome

Sustainable development in agriculture requires strategies to mitigate this problem. According to the US National Research Council, the main aim is to develop a farming system which is more profitable, highly productive, less energy consuming and environment friendly and maintains biodiversity along with ensuring food quality and its safety. The bio-formulations of beneficial microorganism can reduce the use of hazardous chemical fertilizers and pesticide, which could increase the nutritional value of food and help reduce the crop plants from different biotic and abiotic stresses. Many studies have been conducted in the quest of identification, isolation and practical applications of microorganisms to replace the input of hazardous agrochemicals.

14.4.1 Bio-priming for Stress Resistance

Bio-priming is derived from two words 'bio' meaning 'life' and 'priming' meaning 'to prepare'. This concept was first developed for the protection of sweetcorn from the seed decay of *Pythium ultimum* (Callan et al. 1990). The mechanism behind biological control is assumed to be either direct through antagonism of soil-borne pathogens or indirectly by inducing resistance responses of host plant. The mechanisms of biocontrol include antibiosis, parasitism, competition for nutrients and space, cell wall degradation by lytic enzymes and induced disease resistance (Singh et al. 2013). Addition of beneficial rhizosphere microorganisms in the priming process involves either a method for efficient delivery to the crop rhizosphere or ability to manage pathogen. They are useful in adverse soil conditions as well as in maintaining the soil health, reduce the use of hazardous chemicals by decreasing the cost of cultivation and help in enhancing the efficacy of biological control agents. The effective microbial strains are formulated using different organic

and inorganic media either through solid or liquid fermentation technologies. They are delivered as either individual strains or mixtures of strains via seed bio-priming, seedling dip, soil application, etc.

In case of seed bio-priming, they create an outer covering on seed coat and protect the seed from pathogen entry while protecting the endosperm and embryo. Use of seed bio-priming with *Pseudomonas fluorescens* helped in 41.33% reduction in percent disease index of the rice sheath blight disease under field conditions (Suman et al. 2017). Seed bio-priming with *Trichoderma harzianum* yielded 55% reduction in brown leaf spot and sheath blight of paddy (Biswas et al. 2008). Seed bio-priming with *Pseudomonas fluorescens*, *Trichoderma harzianum*, *Trichoderma viride*, *Gliocladium virens* and *Stachybotrys atra* could also effectively manage seed-borne diseases, viz. downy mildew of pearl millet, sheath blight, leaf blast, bacterial leaf blight, glume discolouration, brown spot of rice, loose smut and root rot of wheat, zonate leaf spot, anthracnose, head blight and grain mould of sorghum, ear rot of maize, etc.

Increase in species richness of rhizosphere is the indicator of plant health and productivity (Van Der Heijden et al. 2008; Lau and Lennon 2011; Schnitzer et al. 2011; Wagg et al. 2011). The benefits and mechanisms of microorganisms on plant health and fitness and their application in agriculture are widely studied and documented (Bhattacharyya and Jha 2012; Chaparro et al. 2012; Wu et al. 2013). Moreover, optimization of beneficial microbes and their formulation process requires extensive research to introduce them in sustainable agricultural practices. However, the application of individual microorganism, identification of potential microbes and their application for crop yield improvement represent another important challenge to venture.

14.4.2 Biological Control

Biological control is a process in which population of one species lowers the numbers of another species by mechanisms such as predation, parasitism, pathogenesis or competition. It can be an important component of integrated pest management (IPM) programmes. Natural enemies are already adapted to the habitat, and their conservation can be simple and cost-effective. A microbial consortium is two or more microbial groups living symbiotically. Microbial consortia have several advantages over single species of biocontrol agent such as efficiency, robustness and modularity. Microorganisms under natural environment inhabit in communities, and they may be beneficial to plants. It is clearly understood that microbial consortia lead to enhance defence signalling cascades which leads to transcriptional activation of different metabolic pathways. Many research studies have been conducted to study the plant defence activation with the use of microbial consortium. For obtaining enhanced biocontrol activity, combining more than one biocontrol organism may suppress the plant pathogens by any of its mechanism, which may help us to combat different biotic or abiotic stresses. One microorganism may complement the

biocontrol mechanism of another which might be non-functional for a particular strain, and also, they may be effective against a range of pathogens.

For the management of papaya dieback disease caused by *Erwinia mallotivora*, the consortium development of *W. cibaria* PPKSD19 and *Lactococcus lactis* subsp. *lactis* PPSSD39 showed increased antibacterial activity probably due to the production of bacteriocin-like inhibitory substances (BLIS). The nursery experiment revealed that the application of bacterial consortium of *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 reduced disease severity to 19% and enhanced the biocontrol efficacy to 69% of infected papaya plants after 18 days of treatment showing that *W. cibaria* PPKSD19 and *L. lactis* subsp. *lactis* PPSSD39 are potential candidate as biocontrol agents against papaya dieback disease (Mohd Taha et al. 2019). For the control of basal stem rot disease of oil palm, two potential biocontrol agents (BCAs), AAT0115 and AAB0114 strains, were assessed for their plant growth-promoting (PGP) performance. The two ascomycetous *Talaromyces apiculatus* (Ta) AT0115 and *Clonostachys rosea* (Cr) AAB0114 are biocontrol fungal species with PGP characteristics. These two strains showed to be effective in reducing the linear mycelial growth of *G. boninense* in in vitro condition. Inoculation of either individual Cr and Ta—as well as Cr + Ta consortium—induced a significant increase in leaf area and bole girth of oil palm seedlings after 5 months of inoculation in nursery conditions. Co-inoculation of Cr and Ta resulted in suppression of BSR as compared with Cr or Ta individually (Goh et al. 2020). Srinivasan and Mathivanan (2009) tested two plant growth-promoting microbial consortia, viz. PGPMC-1 consisting of *Bacillus licheniformis* strain MML2501 + *Bacillus* sp. strain MML2551 + *Pseudomonas aeruginosa* strain MML2212 + *Streptomyces fradiae* strain MML1042 and PGPMC-2 consisting of *B. licheniformis* MML2501 + *Bacillus* sp. MML2551 + *P. aeruginosa* MML2212 against sunflower necrosis virus disease (SNVD) along with farmers' practice of insecticidal spray (imidacloprid + mancozeb) and control in farmers' fields. This resulted in significant reduction of disease, enhanced seed germination and plant height and other yield parameters. A consortium of five bacterial isolates protected *Nicotiana attenuata* from a sudden wilt disease by several mechanisms. Three members of the consortium, *Pseudomonas azotoformans* A70, *P. frederiksbergensis* A176 and *Arthrobacter nitroguajacolicus* E46, form biofilms when grown individually in vitro, and the quantum of biofilm increased synergistically in the five-membered consortium, including two *Bacillus* species, *B. megaterium* and *B. mojavensis*. *B. mojavensis* produces the antifungal compound surfactin which inhibits the fungal growth. However, isolates A70 and A176 produce siderophores under in vitro conditions (Santhanam et al. 2019).

14.4.3 Volatile Organic Compounds (VOCs) as Bio-fumigants

Volatile organic compounds are low molecular weight compounds with high vapour pressure which may evaporate and disperse easily. They act as either intraspecies or interspecies signal molecules in air, water and soil (Bailly et al. 2014). Metabolic activity of all the organisms leads to production of certain by-products which are volatile in nature. A wide range of volatile compounds are reported to be produced from bacteria. They have a mixed reaction, i.e. beneficial or deleterious, on growth and development of other organisms when they are exposed. Bacterial volatile compounds (BVCs) are highly diverse in nature and are a mixture of inorganic and organic volatile compounds (Audrain et al. 2015). There are few studies assessing the effects of BVC application under open conditions, and soil applications have been reported, and some potentially promising results were obtained. Here we will discuss the available studies of BVC application into soil and its effects on plant growth and resistance to fungal, bacterial, viral and insect herbivore pathogens.

VOCs produced by *Pseudomonas chlororaphis* subsp. *aureofaciens* SPS-41 are used as bio-fumigants to control *Ceratocystis fimbriata* in postharvest disease of sweet potatoes (Zhang et al. 2019a, b). *Trichoderma*-derived VOCs are employed against late blight pathogen *Phytophthora infestans* in postharvest disease of potato tubers (Elsherbiny et al. 2020). Bio-fumigation was done with volatile organic compounds from *Streptomyces alboflavus* TD-1 and pure chemicals to control *Aspergillus ochraceus.* It showed strong inhibitory effects on the mycelial growth, and abnormalities were found in conidial and hyphal morphology (Yang et al. 2018). It was found that volatile mixtures of chemicals produced from plant growth-promoting rhizobacteria (PGPR) are responsible for inducing immunity and growth enhancement in *Arabidopsis thaliana* (Ryu et al. 2005); this shows its potential exploitation to be used in agriculturally important crops. VOCs released by *Ceratocystis fimbriata* have strong bioactivity against a wide range of fungi, bacteria and oomycetes (Li et al. 2015).

Ryu et al. (2003) reported that 2,3-butanediol produced by *Bacillus* spp. has a role to elicit plant growth and induced systemic resistance (ISR). Many studies revealed the application of 2,3-butanediol into soil of open fields to test its effects under agricultural conditions, and it was found to increase in leaf fresh weight of *Arabidopsis* leaves compared to the control (Ryu et al. 2005). Han et al. (2006) reported that the (2R,3R)-butanediol isomer had greater activity than (2S,3S)-butanediol in eliciting ISR in *Arabidopsis* and tobacco plant. It was also reported that drench application of 100 μM (2R,3R)-butanediol in soil leads to activation of ISR against the anthracnose of *Nicotiana benthamiana* caused by *Colletotrichum orbiculare*. Further when studied at transcriptional-level gene activation, it was found that six pathogenesis-related (PR) genes were upregulated. The jasmonic acid/ethylene signalling pathway is known to modulate ISR; the above drench application studies are the molecular genetic evidence for BVC-induced ISR. Similarly, under greenhouse conditions, drench applications of 2,3-butanediol and

acetoin into soil resulted in 15.2% and 12.4% higher fresh weight of pepper, respectively, than those of control plants (Hahm et al. 2012). Pre-treatment of cucumber seedlings with 10 nM and 0.1 μM 2-butanone stimulated protection against angular leaf spot caused by *Pseudomonas syringae* pv. *lachrymans* in large-scale trials, and total fruit weight was greater for cucumber plants treated with 0.1 μM 2-butanone. Huang et al. (2012) screened the volatile profile of *Bacillus cereus* C1L and found that dimethyl disulphide is a promising chemical for ISR induction. The effectiveness of dimethyl disulphide was examined by drenching the soil of tobacco and maize. It was found that systemic resistance in tobacco and maize was augmented against *Botrytis cinerea* and *Cochliobolus heterostrophus*. Song and Ryu (2013) reported that the ketone BVC 2-butanone emitted from *Bacillus amyloliquefaciens* IN937a and *B. subtilis* GB03 triggered ISR in cucumber against an insect herbivore.

14.4.4 Horizontal Gene Transfer (HGT)

Maintenance of ecological fitness is very important for any living organism. Variations like gene addition or deletion and genetic changes occur in living organisms to ensure sustenance. These changes are brought in by mutations or acquisition through gene transfer. The acquisition if carried between different taxa will be called horizontal gene transfer. It contributes towards improved fitness and survival of introduced or native microflora. Mobile genetic elements are acting as vehicles to carry out HGT. Plasmids, insertion sequence elements, phages and others form the group of mobile genetic elements. HGT occurring through plasmids is a widely accepted phenomenon. The predominance of plasmids in bacteria is indicative of such process occurring naturally and artificially. Plasmids add to bacterial life by helping bacteria to acquire elements needed for adaptation to changing environment. They help in fast changing of bacteria to cope up with continuously changing environment. It further can help in emergence of new strains with novel characters. Technology today like metagenomic approaches has helped to explore this large and untapped diversity of resident MGEs in soil- and plant-associated bacteria. Intraspecies variability and reshaping of bacterial genome distribution of functional traits are ensured by the events occurring through MGEs. Many events of HGT help to sustain changes in biotic interactions occurring in that vicinity. The interactions include (1) antibiotic production by microorganisms, (2) propagation of antibiotic resistance, (3) release of xenobiotics or new secondary metabolites, (4) dissemination of degradative gene and pathway assemblies and (5) symbiotic or pathogenic interactions.

Bacterial community adaptation by circulation of multiple antibiotic resistances is the most widely studied HGT event. HGT helps the community to adapt to strong selection pressures. HGT can happen through three processes of transformation, transduction and conjugation in bacteria. Transformation occurs between highly recipient and donor bacteria; bacteriophages are required for transduction, and for

conjugation an intimate structure will develop. During biofilm formation, the HGT event rate is higher and it can happen through all the three processes. For conjugation to occur in the environment it should be nutrient rich. Recently, the reports of HGT through bacteriophages are reported and gaining high momentum. HGT is a mechanism of survival and evolution (Maheshwari et al. 2017). Majority of such events occurring in soil go unnoticed due to the diversity of microbial life. The community functioning trait is enabling the survival of organisms in changing environment.

14.4.5 Transgenics

The role of soil microorganisms in development of transgenic plants for pest management has been immense, both as an intrinsic tool for the very process of agro-transformation and as a reservoir of a vast number of homologous genes coding for a family of proteins acting against an entire order of pests which can even be subjected to pyramiding in transgenic host plants leading to a broad-spectrum resistance. These two instances as mentioned above are exemplified by *Agrobacterium tumefaciens* and *Bacillus thuringiensis* (Bt), respectively. The *Agrobacterium* populations inhabit different ecological niches (rhizosphere, bare soil, host plants), and therefore, for all practical intents and purposes, we may treat it as a soil bacterium here. Genetic transformation of its host by *Agrobacterium* takes place by transfer of a well-defined segment of DNA from its tumour-inducing (Ti) plasmid to the genome of the host cell. The transferred DNA (T-DNA) harbours a set of opine-catabolism genes and oncogenes, and their expression in plant cells results in transformation of the tissue, and it starts exhibiting neoplastic growth along with production of amino acid derivatives and opines exploited by the bacteria exclusively as a source of nitrogen. Transformed (recombinant) *Agrobacterium* strains, which have their native T-DNA excised and replaced with genes of interest, are the most effective and efficient vehicles employed today for foreign genes' introduction into plants and transgenic plant species production.

Having discussed the importance of a soil bacterium as an intrinsic tool for the very process of agro-transformation (analogous to a vehicle), let's shift our gaze to a soil bacterium which has acted as a reservoir of a family of genes (analogous to passengers to be accommodated in a vehicle for transformation to the destination) coding for variants of proteins acting against different orders of pests. *Bt* crops, predominantly cotton and maize hybrids, carrying out transgenic expression of *cry* genes which have been derived from a soil bacterium called *Bacillus thuringiensis*, have been planted extensively. Pesticide usage was reduced, and significant increases in profits and yields were reported. *Bt* toxins which are occurring naturally and showing activity against a wide range of pest species have been discovered and are available potentially for the *Bt* crops to be engineered into broad-spectrum control of pests. Newer and newer generations of *Bt* crops and products incorporating an ever-expanding range of *cry* toxins targeting other facets of arthropod biology are being developed and introduced commercially, and the fact is that the earliest

way of controlling pests by *Bacillus thuringiensis* was through isolation and crystallization of naturally occurring Bt toxins and their spray in the field. Besides, reports have suggested that administration of genetically modified endophytic bacteria to plants can bolster their resistance to disease. Report of other soil microorganisms as source of genes of interest for development of transgenic plants showing disease resistance is hard to find, but the search is unending and needs to be undertaken very patiently and rigorously because according to an estimate, 1 g of soil is an immense biochemical gene library producing diverse genetic instructions, which have been present for almost 4 billion years on the earth, and the DNA therein can extend to 1598 km (Trevors 2010). Surely, this tremendous genetic information helps us in finding novel genes which confer disease resistance for further development of transgenics which may promise sustainable crop protection in the future.

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Chapter 15
Bacterial Inoculants for Control of Fungal Diseases in *Solanum lycopersicum* L. (Tomatoes): A Comprehensive Overview

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Abstract Globally, *Solanum lycopersicum* L. (tomatoes) is the second most widely grown vegetable. This crop is sensitive to over 200 diseases caused by a variety of phytopathogenic microorganisms, specifically, soil-borne fungi. The major fungal pathogen causing diseases in tomatoes are *Fusarium oxysporum* f. sp. *lycopersici*, *Botrytis cinerea*, *Verticillium dahliae*, *Sclerotium rolfsii*, *Colletotrichum* sp., *Alternaria* sp. *Rhizoctonia solani*, etc. Even though a wide range of chemical fungicides is now available to combat fungal diseases, the overuse of these chemicals has been shown to leave negative/adverse influences on the texture, yield and nutritive value of the fruits. In this regard, to manage the fungus-induced tomato diseases, plant growth-promoting (PGP) bacteria are one of the most environmentally friendly, effective, safe and economically sound solutions. A variety of beneficial soil microorganisms (BSMs) are currently being employed as soil or plant inoculants in several crop plants, including tomatoes, as biocontrol agents (BCAs). These BCAs also work as growth regulators, in addition to preventing fungal diseases. The current chapter discusses the application of beneficial and antagonistic BCAs, their effectiveness as well as bacterial-mediated mechanisms involved in the management of diseases in tomatoes. The specific mechanisms are antibiosis, competition, production of cellulolytic enzymes, cyanogenic compounds (HCN) and siderophore and induced systemic resistance (ISR). The ability of PGP rhizobacteria to antagonize a pathogen and suppress the disease through multiple pathways has been intensively studied to use them as effective BCAs. As a result, this chapter highlights a full explanation of various bacterial-mediated biocontrol mechanisms used by BCA. As environmental and health issues highlight the need to transition to a more sustainable agriculture system, the use of indigenous PGP rhizobacteria in plant disease prevention is gaining attraction. It's also recommended that using a bacterial consortium guarantees that BCA performs consistently in field settings.

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15.1 Introduction

Tomatoes (*Lycopersicum esculentum* Mill.), commonly known as 'golden apples', are the world's second most widely farmed vegetable crop after potatoes, due to their flavourful fruit and high nutritional content (Costa and Heuvelink 2018). Vitamin A, vitamin C and β-carotene, as well as vital minerals, are abundant in tomatoes (Çolak et al. 2020). It is also high in phosphorus (P), potassium (K), magnesium (Mg) and iron (Fe), all of which are needed to keep nerves and muscles functioning normally (Ali et al. 2020). Tomatoes contain β-carotene (a precursor to vitamin A) and phytosterols, making them the third-best source of vitamin C and the fourth-best source of vitamin A in human diet (Poiroux-Gonord et al. 2010). A strong antioxidant found in natural lycopene is a phyto-nutrient found in red, ripe and cooked tomatoes that helps prevent heart-related diseases and sarcoma (Kaur and Kaur 2015). Other preventive processes, such as anti-inflammatory and antithrombotic functions, are also attributed to this fruit. The short growing season, cheap cost and great economic returns have enticed growers to cultivate the crop throughout the year, even in hotter climates (Villareal 2019). Because of the diverse nature of the fruit, tomatoes are often considered a cash and industrial crop in several countries like India, the USA, China, Turkey and Egypt (Costa and Heuvelink 2018). China is the world's biggest tomato producer, with yearly production of approximately 34 million tonnes, according to international statistics (Nicola et al. 2009). Tomato output in the world is expected to be 145 million tonnes per year, covering 4.36 million ha (Chohan et al. 2017).

Tomatoes are grown all over the world due to their resilience to a wide range of soils and climates (Morganelli 2007), but their softness makes them vulnerable to insect pests and various abiotic (pesticides, heavy metals, salinity and drought) and biotic stresses (Atkinson et al. 2011). Diseases caused by phytopathogens are the primary limiting factors in overall crop losses around the world, and they are becoming increasingly important as the global population grows. Over 200 pathogen-caused illnesses have been recorded in tomatoes around the world (Watterson 1986). Seed-borne infections, on the other hand, can quickly travel from one location to another and act as the first source of inoculum (Rennie and Cokerell 2006). High-quality seeds, therefore, play an important role in producing long-term, lucrative veggies, and tomatoes are no exception to this. Microorganisms such as fungi, bacteria, viruses and nematodes cause seed-borne illnesses. Among disease-causing parasitic microbes, fungi are the most commonly seen on seeds. As a result, infected seeds have a negative impact on seed health, limiting germination capacity, poor seedling vigour, transmitting fungus pathogen to seedlings, speeding up storage deterioration, transferring pathogens into new areas and expanding the inoculum source in the field. Fungi, a crucial category of microorganisms, are responsible for a number of seed-borne illnesses of tomato, which result in significant yield losses.

Several seed-borne fungi, such as *Aspergillus flavus*, *Rhizopus solani*, *Curvularia* spp. and *Fusarium stolonifer* induce problems in tomato seeds, including wilt, necrosis, rotting and toxification of seeds (Neergard 1997). Late blight, a disease of tomato (caused by *Phytophthora infestans*), is one of the most destructive tomato diseases, resulting in significant financial losses (Fry 2020). *Sclerotinia* rot (caused by *Sclerotinia sclerotiorum*) is another major fungal infection that is threatening tomato crop productivity (Jnr 2000). Several studies have been published on *Fusarium* species-caused wilt, crown and root rot infections in tomatoes (Yezli et al. 2019). The wilt (*Fusarium oxysporum* f. sp. *lycopersici*) and early blight (*Alternaria solani*) diseases are regarded the most damaging fungal diseases that affect tomato plants.

Since the fungal diseases are pandemic, simple cultural cleanliness is ineffective. To protect the fungal diseases, chemical fungicides are often used for better yield and productivity of tomatoes. Even though agrochemicals have long been employed as a reliable method of harvest insurance, their increased use has resulted in several undesirable consequences, including disease resistance and non-target natural effects. Furthermore, the increased use of fungicides to combat tomato diseases has resulted in an increase in health risks due to phytotoxic residues and environmental consequences. As a result, it is strongly recommended that alternate disease management methods be used in place of agrochemical substances.

Managing fungal diseases using biological means is seen to be a viable method for reducing disease severity. In comparison to the previously mentioned use of chemical fungicides, the use of soil beneficial bacteria that colonize the underlying foundations of harvest plants and suppress the soil-borne illnesses is becoming an elective alternative. In this regard, PGP bacteria can be used as soil inoculants to reduce soil-borne illnesses, which is an organic solution. Because they have the potential to colonize the rhizosphere swiftly and spread down the root from a single seed treatment or soak application into the soil, PGPR with the viability of their biocontrol activity typically provide long-term protection from soil-borne diseases at the root surface. A huge number of PGPR have been recovered and characterized in the hopes of developing them as biocontrol agents for tomato illnesses. For example, Kilani-Feki et al. (2016) also employed *B. subtilis* strains to suppress the *Botrytis cinerea*, the pathogen that causes tomato fruit rot. In another study, biocontrol agents (BCAs) were recovered to combat the tomato wilt disease. It was observed that *Ochrobactrum intermedium* and *B. amyloliquefaciens*, among strains, potentially inhibited the disease incidence and increased the seedling growth and vigour indices of tomatoes (Gowtham et al. 2016). Furthermore, the effectiveness of *P. fluorescens* strains against a variety of fungal diseases (leaf blight, damping-off, stem canker, and root rot) in tomatoes (Singh et al. 2017) has been reported. Under in vitro and pothouse settings, BCAs, viz. *P. aeruginosa*, *P. fluorescens*, *B. amyloliquefaciens* and *B. subtilis* were able to successfully suppress the canker and wilt disease of tomato (Abo-Elyousr et al. 2019). Recently, a new technique for inducing systemic resistance (ISR) in plants by employing PGPR has been investigated by Attia et al. (2020). They confirmed that PGPRs were found to be effective in reducing the growth of *A. solani* (causing early blight disease) in tomato plants. In comparison to

non-treated plants, they achieved an 84.3% protection rate. Effective selection, screening and safety investigation of promising PGPR strains are required for incorporating soil microbes for disease control/plant growth promotion in cropping systems and eliminating the need for chemicals. Furthermore, information on disease targeting, mass-scale production costs and procedures of registration must all be updated to improve the market standing of these BCAs. The current chapter aims to look into the potentiality of antagonistic PGP bacteria (BCAs) in managing the major fungal diseases of tomatoes. This chapter also discusses the challenges and benefits of commercializing these bacteria in the agriculture sector.

15.2 Prevalence of Seed-Borne Mycoflora: A Historical Perspective

Seed-borne fungi are microorganisms that can host the seeds both within and externally, causing diseases or contaminating the environment (Amza 2018). Phytopathogenic fungi may cause post-germination death, rendering them poisonous and lowering their quality for human food and seed production. The conidia, oospores, sclerotia, hyphae and chlamydospores are some examples of the main forms in which they are present (Arora 1986). The tomato, which is employed as a model crop in genetics, is susceptible to a variety of seed-borne fungal diseases.

A total of 12 phytopathogenic fungi were recovered from the fruits and seeds of *Solanum lycopersicum*. It was found that the majority of the pathogens were habitants of fruits; however, the species of *Cladosporium* were detected in seeds (Dhekle and Bodke 2013). In reality, a considerable number of fungal isolates belonging to genera *Fusarium*, *Pythium, Botrytis*, *Alternaria* and *Rhizoctonia* were found in tomato seeds, causing several seeds-borne diseases. Mycoflora are isolated as surface contaminants, internally seed-borne flora, and are known to cause major field diseases depending on the presence of fungi on the seed coat or in the seed. Various fungal phytopathogens, viz. *F. oxysporum*, *Alternaria solani*, *Aspergillus flavus*, *A. fumigatus*, etc., have been detected and identified from infected tomato plants and reported to cause serious seed damage (Fig. 15.1) as reported by several workers. The investigation of grey mould disease in tomato leaves was done using a hyperspectral imaging technique based on competitive adaptive reweighted sampling (CARS) and correlation analysis (WANG et al. 2017). Similarly, tomato plants were infected by fungal pathogens like *F. semitectum* (1–3% infection), *F. moniliforme* (0.5% infection), and *Curvularia lunata* (0.5–7.5% infection) and *Bipolaris* spp. (1.5% infection) (Bhatti et al. 2010). The duration of storage has a significant impact on the prevalence of various mycoflora. On a modest scale, *Phytophthora infestans* has caused the full destruction of tomato harvests around the world (Panthee and Chen 2010). *Hormonema* spp., one of the most prevalent genera on tomato seeds, were discovered by Nishikawa et al. (2006) while assessing 109 species of seed-borne fungi from three cultivars of tomato. The seed could be

Fig. 15.1 Major tomato diseases caused by fungal pathogens. Early blight (*Alternaria solani*) (**a**), late blight (*Phytophthora infestans*) (**b**), buckeye rot (*Phytophthora*) (**c**), septoria leaf spot (*Septoria lycopersici*) (**d**), anthracnose (*Colletotrichum*) (**e**), verticillium wilt (*Verticillium dahliae*) (**f**), southern blight (*Sclerotium rolfsii*) (**g**), fusarium wilt (*Fusarium oxysporum* f. sp. *lycopersici*) (**h**) and grey mould (*Botrytis cinerea*) (**i**) (source: https://www.google.com/)

diseased within or be contaminated on the exterior. The fungus *F. oxysporum* f. sp. *lycopersici* causes fusarium wilts, one of the most destructive diseases of tomatoes. Similarly, previous researchers found a total of 17 fungal phytopathogens linked with tomato var. local seeds, with *Aspergillus niger*, *A. flavus*, *Fusarium moniliform*, *Rhizopus nigricans*, *Curvularia lunata* and *Alternaria alternata* being the most prevalent. In the Indian state of Gujarat, 12 fungi were discovered with four tomato cultivar seeds. *A. alternata*, *A. flavus* and *A. niger* were the most common, whereas *A. amstelodami* and *Cunninghamella echinulata* were discoveries.

15.3 Management of Fungal Diseases in Tomatoes

In the fight against crop diseases, seed health is crucial. The rise of endemic diseases as a result of changing global environment poses a difficulty in maintaining the health of the plant. As a result, timely and precise diagnosis of the problem, as well as pathogen surveillance, gives time for mitigation actions to be implemented. Treatment of seeds to destroy pathogens carried within or on the seed has been demonstrated to be effective in preventing epiphytotic plant diseases. Seed-borne fungi, on the other hand, are easy to control compared to airborne or soil-borne

fungi. Farmers are encountering financial difficulties as a result of significant crop losses caused by seed-borne mycoflora on their crops. Controlling seed-borne infections via various approaches is a crucial element in every agricultural crop production and protection programme. To eliminate pathogens from seeds both inside and externally, as well as to protect seeds from soil-borne diseases, a variety of chemical, biological, physical, and mechanical techniques have been applied (Shahid et al. 2017).

15.3.1 Use of Chemical Fungicides (Chemical Method)

Mycotoxins released by seed-borne fungal pathogens cause serious issues in humans. Hence, seeds should be treated with appropriate chemical before planting. Chemical fungicides are used since pre-historic times for the management of phytopathogenic fungi and for obtaining a better yield of crops. Historically, treating seeds with chemical fungicide using spraying, drenching of soils and dusting has been important (Copping and Duke 2007). These methods could protect the seeds and seedlings from the soil-dwelling fungal pathogens causing damping-off and rot diseases (Divya Rani and Sudini 2013). Amini and Sidovich (2010) conducted in vitro and in vivo experiments where they used six chemical fungicides, viz. fludioxonil (FLN), bromuconazole (BMZL), benomyl (BML), carbendazim (CBZM), azoxystrobin (AZBN) and prochloraz (PCZ), against the fusarium wilt disease in tomato crop. When administered to seedlings at prescribed levels, PCZ and BMZL were found to be the most effective against *Fusarium oxysporum* f. sp. *lycopersici*. Among the fungicides tested, FLN and BMZL showed a phytotoxic impact on tomato seedlings. Similarly, in another investigation, Al-Kassim and Monawar (2000) treated (in vitro) five vegetable seeds including *Solanum lycopersicum*, *Solanum melongena*, *Abelmoschus esculentus*, etc., in Gazan province with 0.2% of chemical fungicides like benomyl, cozib and mancozeb before incubation. The majority of the isolated fungi were inhibited by all of the tested fungicides. Benomyl, on the other hand, was the most effective against all of the fungi found on the seeds of the tomato.

Major fungal diseases of tomatoes, their symptoms and chemical control measures

S. no.	Disease	Causal agent	Symptoms	Chemicals used	Effectiveness	Reference
1	Early blight	*Alternaria solani*	• Brown-black spots (in the form of lesion 1/2-inch diameter) • Formation of blotches (irregular) • Defoliation, browning of infected leaves • Appearance of lesion (dark) on stems • Girdling of the stem or collar rot	Mancozeb 80WP, Bavistin 50WP, Indofil M-45, Sulcox 50WP and Tall-25EC, chlorothalonil (0.2%), kasugamycin (0.2%), azoxystrobin (0.1%), propiconazole (0.1%), pyraclostrobin (0.2%), perfekt (0.2%), metalaxyl (0.2%), mancozeb (0.25%)	Suppressed the fungal growth Reduced the disease incidence	Roy et al. (2019), Arunakumara et al. (2010)

(continued)

S. no.	Disease	Causal agent	Symptoms	Chemicals used	Effectiveness	Reference
2	Septoria leaf spot	*Septoria lycopersici*	• Disease infection on lower leaf • Symptoms also occur on stems and blossom • Symptoms on fruits appear in the form of small-sized water-soaked spots • Development of greyish white centres with dark edges • Yellowing, withering of leaves and ultimately fall off	Fungicides pyraclostrobin (116.6 ppm), fluxapyroxad (58.5 ppm), mancozeb (4000 ppm), difenoconazole (125 ppm), chlorothalonil (1500 ppm), propineb (2100 ppm), fluazinam + thiophanate-methyl (375 + 375 ppm) and metiram + pyraclostrobin (1100 + 100 ppm)	Suppressed the fungal growth Reduced the disease incidence	Monteiro et al. (2021)
3	Late blight	*Phytophthora infestans*	• Younger/older leaves infected • Appearance of pale-green water-soaked spots starting at leaf tips that enlarge rapidly, forming irregular, greenish-black blotches • Development of white moulds at the margins of infected areas • Under the favourable condition, whole plants rapidly defoliated • Infection on petioles and stems occurs as brown streaks	Fungicides oxathiapiprolin, chlorothalonil, azoxystrobin, mandipropamid and mefenoxam were effective against the disease	Chemicals single or in mixture controlled the disease Suppressed the growth of pathogens Improved the growth and yield of tomato	Cohen et al. (2018)
4	Fusarium wilt	*Fusarium oxysporum* f. sp. *lycopersici*	• Development of leaves • Affected leaves soon wilt and dry up, but they remain attached to the plant • The wilting continues successively on younger foliage and eventually results in plant death • The stem remains firm and green on the outside but exhibits a narrow band of brown discolouration in the vascular tissue • Brown streaking in the vascular tissue of infected plants becomes plugged during the attack by the fungus, leading to wilting and yellowing of the leaves	Chemical fungicides difenoconazole (200 mg/L), benomyl, carbendazim, prochloraz, fludioxonil, bromuconazole and azoxystrobin were effective		Amini and Sidovich (2010)
5	Verticillium wilt	*Verticillium dahliae*	• Leaf edges and areas between the veins turn yellow and then brown • Infected plants often have a characteristic V-shaped lesion at the edge of the leaf	Nano-fungicide (leaf extract olive oil-loaded chitosan nanoparticles)	Diminished the disease symptoms	Mazzotta et al. (2022)

(continued)

S. no.	Disease	Causal agent	Symptoms	Chemicals used	Effectiveness	Reference
			occurring in a fan pattern • As the disease progresses, younger leaves begin to wilt and die, until only a few healthy leaves remain at the top of the plant			
6	Anthracnose	*Colletotrichum*	• Symptoms appear first as small, circular, slightly sunken lesions on surface of ripening fruits • Spots quickly enlarge, become bruise like depressions and develop a water-soaked appearance directly beneath the skin (epidermis) of the fruit • As these spots expand, they develop dark centres or concentric rings of dark specks the rings consist of numerous small spore-producing bodies	Azoxystrobin-based fungicides Onestar 23% SC and Amistar 23% SC	Decreased the incidence of disease (69–71%)	Saxena et al. (2016)
7	Buckeye rot	*Phytophthora*	• Symptoms appear as water-soaked greyish-green/brown spots • Further, spot enlarges and develops into a lesion with a target-like pattern of concentric rings of narrow dark-brown and wide light-brown bands	Application of fungicides metalaxyl, cymoxanil, mancozeb and copper oxychloride	Reduced the incidence of disease	Gupta and Bharat (2008)
8	Southern blight	*Sclerotium rolfsii*	• Formation of wilting on plants • Development of water-soaked lesion on stems • Produced sclerotia are white, later becomes dark-brown (spherically structured) • White mycelium and sclerotia at stem base of infected plants are the main symptoms of disease	Fungicides pyraclostrobin, quintozene and fluxapyroxad were used	Chemicals significantly reduced the disease incidence	Keinath and DuBose (2017)
9	Grey mould	*Botrytis cinerea*	• Appearance of grey-brown velvety mould covering on stems/younger leaves • Grey spores cover dying flowers and the calyx of fruit	Difen Super, 55% WP fungicide concentration of 0.08% with active ingredient, difenoconazole, and fungicide Skor, 250 g/l EC with concentration of 0.05% and 0.07%	Effective against the pathogen	Zuparov et al. (2020)

15.3.2 Biological Management

Chemical fungicides have a significant impact on seed-borne fungal infections, but they also have a negative impact on beneficial microbial diversity in soils as well as crop productivity. Furthermore, irregular fungicidal usage is not only harmful to animals and humans but also leads to development of resistance among the pathogens targeted. Because fungicides are harmful to non-target organisms (Marinho et al. 2020), scientists are turning to more environmentally benign and cost-effective means of disease management, such as acid treatments, and the use of antagonistic microbes (Droby et al. 2022), and plant extracts (Shuping and Eloff 2017). These are interesting and viable options for releasing plant growth regulators that influence overall crop development and improve morpho-physiological features. The method of biocontrol has been utilized for over 2000 years, and it has been widely used in managing pests since the end of the eighteenth century. The types of biological control can be classified into natural, conservation, inoculative (classical) and augmentative biocontrol. Natural biocontrol has been used to reduce pests since evolution, whereas conservation biocontrol comprises human measures to stimulate and protect the performance of natural foes. The inoculative mode of biocontrol is the most extensively used type of biocontrol, in which natural enemies are dispersed into new places where the pest has been mistakenly introduced. Natural enemies are mass-produced in bio-factories and sent into the market for fast pest control, making augmented biocontrol more appealing. This type of plant protection is regarded to be both environmentally beneficial and food-safe. Moreover, gaining a better knowledge of biocontrol mechanisms via interactions between BCAs and phytopathogens may aid in the improvement and development of biocontrol systems.

15.4 Plant Growth-Promoting Rhizobacteria: Tapping for BCA

Rhizobacteria are a varied collection of bacteria that colonize the rhizosphere environment. In the root zone, they are strong microbial competitors. They either directly or indirectly influence plant development. The direct method entails PGPR's positive effects that directly boost plant growth. These systems help plants grow, but the ways they do so differ from species to species and strain to strain (Noori and Saud 2012). Through biogeochemical cycling, the PGPR nourishes the plant by converting the nutrients into the soil. They also help plant growth by facilitating the transfer of these nutrients into the plant. They promote plant growth by synthesizing plant hormones such as indole acetic acid (Ahmed et al. 2021), cytokinins (Liu et al. 2013), fixing nitrogen from the atmosphere (Malik et al. 1997), solubilizing minerals like phosphorus (Gomez-Ramirez and Uribe-Velez 2021) and generating siderophores that can solubilize and sequester iron (Sultana et al. 2021) and provide nutrients to plants (Etesami and Adl 2020) in addition to combating soil-borne plant diseases (Hamid et al. 2021). It plays a significant effect in repelling

phytopathogenic bacteria in addition to promoting plant growth (Hassan et al. 2019). *Bacillus subtilis* has a biocontrol efficiency of more than 50% on tomato plants against the plant disease caused by *Ralstonia solanacearum* in greenhouse conditions (Chen et al. 2013). *Rhizoctonia solani*, which causes damping-off in tomatoes, was controlled by a strong biocontrol agent, *Priestia endophytica* FH5 (Zhou et al. 2021). The biofortified vermicompost prepared from chosen BCAs (like *B. subtilis* and *P. fluorescens*) could be used to successfully manage the wilt diseases in tomatoes caused by *Fusarium oxysporum* (Basco et al. 2017). In a similar study, *S. pratensis* strain LMM15 (having the potential of BCA) was sprayed on tomato leaves 1 day before fungal inoculation. After BCA spraying, it was observed that the occurrence of grey mould disease was reduced by approximately 46%. Furthermore, the applied biocontrol agent significantly increased the plant stressor metabolites (proline and lipid peroxidation) as well as defence enzymes in shoot tissues (Lian et al. 2017). These strains of actinomycetes drastically reduced the pathogen proliferation while also improving tomato growth characteristics (Goudjal et al. 2014).

15.5 Mechanisms Involved in Disease Suppression (Indirect Mechanisms)

The PGPR's antagonistic characteristics against a variety of diseases expand their potential as biocontrol agents (Fatima et al. 2022). Several genera of beneficial soil bacterial strains including *Pseudomonas* (Kabdwal et al. 2019), *Bacillus* (Ni and Punja 2019), *Enterobacter* (Xue et al. 2009), *Serratia* (Youssef et al. 2016), *Klebsiella* (Gaur et al. 2017), *Azotobacter* (Alsudani 2022), etc. are familiar as biological control agents (BCAs) used in reducing tomato diseases. Many reports claim that using the PGP consortium as biological control agents has some advantages over other disease control methods, such as being an environmentally safe and non-toxic indigenous microorganism whose application is both environmentally safe and favourable to human health. Induction of systemic resistance (ISR), antibiotic production, competition, secretion of cellulolytic/hydrolytic enzyme and production of HCN and siderophores are all essential mechanisms involved in BCA's antagonistic effects (Narayanasamy 2013). Furthermore, they can help plants cope with numerous stressors such as salinity, drought, hunger, heavy metal toxicity and so on, allowing them to thrive in such environments. Even though various free-living PGPR are regarded as plant development beneficial microbes, not all the strains within the same species have the same metabolic capacity to boost plant growth. It's crucial to understand the rhizosphere microbiota's capabilities, as well as its mechanisms of action, to ensure long-term crop production (Babalola et al. 2021). The PGPR's antagonistic characteristics against a variety of diseases expand their potential as BCAs (Verma et al. 2019). Various genera of *Bacillus* including *Bacillus megaterium, B. subtilis* and *B. polymyxa*, *P. fluorescens* and *T. harzianum* were co-inoculated with *Azospirillum* sp. and *Azotobacter* sp. and were reported to effectively control the disease caused by fungal pathogens (Saad et al. 2016).

Some biocontrol agents (BCAs) involved in the management of major fungal diseases of tomatoes

S. no.	Major fungal disease	Causal agent	Biocontrol agent involved	Mechanism involved	Effectiveness	Reference
1	Fusarium wilt disease	*Fusarium oxysporum* f. sp. *lycopersici*	*Bacillus* sp.	Production of cell wall-degrading enzymes like β-1,3-glucanase, protease, chitinase, ammonia, siderophore, HCN production, bioactive volatile and non-volatile metabolites	• Reduced the disease severity up to 55%. Enhanced the growth and physiological traits of plants	Jangir et al. (2018)
2	Tomato vascular wilt	*Fusarium oxysporum*	*Bacillus velezensis* NKG-2	Volatile organic compounds (VOCs)	• Suppressed the disease severity • Increased the growth of plants	Myo et al. (2019)
3	*Botrytis cinerea*	Grey mould disease	*Bacillus subtilis* L1–21	Antifungal metabolites	• Completely inhibited (100%) the fungal growth • Increased the plant growth and improved the fruit quality	Bu et al. (2021)
4	Alternaria rot disease	*Alternaria alternata*	*Bacillus atrophaeus*	Antifungal metabolites O-anisaldehyde And lipopeptides	• Reduced the germination of the spore. • Decreased the disease severity of *Alternaria* in tomato fruit.	Chacon-Lopez et al. (2021)
5	Black scurf disease	*Rhizoctonia solani*	*Bacillus subtilis Hussain T-AMU*	Lipopeptide, biosurfactant	• Inhibited the growth of the pathogen • Inhibited the incidence and severity of disease	Hussain et al. (2021)
6	Alternaria leaf blight	*Alternaria solani*	*Bacillus velezensis* NKMV-3	Production of lipopeptide (antibiotic synthesis genes), *iturin C*, *surfactin A* and *fengycin B* and *D*	• Controlled the growth of the pathogen	Vignesh et al. (2022)
7	Fusarium wilt disease	*Fusarium oxysporum* sp. *lycopersici*	*Bacillus inaquosorum* KR2-7	Production of BGCs fengycin, surfactin and bacillomycin F, bacillaene, macrolactin, skf, subtilosin A, bacilysin and bacillibactin	• Reduced the disease severity • Increased defence-related enzyme activities	Kamali et al. (2022)
8	Early blight disease	*Alternaria alternata*	*Bacillus s*p.	Antibiosis, production of volatile organic compounds	• Decreased the disease severity	Pane and Zaccardelli (2015)

(continued)

S. no.	Major fungal disease	Causal agent	Biocontrol agent involved	Mechanism involved	Effectiveness	Reference
	Wilt disease	*Verticillium dahliae*	*Bacillus subtilis*	Various antibiotic metabolites and VOCs	• Suppressed the fungal infection, increased tomato yield	Rahman et al. (2021)
9	Foliar blight disease	*Botrytis cinerea*	*B. nakamurai, B. pseudomycoides, B. proteolyticus, B. thuringiensis, E. asburiae* and *E. cloacae*	Antifungal VOCs such as 3-methylbutan-1-ol, sulphur-containing compounds, 2-heptanone and dodecanal	• Increased the growth and yield of tomato	Chaouachi et al. (2021)
10	Anthracnose disease	*Colletotrichum capsici*	*Bacillus* sp. *strain M10*	Extracellular enzyme production	• Suppressed the fungal growth	Srikhong et al. (2018)
11	Fusarium wilt	*Fusarium oxysporum* f. sp. *lycopersici*	*Bacillus pumilus*	Loss of protoplasm in fungal cell wall	• Reduced (73%) disease incidence • Increased root (60%) and shoot (84%) of tomato	Heidarzadeh and Baghaee-Ravari (2015)
12	Early blight and anthracnose disease	*Alternaria solani*, *Colletotrichum coccodes*	*Pseudomonas fluorescens A506*	Antifungal antibiotics and VOCs	• Suppressed anthracnose on fruit and early blight on detached leaves, controlled the disease in tomato	Cuppels et al. (2013)
13	Early blight disease	*Alternaria solani*	*Pseudomonas fluorescens*	Antifungal antibiotics and VOCs	• Inhibited (45.55%) the fungal growth	Koley et al. (2015)
14	Grey mould disease	*Botrytis cinerea*	*Bacillus licheniformis*	Antifungal compounds	• Inhibited the fungal growth • Controlled the disease incidence (90%) in tomato	Lee et al. (2006)
15	Foot rot disease	*Fusarium oxysporum* f. sp. *radicis-lycopersici*	*Collimonas fungivorans*	Antifungal compounds, cell wall-degrading enzymes	• Suppressed the fungal growth	Kamilova et al. (2007)
16	Black scurf and wilt disease	*Rhizoctonia solani, fusarium oxysporum*	*Bacillus subtilis* (TD11), *Bacillus cereus* (TD15)	Secretion of antifungal metabolites Induced systemic resistance (ISR)	• Inhibited the growth of *Rhizoctonia solani* (40%) and *Fusarium oxysporum* (80%) • Controlled (50%) the disease incidence in tomato	Malik et al. (2022)
17	Root rot disease	*Sclerotium rolfsii*	*Bacillus* sp.	Production of hydrogen cyanide (HCN) and extracellular enzymes	• Suppressed the fungal growth • Reduced the disease incidence	Kumar et al. (2012)

(continued)

S. no.	Major fungal disease	Causal agent	Biocontrol agent involved	Mechanism involved	Effectiveness	Reference
18	Fusarium wilt disease	*Fusarium oxysporum*	*Pseudomonas* sp.	Siderophore production	• Suppressed the fungal growth Reduced the disease incidence	Arya et al. (2018)
19	Fusarium wilt disease	*Fusarium oxysporum*	*Streptomyces* SNL2	Synthesis of phenolate siderophore	• Reduced the prevalence of wilt disease by 88.5%	Goudjal et al. (2016)
20	Early blight of tomato	*Alternaria solani*	*Lysinibacillus fusiformis* L-2, *Bacillus subtilis* B-1 and *Achromobacter xylosoxidans* A-3	Production of siderophore and other antifungal compounds	• Suppressed the fungal growth • Controlled the disease • Improved the growth features of tomato crop	Attia et al. (2020)
21	Fusarium wilt disease	*Fusarium oxysporum*	*B. pumilus*	Siderophore production	• Suppressed the fungal growth. Reduced the disease incidence	Heidarzadeh and Baghaee-Ravari (2015)
22	Fusarium wilt	*F. oxysporum* f. sp. *lycopersici*	*Burkholderia contaminans* AY001	Systemic induced resistance (ISR), production of antimicrobial compounds, including di (2-ethylhexyl) phthalate and pyrrolo [1,2-a] pyrazine-1,4-dione, hexahydro-3-(phenylmethyl)	• Enhanced the growth of tomato plants • Increase the disease resistance • Improved the yield attributes	Heo et al. (2022)
23	Fusarium wilt	*Fusarium oxysporum*	*Pseudomonas aeruginosa*	Production of antifungal metabolites like siderophore, HCN and ammonia	• Inhibited the fugal growth (75%) • Increased the growth parameters	Parasuraman et al. (2022)
24	Early blight	*Alternaria solani*	*Pseudomonas aeruginosa*	Production of antifungal metabolites like siderophore, HCN and ammonia	• Inhibited the fugal growth (75%) Increased the growth parameters	Parasuraman et al. (2022)
25	Early blight disease	*Alternaria alternata*	*B. atrophaeus and Brevibacterium frigoritolerans*	Induced systemic resistance (ISR) Secretion of extracellular enzymes	• Suppressed the fungal growth • Reduced the disease incidence • Increased the plant growth and biomass	Chacon-Lopez et al. (2021)
26	Verticillium wilt disease	*Verticillium dahliae*	Pseudomonas stutzeri	Synthesize antifungal metabolites like HCN, siderophore and other extracellular enzymes	• Enhanced the growth characteristics (stem length, number of leaflets, leaf area and root	Essalimi et al. (2022)

(continued)

S. no.	Major fungal disease	Causal agent	Biocontrol agent involved	Mechanism involved	Effectiveness	Reference
					weight) and biochemical parameters (nitrate reductase activity, proline and chlorophyll content) of tomato.	
27	Fusarium wilt disease	*Fusarium oxysporum* f. sp. *lycopersici*	*Pseudomonas fluorescens* Pf1	Secretion of essential plant growth-regulating substances, synthesize antifungal metabolites	• Decreased the disease incidence • Improved the germination, vigour indices, growth and biomass of tomato	Johnson et al. (2022)
28	Collar rot disease	*Sclerotium rolfsii*	*Pseudomonas fluorescens* Pf1	Synthesize antifungal compounds	• Suppressed the growth of pathogen • Reduced the disease incidence	Johnson et al. (2022)

15.5.1 Production of Antibiotics

The chemical compounds which are produced by a species of microbes and are used to kill other species of microbes are known as antibiotics. To survive predation, competition and other threats, microorganisms interact with each other and produce these chemical molecules (Kaya and Koppenhöfer 1996). Bacterial antagonists decrease phytopathogens by secreting inhibitory compounds into the extracellular environment (Freitas et al. 2022). It is a highly effective and extensively researched aspect of biocontrol. The 2,4-diacetylphloroglucinol (DAPG), pyrrolnitrin, phenazine, tensin, pyoluteorin, tropolone, oomycin A, cyclic lipopeptides and hydrogen cyanide (HCN) are some of the well-known antibiotics for biological control (Saeed et al. 2021). The majority of antibiotics produced by *Bacillus* sp. are effective against plant harmful fungi *Fusarium oxysporum* and *Alternaria solani* (Zhang et al. 2020). *B. subtilis* has been reported as a strong biocontrol agent, and it inhibited the fungal growth by producing a wide range of antibiotics, including bacilysin, bacillomycin, fengycin, zwittermicin and difficidin (Kim et al. 2010; Telang 2010). *Bacillus* strain TNAM5 was discovered to be effective in suppressing FOL by producing diffusible and volatile organic (VO) antifungal compounds, ammonia and HCN (Prashar et al. 2013; Kumari et al. 2021). Phichai (2014) discovered that *B. subtilis* could generate antifungal metabolites (bacitracin, subtilin, bacillin and bacillomycin) that inhibited *Alternaria* spp. However, because antibiotic resistance is a problem, having too much reliance on antibiotic-producing bacteria as a BCA could be a disadvantage.

15.5.2 Competition

Effective colonization and increased competition are important factors in biocontrol; thus, the biological control agents (BCAs) should be able to tolerate and multiply in a natural environment (Pal and Gardener 2006). Microorganisms must efficiently compete for accessibility of nutrition and niche to establish themselves in the rhizosphere (Lugtenberg 2015). Biocontrol mainly relies heavily on competition between pathogenic and non-pathogenic microorganisms (Daguerre et al. 2014). Plant-associated bacteria are known to provide more protection to the plant by accelerating rhizosphere penetration than pathogens. Biocontrol chemicals exhaust the few available substrates, rendering them inaccessible to infections. At the same time, they produce metabolic chemicals that are harmful to infections (TariqJaveed et al. 2021). As a result, an effective BCA must be able to safeguard the deeper root sections by colonizing well and suppressing the pathogen at the growing tips. In rhizobacterial populations, species of *Pseudomonads* have been identified as very efficient competitors for root exudates (Kuiper et al. 2002). Antimicrobial chemicals are found in some root exudates, providing a favourable ecological niche for PGPR, which may then detoxify them (Badri and Vivanco 2009). This means that PGPR competence is heavily contingent on their capacity to exploit specific environmental conditions or adapt to changing conditions (Amaya-Gómez et al. 2020). Kuiper et al. (2001) found that root colonizing *Pseudomonads*' competitive colonization ability was strongly influenced by their enhanced intake of putrescine, a tomato root exudate.

15.5.3 Induced Systemic Resistance (ISR)

Beneficial bacteria living in the rhizosphere region interact with the host plant to help it fight against different phytopathogens (Kumar and Jagadeesh 2016). The enhanced physiological state of defence elicited by broad-spectrum biotic and abiotic stimuli is known as induced resistance. The induction of systemic resistance is characterized by (1) induced systemic resistance (ISR) and (2) systemic acquired resistance (SAR). ISR occurs when plants' intrinsic defence mechanisms are triggered in response to biotic threats (Pineda et al. 2013). Plants in SAR develop greater resistance to uninfected plant sections while dealing with a wide range of diseases (Pieterse et al. 2001). ISR is primarily mediated by the jasmonate or ethylene-sensitive pathway, whereas SAR is mediated by salicylic acid (SA) (Mandal and Ray 2011). Rhizobacteria invading plant roots can induce resistance to a wide range of illnesses. It has been reported that some strains of *Bacillus* (Choudhary and Johri 2009), *Pseudomonas* (Jisha et al. 2019), *Burkholderia* (Ahmad et al. 2022) and *Serratia* (Singh and Jha 2016) cause ISR in response to various infections. By evoking such induced resistance, BCAs display the inhibition of diseases caused by fungal, bacterial, viral and, in some cases, insects and nematodes. The genus

Bacillus is an excellent BCA and the most widely used biopesticide for controlling plant diseases (Miljaković et al. 2020). Some species of *Pseudomonas* also play an imperative role in ISR. For example, *P. fluorescens* was found to provide ISR to tomato plants against the diseases causing fungal pathogens (such as *P. infestans* and *F. oxysporum*) (Santoyo et al. 2012). The use of potential *Pseudomonas fluorescens* VSMKU3054, an efficient BCA, gave clear evidence of ISR-mediated biocontrol of tomato grey mould disease (Suresh et al. 2022). In a study, Chunyu et al. (2017) evaluated the efficiency of PGPR strain *B. amyloliquefaciens* SQRT3 to prevent the tomato crop against pathogens by inducing a systemic resistance mechanism. In a crop-based study, it was observed that by activating the various defence enzymes (PPO, POX, GLU, CHI and PAL), and induction of systemic resistance, antagonistic PGPR strains *S. marcescens*, *Streptomyces cereus* and *Bacillus cereus* increased the resistance of wilt disease in tomato (Ferraz et al. 2015). Reduction in the severity of disease and enhanced upregulation of defensive enzymes triggered by the combined inoculation of *Bacillus atrophaeus*, *B. subtilis*, and *Burkholderia cepacia* exhibited a direct biocontrol and ISR mode of action for suppression of vascular disease in tomato crops (Shanmugam and Kanoujia 2011). Similarly, by activating and upsurging the activities of peroxidase, PPO and phenylalanine ammonia-lyase, the biocontrol agent *Pseudomonas putida* stimulated the systemic responses in tomatoes against early blight disease (Ahmed et al. 2011). In another study, antagonistic bacteria *B. subtilis* OTPB1 suppressed the early blight of tomato (caused by *A. solani*) due to increased systemic response. The bacterial-inoculated tomato seedlings had significantly higher levels of defence-related enzymes (peroxidase, PO; polyphenol oxidase, PPO; and superoxide dismutase, SOD) than uninoculated control seedlings (Chowdappa et al. 2013). In the external environment, bacteria produced exopolysaccharides (EPS). The EPS aids in drought resistance, stress resistance and phytopathogen defence (Fig. 15.2). In various crops, the significant function of bacterial EPS as an elicitor for the generation of systemic resistance has already been established. The exopolysaccharide synthesized by *Bacillus* sp. EPS has been shown to effectively minimize the occurrence of wilt disease in tomatoes caused by *F. oxysporum* (Thenmozhi and Dinakar 2014).

15.5.4 Production of Cellulolytic/Cell Wall-Degrading Enzymes

Cellulolytic/cell wall-degrading enzymes secreted and excreted by certain bacteria can disrupt pathogen development and/or activity. The secretion of these enzymes is a cost-effective way to stop pathogen proliferation through the lysis of pathogenic cell walls. Several bacterial genera produce and release lytic enzymes that are capable of hydrolyzing a wide range of polymeric materials (proteins chitin, cellulose, hemicellulose and DNA). This technique allows the phytopathogen to be directly parasitized. The release and expression of these enzymes by various

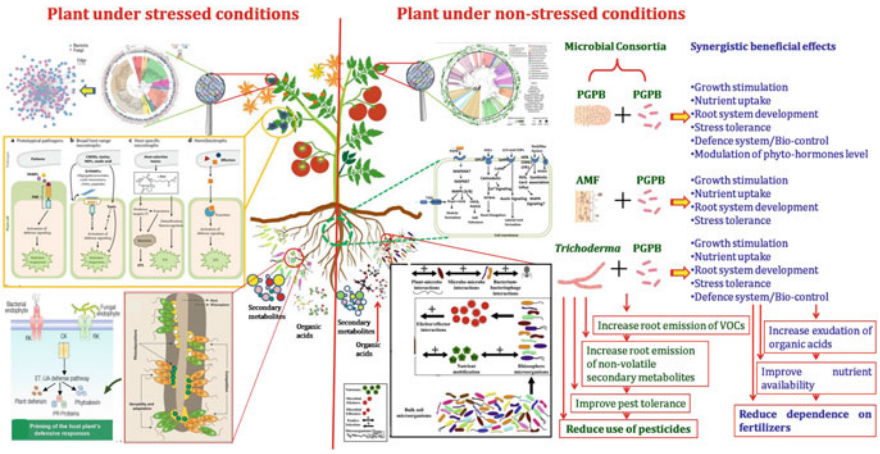

Fig. 15.2 An overview of microbe-mediated modulation of physio-biochemical and molecular mechanisms of biotic stress tolerance in plants. Photograph clearly depicts that plant secreted chemically different biochemicals/secondary metabolites which regulate recruitment of microbial strains under stressed and non-stressed conditions. These microorganisms further regulate the defence mechanisms in plants. Figure showed the multi-trophic interactions in the rhizosphere which define the active rhizosphere effects and how the microbiome served as the first line of defence and maintain defence level and plant growth under stressed conditions

microorganisms can occasionally directly decrease plant pathogen activity. Polymeric components (such as cellulose, hemicelluloses, chitin, proteins and DNA) found in fungal cell walls can be digested by lytic enzymes including cellulase, chitinase and protease, among others. *Pseudomonas* sp., among soil bacteria, has gotten increased attention since it can produce a variety of cell wall disintegrating enzymes, including chitinase, protease/elastase and 1,3-glucanase (Wang et al. 2021).

Chitinases and 1,3-glucanases are two of the most important hydrolytic enzymes involved in the breakdown of fungal cell walls. Mycolytic enzymes produced by rhizobia, particularly chitinases, are known to hydrolyze chitin, a significant component of fungal cell walls (Chavan and Deshpande 2013). Production of extracellular cellulolytic enzymes (cellulase, chitinases, protease and β-1,3-glucanase) by *Bacillus* sp. recovered from *Solanum lycopersicum* rhizosphere inhibited the fungal pathogens and proved to be a viable bioresource for the agricultural business (Kumar et al. 2012). In a study, Ramyabharathi and Raguchander (2014) found that antagonistic PGPR strain *B. subtilis* EPCO16 produced HCN and volatile organic compounds (VOCs) together with proteolytic and extracellular enzymes chitinase and β-1,3-glucanase. This strain retarded the growth of *F. oxysporum* f. sp. *lycopersici* causing fusarium wilt disease in tomatoes and proved to be a successful biocontrol agent in managing the fungal diseases. Further, strains of *Pseudomonas* (*P. fluorescences* NRC1 and *P. fluorescens* NRC3) inhibited the phytopathogens

Phytophthora capsica and *R. solani* (the causal agents of root rot disease in tomato) by secreting different cell wall-degrading enzymes and synthesizing antifungal metabolites (Moataza 2006). Under greenhouse circumstances, the strain *Bacillus* sp., which could synthesize different types of extracellular enzymes, reduced the disease incidence of wilt disease (caused by *F. oxysporum* f. sp. *lycopersici*) by 36% in tomato plants, suggesting that it could be an effective biocontrol agent for tomato wilt (Jangir et al. 2018). *P. fluorescens* has also been identified as a potent BCA against the fungal pathogen, stimulating important enzymes such as peroxidase (POX), polyphenol oxidase (PPO) and superoxide dismutase (SOD), as well as 1,3-glucanases (Dorjey et al. 2017). Apart from the extracellular enzymes, the inhibition of phytopathogens could also be aided by other microbial by-products. For instance, at picomolar concentrations, hydrogen cyanide (HCN) efficiently disables the cytochrome oxidase pathway and is extremely hazardous to all aerobic microbes. Hydrolytic enzymes produced by some identified strains of *Bacillus* have been described for the biocontrol of the phytopathogen *F. oxysporum* causing wilt disease in tomatoes (Jadhav and Sayyed 2016). The efficiency of different rhizobacterial strains is dependent on the host plant and soil factors, in addition to the biocontrol methods listed above. Furthermore, their innate capacities and rhizosphere competence play a significant role in the manifestation of their biocontrol features (Weert and Bloemberg 2007). In a field trial, Shanmugam and Kanoujia (2011) evaluated the biocontrol potential of antagonistic PGPR strains, *B. cepacia* and *B. subtilis* against *Fusarium oxysporum* f. sp. *lycopersici* causing wilt disease in tomato. They found that the bacterial strains reduced the incidence of disease by secreting extracellular enzymes. Further, they claimed that leaf and root sample analyses had the highest induction of chitinase and 1,3-glucanase.

15.5.5 Production of Hydrogen Cyanide (HCN)

Hydrogen cyanide (HCN) is an antibacterial and antifungal molecule well known for its ability to prevent disease (Haas and Keel 2003). HCN is likely to disrupt the electron transport chain (ETS) and the cell's energy source, resulting in cell death. Cytochrome oxidase is also known to be inhibited by the action of HCN (Cooper and Brown 2008). This detrimental characteristic gives PGPR a competitive advantage over fungal pathogens, and it can be used in plant disease biocontrol (Shaikh and Sayyed 2015). Among rhizosphere microbes, *Pseudomonas* and *Bacillus* spp., for example, produce cyanogenic compound (HCN) as a secondary metabolite (Sivasakthi et al. 2014). Bacterial cyanogenesis has also been described in the species of *Burkholderia* (Shahid and Khan 2018), *Achromobacter* (Oves et al. 2019), *Bacillus* (Hassan et al. 2010), and *Azotobacter* (Shahid et al. 2019). However, fluorescent *Pseudomonas* is the most common HCN produce (Keerthana et al. 2022). The majority of research suggests that HCN-producing bacteria are active

against fungal infections and, thus, serve as a biocontrol agent (Sehrawat et al. 2022; Haddoudi et al. 2021; Hernández-León et al. 2015; Pandya and Saraf 2014). In a study, Kumar et al. (2012) found that HCN-synthesizing *Bacillus* sp. suppressed the disease-causing pathogen *Sclerotium rolfsii.* Similarly, PGPR strain *Beijerinckia fluminensis* suppressed the growth of major fungal phytopathogens while assessed under in vitro conditions (Al-Shwaiman et al. 2022).

15.5.6 Production of Siderophore

Iron (Fe) is a necessary micronutrient for all living things and is found in the soil mostly in the form of ferric ion (Fe3+), which is only sparingly soluble. As a result, plants are unable to absorb it. Plants can utilize the microorganism-produced siderophores to absorb iron (Radzki et al. 2013). Beneficial soil microbial diversity synthesizes siderophores (low-molecular-weight Fe-chelating molecules) having high affinity and selectivity for binding and forming a complex Fe complex (III) (Colombo et al. 2014). It functions as a ligand, allowing iron to be sequestered and transported into the cell. In recent times, this characteristic has gotten a lot of attention, and the in vitro assessment of siderophore synthesized by identified PGPR has been reported (Parray et al. 2016). Rather than Gram-positive PGPR transport systems, siderophore-mediated iron transfer mechanisms are best researched in Gram-negative PGPR transport systems. Among different varieties of 500 siderophores, only 270 have been structurally studied. Siderophores have been proposed as an environmentally acceptable alternative to insecticides.

Since siderophores have a higher affinity for iron than fungal pathogens, they have a competitive edge when it comes to effectively reducing the proliferation of phytopathogen (Govindasamy et al. 2008). When there is a lack of iron, fungal pathogens become unable to reproduce and are pushed out of their biological habitat. As a result, siderophore synthesis is an attractive feature of PGPR as a biocontrol agent. Siderophores produced by *Pseudomonas* are reported to have a stronger affinity than other bacterial siderophores (Patel et al. 2018). Several studies have been published on the biocontrol efficacy of PGPR that produce siderophores. Siderophore-producing strains of *B. subtilis* MF497446 and *P. koreensis* MG209738 exhibited strong effectiveness against the fungal pathogens, reduced the disease incidence and improved the crop growth (Ghazy and El-Nahrawy 2021). Under field circumstances, Attia et al. (2020) found that siderophore-producing PGPR strains, viz. *Lysinibacillus fusiformis* L-2, *Bacillus subtilis* B-1 and *Achromobacter xylosoxidans* A-3 suppressed the early blight disease of tomato caused by *Alternaria solani*. Similarly, the indigenously isolated *Pseudomonas* strain reduced the incidence of fusarium wilt disease in tomato crops by producing siderophore (Arya et al. 2018). In addition, siderophore-synthesizing *Streptomyces* SNL2 reduced the prevalence of wilt disease by 88.5% (Goudjal et al. 2016).

15.6 Future Perspective

Managing sustainable natural reserves can help ensure food security for the world's growing population. PGPB have been shown to play an important function in agricultural management in several studies. Though, there is still a knowledge vacuum underpinning microbe-plant symbiosis under various stress circumstances, especially pathogen stress. Understanding the rhizosphere ecology that governs pathogen and antagonist dispersion could help improve biocontrol efficiency against plant-based pathogens. Future studies will necessitate intense rhizosphere engineering based on the successful discovery and separation of novel metabolites, which could establish a unique environment for plant-microbe interactions. Instead, exploring and applying the combined inoculation over a single strain, on the other hand, could be an efficient means for the suppression of fungal diseases. Furthermore, genetic alterations to improve biocontrol values could be a new study area for managing plant diseases. Transforming the strains with higher quantities of antifungal and growth-promoting essential metabolites, for example, maybe an excellent option. The use of cutting-edge tools to explore microbiological, biochemical and molecular interactions between plants and interacting microorganisms may provide in-depth knowledge for a better understanding of interactions between plants and microbes. To summarize, the forthcoming challenge will be to improve the effectiveness and long-term resilience of biological control in the field. If this issue is handled, biocontrol efficacy could be increased by leveraging expertise to design better screening processes, formulations and application procedures, as well as innovative integrated disease management strategies.

15.7 Conclusions

Tomato is a highly nutritious vegetable crop grown all over the world and ranks second only to potatoes in terms of consumption. Tomato seed-borne infections, on the other hand, are a major source of concern in the seed industry because they have a negative impact on seedling germination and vigour, resulting in a significant reduction in yield and product quality. The current research shows that PGPR not only have different biological promotional effects on tomato plant development parameters but also operate as biocontrol agents (BCAs) to protect the plant from diseases. It should become increasingly more effective and cost-effective to replace fungicides with a biological pesticide. Before commercialization, molecular analysis can help stabilize the effects of PGPR in biological control and determine potential risks. To use PGPR effectively for disease reduction or crop protection in the future, a logical selection of organisms will be required, as well as technical improvements in upscaling and formulation procedures will be needed. The PGP genomic products may be enhanced through the genetic engineering of PGPR. As a result, a single bacterial strain or a consortium with varying features will reduce pathogen attacks

while also promoting plant growth, which will stimulate producers. Although investigations should focus on the relative contributions of each mechanism responsible for PGPR strains' significant biocontrol activity, it has become obvious that they use numerous pathways to operate as an effective biocontrol agent. An in-depth study of microbial interactions with plants, as well as exploitation of microbial ecology in the soil and rhizosphere, will aid in revealing the many dimensions of disease suppression by these biocontrol agents. Furthermore, for maximal commercial utilization of these strains, cautiously conducted skilful field trials of tomato plants inoculated with BCAs are required. Finally, the success of the microbial inoculant industry, particularly those that use PGPRs, will be determined by factors such as product marketing and substantial research. Furthermore, to incorporate PGPR strains into the agriculture industry, they will need to be optimized for improved fermentation and formulation procedures.

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Chapter 16
Prior Weakening as a Tool to Control Soilborne Plant Pathogens and Associated Disease Pressure

Ritu Mawar and Satish Lodha

Abstract Soilborne plant pathogens cause heavy losses in commercially important crops grown worldwide. Several management strategies have been advocated to minimize losses due to these pathogens based on several research findings. Organic amendments alone or combined with biocontrol agents, soil solarization, soil steaming and fumigation have been suggested as important management approaches used in different parts of the world. Unfortunately, the use of a single management strategy has not been found effective in completely eradicating propagules of soilborne plant pathogens at soil depths where propagules of these pathogens survive. In the last two decades, studies were conducted world-over to improve pathogen control by exposing resting structures to sublethal doses of heating. These doses incidentally eliminate only a part of inoculum, which may also affect the surviving and possibly weakened propagules of soilborne pathogens. If any effective management strategy is subsequently applied after a requisite weakening is achieved, it may require less dose, duration and money for improving the pathogen control. The effects of different intensities of sublethal doses were studied in many parts of the world under field and laboratory conditions on different pathogens. These have demonstrated that weakening effect achieved by sublethal doses of any stress reduced the survival of pathogenic propagules and caused a pronounced effect on remaining viable, but weakened propagules, which in turn reduced disease incidence on crops. Several mechanisms have been studied to investigate the cause of the weakening effect. These include direct heat, heat shock proteins, toxic volatiles, cracking in spores, dehydration, loss of energy, delayed germination and mortality. This caused increased microbial antagonism, which was considered as the most important mechanism, which operated singly or in a sequence for further exerting an irreparable effect on resting structures of soilborne plant pathogens. This tool provides a novel approach for improving pathogen control in those regions where adequate sublethal heating or any other method of stress is possible due to hostile climatic conditions. Studies conducted on the effectiveness of prior

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weakening on different soilborne plant pathogens and the mechanisms involved are reviewed in this paper.

Keywords Sublethal heating · *Brassica* amendments · *Macrophomina phaseolina* · *Fusarium* · Heat shock proteins · Microbial antagonism · Melanin

Abbreviations

AO	Acridine orange
BCAs	Biological control agents
CS_2	Carbon disulphide
FDA	Fluorescein diacetate
Foc	*Fusarium oxysporum* f. *sp. cumini*
Fom	*Fusarium oxysporum* f. sp. *melonis*
Fon	*Fusarium oxysporum* f. sp. *niveum*
GSLs	Glucosinolates
HSPs	Heat shock proteins
MB	Methyl bromide
MC	Mustard oil cake
MR	Mustard residue
PCD	Programmed cell death
PI	Propidium iodide
PS	Phosphatidylserine
SEM	Scanning electron microscope
SI	Summer irrigation
SLH	Sublethal heating
V	*Verbesina encelioides*

16.1 Introduction

Soilborne plant pathogens including different genera of fungi, nematodes, bacteria, parasitic plants, arthropods and other organisms often cause enormous losses in major crops by way of affecting yield and quality. In intensive agriculture, economically important crops are planted sequentially on the same piece of land resulting in a rapid build-up of pest populations in the soil. Inoculum densities in the soil are directly proportional to disease intensity in the field (Lodha 1995). These growing conditions, therefore, require the use of effective management strategies through which inoculum density of soil pests and disease intensity can be restricted below the economic threshold level to get the profitable harvest. In addition to being effective, many other factors are considered. These include the presence of adequate inoculum

density and soil type. Besides these, social, economic and legal implications are also to be considered for the use of sequences of management approach.

16.1.1 Management Strategies

The main thrust of soil disinfestations involves eradicating soilborne pests in the agricultural fields, before planting, uniformly to the desired depth, with minimal disturbance of the biological equilibrium and without much affecting physicochemical properties. Several management approaches have been suggested from various parts of the world and were found successful in reducing inoculum density and induced diseases.

Soil fumigation affects the survival of soilborne plant pathogens drastically but also simultaneously induces a biological vacuum, which is considered environmentally hazardous besides deteriorating soil health. A number of fumigants are in use world-over. In most of the countries, methyl bromide, a toxic fumigant, was used for soil disinfestations for a long time. It was found to be an ozone-depleting pesticide and is extremely toxic, kills most living organisms exposed to it. In 1987, governments of different countries agreed on the Montreal Protocol on substances that deplete the ozone layer to protect human health and environment against depletion of the stratospheric ozone layer resulting from human activities. An alternative solution to the problem could be the application of reduced doses of fumigants mostly in combination with non-chemical methods such as soil solarization, sublethal heating, *Brassica* amendments and microbial antagonism to ensure optimum control.

Organic amendments are incorporated in the soil to improve soil fertility, moisture-holding capacity, aeration, porosity and tilth of the soil. Besides these attributes, the organic amendment enhances microbial population including antagonists in the soil with increased microbial activity to reduce surviving pathogenic propagules of soilborne plant pathogens. Studies have shown that their use has controlled or reduced pathogens and induced diseases. Farmers of every region are traditionally using these to restore the fertility of the soil as well as to check pest and disease incidence on cultivated crops. Since ancient times, attempts were in practice to harness solar energy for pest and disease control. Farmers of peninsular India were exposing field soil to control soilborne plant pathogens. The practice of deep summer ploughing to control cereal cyst nematode *Heterodera avenae* was suggested in wheat and barley (Mathur et al. 1987). Increased yield of wheat was found to be directly related to the increased number of summer ploughings. Similarly, summer ploughing has been recommended for partial control of the cumin wilt pathogen (Champawat and Pathak 1990). This method of control brings soil from lower depth, which is exposed to a long duration of summer heat. Soil solarization or polyethylene mulching to control soilborne pests was first demonstrated in Israel (Katan et al. 1976). Subsequently, this method has gained considerable importance in the whole world in these decades as a regular practice to eliminate propagules of soilborne pests.

Biofumigation is a term coined to describe the suppression of soilborne pests and pathogens by *Brassica* rotation or green manure crops. Glucosinolates (GSLs) are present in various quantities in tissues of many dicotyledonous plants. Enzymatic hydrolysis of GSLs in the presence of enzyme myrosinase results in the production of various compounds, some of these possess antimicrobial activity. Cruciferae is one such plant family with a high content of GSLs in their tissues and is also characterized by a high content of other sulphur-containing compounds. Antifungal volatile compounds such as allyl isothiocyanates have been found in leaf extracts of various *Brassicaceae* species. The generation of biotoxic compounds from decomposing *Brassica* amendments increases with increased temperature. In various countries, this method has been in use alone or coupled with solarization for improving control of pathogens and is termed as bio-solarization.

Considerable interest has been generated for the use of biological control agents (BCAs) reflecting increasing environmental concern over the prevailing use of pesticides. This method has further gained importance due to occurrence of fungicide resistance in some pathogens. In the case of many soilborne plant pathogens, reliable chemical means of control or resistant genotypes in the host are also not available. Among the beneficial biotic agents in soil that may be influenced by soil disinfestation are those that promote plant growth and health. Soil solarization often reduces mesophilic microorganisms, but mesophilic and thermophilic organisms continue to survive. This way these may contribute to suppressing resting and germinating structures of soilborne plant pathogens. Many fungi, bacteria and actinomycetes have been discovered and are in practice as a component of integrated management of plant pathogens.

16.1.2 Prior Weakening

The original concept of weakening of pathogenic propagules as a prerequisite approach to improve pathogen control was initially postulated by Davey and Leach (1941). These workers studied the effect of fumigant formalin and found increased colonization of sclerotia of *Sclerotium rolfsii* by species of *Trichoderma* and concluded that bioagent was able to colonize the sclerotia of the pathogen only after a required weakening effect was exerted by the fumigant. Subsequently, though the tested doses of carbon disulphide (CS_2) were not having a toxic effect against *Armillaria mellea,* the control was assigned to the weakening effect, which allowed parasitization by *Trichoderma viride* to kill the pathogenic propagules. Therefore, it was concluded that moderate soil sterilization was superior as it could kill most of the microorganisms present in the soil. Garrett (1956) suggested that the target fumigant could cause weakening in resistance of pathogenic propagules, which were finally prone to the attack of *T. viride*. Similarly, the survival of *A. mellea* was weakened by a sublethal dose of heating resulting in the indirect killing of the pathogen by *Trichoderma* spp., which was a predominant colonizer of the weakened roots (Munnecke et al. 1976). Concluding this concept, Baker and Cook (1974)

suggested that if any strategy just makes the pathogen weak enough, then it can become more vulnerable to be an effective tool for use as a prerequisite for another management strategy. This elucidates exposure of field to summer heat or deep summer ploughing by farmers of the Indian subcontinent to have weakening effect on pathogenic propagules.

This hypothesis, though postulated many years back, was not tested against many other soilborne plant pathogens in improving control and, thus, remained ignored world-over, perhaps due to suitability and effectivity of other management approaches like chemical fumigants. Only in recent decades, this aspect began to gain renewed interest, when adequate control was not achieved by the application of other management strategies or when pathogenic propagules survived to undetected levels at the desired soil depth. The studies were again concentrated on its combination with other management approaches like biological control, biofumigation, organic amendments and soil solarization particularly against relatively heat-tolerant or important pathogens. The main interest developed because the weakened propagules became more vulnerable to enhanced colonization by existing BCAs and other microorganisms in the soil, which could improve the mortality of weak pathogenic propagules of soilborne plant pathogens due to their increased vulnerability.

It is expected that a requisite weakening achieved by sublethal doses of heat or any other killing agent may improve the use of optimum or often reduced doses of other strategies, thereby resulting in improved reduction in pathogenic propagules even for sclerotia of a heat-tolerant pathogen like *Macrophomina phaseolina* (Mawar and Lodha 2009). The fate of weakened propagules is mainly dependent on the time, duration of exposure and intensity of sublethal doses, which can render these propagules vulnerable to such a level that these are rapidly colonized and antagonized by BCAs. The sequence of the application when two or more agents (management approaches) are applied at sublethal doses should also be taken into consideration (Eshel et al. 2000). It needs to be carefully assessed the effect of weakening to the desired level compared to their non-treated counterparts. The effect of weakened propagules has been studied and successfully demonstrated with a number of soilborne plant pathogens in different countries mainly in a sequence with soil solarization, organic amendments or BCAs (Table 16.1). However, some other aspects also caused weakening including the relation of energy stress like fungistasis, propagule debilitation, recoupment, autolysis, re-germination, persistent structure formation and hyphal extension (Lookwood 1990). Factors that can increase energy stress will decrease disease incidence due to increased loss of energy reserves, ammonia, herbicides, temperature and moisture stresses and germination lysis. In the forthcoming sections, such examples are dealt with for studied individual pathogens. The death rate of a population depends both on the doses and exposure time, causing various degrees of reduction in viability of pathogenic propagules or pronounced weakening. Studies have demonstrated that the weakening of propagules of various pathogens following sublethal treatments resulted in reduced survivability and pathogenicity and proportionate reduction in disease incidence in the field.

Table 16.1 Weakening effect of sublethal heating tested on pathogens

Sl. no.	Pathogens	References
1.	*Fusarium oxysporum.* f. sp. *cumini*	Israel et al. (2011)
2.	*Fusarium oxysporum.* f. sp. *niveum*	Freeman and Katan (1988)
3.	*Fusarium oxysporum* f. sp. *ciceri*	Arora et al. (1996)
4.	*Fusarium oxysporum* f sp. *basilici*	Eshel et al. (2000)
5.	*Macrophomina phaseolina*	Lodha et al. (2003), Mawar and Lodha (2009)
6.	*Sclerotium rolfsii*	Lifshitz et al. (1983), Stapleton and Duncan (1998), Eshel et al. (2000)
7.	*Meloidogyne incognita*	Stapleton and Duncan (1998)
8.	*Pythium ultimum*	Stapleton and Duncan (1998)
9.	*Verticillium dahliae*	Tjamos and Fravel (1995)
10.	*Ralstonia solanacearum*	Yamfang et al. (2013)
11.	*Clostridium perfringens*	Skanou et al. (2018)

16.2 Direct Field Exposure to Sublethal Heating (SLH)

16.2.1 M. phaseolina

M. phaseolina (Tassi) Goid. causes various types of symptoms on over 500 species of plants (Farr and Rossman 2014). Valuable crops are host of this ubiquitous pathogen in most of the tropical and subtropical countries. Luna et al. (2017) reviewed the present status of charcoal rot in soybean and reported yield loss of more than 1.9–2.0 million tonnes during 2003–2012. In the Indian arid and semi-arid regions, certain specific bio-ecological factors contribute to the occurrence of *M. phaseolina* induced diseases on crops to the extent that 80% incidence of charcoal rot was reported on cowpea (Lodha et al. 1986).

During different studies on soil solarization, a residual inoculum of *M. phaseolina* was left behind at lower soil depth, which in turn recolonized the host debris and increased its inoculum to cause the disease (Mihail and Alcorn 1984). Efforts were made to integrate other management strategies to augment the effectiveness of soil solarization. Amending soil with *Brassica* residues has been found to improve a significant reduction in viable counts of *M. phaseolina* (Lodha et al. 1997: Israel et al. 2005). Combining SLH before the application of other management strategies caused a discernible weakening in survived resting structures of the pathogen in the heated soil. These weakened propagules are rendered more vulnerable when another management approach was subsequently used, which also promoted microbial antagonism.

In a typical hot arid climate of India, the effect of the prior weakening of *M. phaseolina* sclerotia achieved by dry heated soil was used as a tool to make

sclerotia vulnerable for subsequent management strategy. This study aimed to work out the appropriate time for applying a less expensive concentration of *Brassica* residues to improve the control of *M. phaseolina* (Lodha et al. 2003). Effect of different intensities of heat levels was studied where dry sclerotia were exposed to dry heated soil for 0, 30 and 60 days during hot summer days in an open field, while for 30 days sclerotia-infested soil was kept in shade under the dense canopy of trees (moderate heat levels). The initial population of *M. phaseolina* was estimated on a specific medium. The soil in pits of 0 days was amended with *Brassica* amendments as residues of Indian mustard (*Brassica juncea* (L) Czern and Coss) and its oil-cake (MR 0.18 and MC 0. 36%w/w). The equal amount of non-amended soil filled in separate pits served as the control. Irrigation was applied on the following day. In the second and third sets, soil continued to be exposed to dry summer heat. Effect of SLH under open field was observed where 3.3% reduction in viable propagules of the pathogen was estimated in the samples analysed on April 16 at 0–30 cm soil depth, which increased to 6.9% and 11.2% in the subsequent samples from soil retrieved on May 16 and June 16, respectively (Lodha et al. 1997). A significantly higher reduction was estimated in the samples retrieved on July 1, where the reduction was greater at 0–15 compared to 15–30 cm soil depth. In non-amended dry pits, reduction in viable sclerotia of *M. phaseolina* was estimated to be 31% at 0–30 cm soil depth. However, a small increment in soil temperature improved this reduction by 34%. Further, SLH for 60 days did not improve the reduction in the viable counts of the pathogen. In general, significant improvement in viable counts of the pathogen was conspicuous at heat level 2 and 3 (30 and 60 days) compared to heat level 1 (0 day). These studies demonstrated that amending soil with *Brassica* amendments coupled with irrigation after exposing sclerotia to dry heated soil for a long duration resulted in a pronounced reduction in the viable counts of pathogen without leaving any phytotoxic effect on succeeding crop raised in the rainy season.

Subsequently, utilizing dry summer heat effect of variable levels of SLH on the sclerotial population of *M. phaseolina* was ascertained (Mawar and Lodha 2009). Laboratory-produced inoculum was mixed in soil and kept for stabilization for 7–8 days. Infested soil was exposed to four levels of heat: (1) dry heating for 30 days (A, May), (2) 60 days (B, June), (3) 0 day (C, infested soil kept in the laboratory) and (4) 60 days (D, shaded conditions). On March 31, all the pits of A, B and D were filled with 9 kg of pathogen infested soil, while the soil of set C was kept separately at room temperature (30–35 °C). The soil in pit A was amended on April 30 with MR (0.18%w/w) and MC (0.04%w/w). Pits filled with the only pathogen infested soil with irrigation and dry infested soil served as the corresponding control. On May 1, irrigation was applied. Similarly, infested soil from set B and D was withdrawn from pits, and same day infested soil of set C was brought from laboratory to the field. Infested soil of each set was amended with an equal quantity of *Brassica* amendments except that of the wet control. Irrigation was applied on June 1 in all the sets. During the experimental seasons, temperatures ranged between 38 and 45 °C in irrigated plots, while in dry soil these ranged between 42 and 47 °C at 0–30 cm soil depth.

The combined effect of dry heating reduced pathogenic propagules exposed to different heat levels at 0–30 cm soil depth by 9.8–18.4% and 6.9–15.1% in 1998 and 1999, respectively. The reduction in viable counts of *M. phaseolina* was significantly higher at 16–30 cm soil depth compared to 0–15 cm soil depth except at heat level C, but in the year 1999 reduction in population density was invariably higher at upper soil depth. Irrigation in dry but heated soil caused 37.5–39.9% reduction at the heat level A, where the soil was exposed to 30 days of dry heating. This reduction improved to 39.1–42.3% when the dry exposure was given for 60 days at both the soil depths in 1998. When *Brassica* amendments were combined, a dramatic increase in reduction of *M. phaseolina* counts was estimated that was significantly greater in MR + MC + SI and at a lower soil depth compared to MR + SI and at higher depth in 1998. However, in the second season, reduction in viable counts of *M. phaseolina* was higher at upper soil depth. Exposure of infested soil for 60 days augmented the reduction by 69.5–81.3% (MR) and 83.6–90.4% (MR + MC + SI) at 0–15 and 16–30 cm soil depth and was significantly better than that achieved at heat level A.

In the Sahelian region, effects of soil solarization alone or in combination with organic amendments were studied on the survival of *M. phaseolina* and charcoal rot of cowpea (Ndiaye et al. 2007). Amendments alone caused 16% or 35% reduction in viable propagules. However, combining amendments followed by solarization improved reduction by 46–66%. Combining two management approaches in a sequence reduced the incidence of charcoal rot by 78–96%. The pronounced effect of these strategies on the disease incidence compared to the inoculum density was elucidated by a weakening effect caused on survived, but weakened propagules. This study also explains that the sequence of use of management strategies is often important in improving pathogen control.

When compost was prepared from *M. phaseolina*-infected guar [*Cyamopsis tetragonoloba* (L.) Taub.] residues, it was observed that sclerotia of the pathogen were not completely inactivated during the heating phase at 30 cm depth in compost pit at 48–51 °C temperature (Lodha et al. 2002). However, when matured composts were exposed to a summer heat for 7 days, a 53–61% reduction in counts of *M. phaseolina* occurred in exposed composts. This reduction was attributed to the combined effect of fungi toxic compounds and increased microbial antagonism on weakened propagules of *M. phaseolina*. The release of such volatiles was shown to reduce the density of viable propagules in another study (Sharma et al. 1994).

16.2.2 Fusarium oxysporum *f. sp.* cumini

Cumin (*Cuminum cyminum* L.) is believed to be a native from the east Mediterranean to East India. India is the largest producer and consumer of cumin seed in the world. Losses caused by wilt incited by *Fusarium oxysporum* f. sp. *cumini* (*Foc*) makes the cultivation of this crop often unprofitable. Low organic matter, low microbial population and poor moisture retention capacity along with repeated

cultivation of susceptible genotypes have made the sandy soils of the region conducive to wilt pathogen. Resting structures of the *Foc*, the chlamydospores, survive in the soil for more than 10 years. In the absence of resistant sources against *Foc*, to reduce the population of pathogens below the economic threshold level, integration of cultural, chemical and biological control measures is the only way to manage this disease.

Soil solarization was found to be an effective technique in reducing soil population density of *Foc* and induced wilt as ample solar irradiations and high soil temperatures are available during crop-free period in a hot arid region (Lodha 1995). The use of organic amendments like MC and MR has shown excellent results in managing the wilt of cumin (Sharma et al. 1994; Mawar and Lodha 2002: Israel et al. 2005). These amendments increased microbial population and activity in the soil, besides enhancing the population of antagonists.

The efficiency of *Brassica* amendments was further improved when *Foc*-infested fields were continuously exposed to dry summer heat for 60 days before the application of amendments and irrigation (Mawar and Lodha 2009). This improvement in the control was attributed to interactive effects of SLH achieved in dry soil exerting a weakening effect on *Foc* propagules followed by the use of MR and irrigation. In all, 10–14% reduction in viable propagules was evident merely by increasing exposure time. Since a part of the MC is consumed as a supplemental feed for livestock, other inexpensive and readily available on-farm wastes were explored.

Subsequently, an integrated schedule of management approaches was attempted. Thus, integrating SLH, MR (2.5 t ha^{-1}) and an obnoxious weed *Verbesina encelioides* (V) (0.5 t ha^{-1}) and irrigation resulted in almost equal pathogen control that was achieved by MR + MC. The effects of varying heating regimes and the duration of SLH were ascertained on the efficiency of organic amendments for reducing viable propagules of *Foc* in the field (Israel et al. 2011). Increased duration and amount of heat improved reduction in *Foc* propagules. The weakening of propagules achieved by exposure to dry heat was integrated into a sequence with other management approaches like organic amendments and polyethylene mulching of moistened soil (Fig. 16.1). Organic amendments include residues of *V. encelioides* (0.18%), onion residues (0.18%), MR (0.18%) and MC (0.04%) alone or in combinations. At heat level 4, MR + MC amendments reduced viable propagules (71.3%) of *Foc*, which was less than achieved with *Verbesina* (54.3%) or onion (64.4%) residues. However, reduction improved (58.6–66.6%), when MR have combined these on-farm wastes. Reduction further improved when a small dose of MC was combined with MR. Improvement (27.1–36.9%) in reducing counts of *Foc* was more conspicuous at heat level 3 (unshaded conditions) compared with that achieved at level 4. A maximum reduction in viable counts was obtained with MR + V and was significantly equal to the reduction achieved with MR + MC.

Exposing pathogen infested soil to dry heat for 56 days at heat level 2 improved (0.8–2.9%) reduced viable counts of *Foc* compared to heat level 3. Similar to heat level 3, 97.1% reduction achieved with MR + V was significantly better than the other combinations of amendments followed by MR + MC with only 0.8% improvement in reduction overheat level 3. Elevated temperatures at heat level

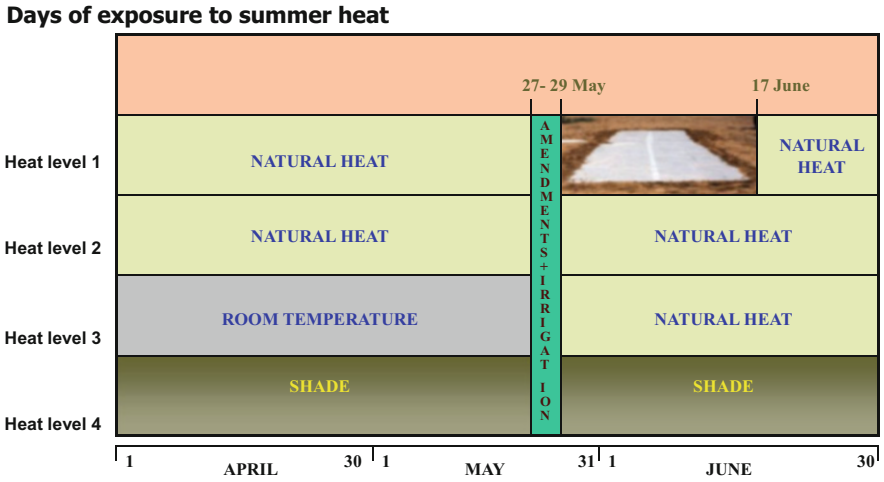

Fig. 16.1 Schematic layout of the experimental plan indicating varying intensities of sublethal heating against viable propagules of *Fusarium oxysporum* f. sp. *cumini* in soil achieved by different days of summer exposure before application of amendments and irrigation. (1) 56 days of dry summer exposure before the incorporation of amendments, application of irrigation and 20 days polyethylene mulching. (2) Summer exposure for 56 days+ amendments +summer exposure. (3) Infested soil kept at room temperature for 56 days before summer exposure. (4) Infested soil kept under shade

1 (polyethylene mulching) eliminated viable counts of *Foc* in amendment treatments except for onion residues (Fig. 16.2). In all, the increased reduction in heat level over 2 ranged between 2.9% and 8.7%. A combination of solarization with amendments after summer exposure to infested soil also improved reduction at the lower soil depth.

16.3 Indirect Exposures by Simulation

16.3.1 Fusarium oxysporum *f. sp.* niveum

Watermelons (*Citrullus lanatus*) suffer heavily due to wilt incited by *Fusarium oxysporum* f. sp. *niveum* worldwide. Chlamydospores, as a resting structure, can survive in the soil for several years. In a survey, successive cropping of watermelons for seven seasons caused 90% or more yield losses (Callaghan et al. 2016). An effort was made to ascertain the weakening effect exerted by SLH on *Fusarium* propagules (Freeman and Katan 1988). Two pathogenic strains of *Fusarium oxysporum* f. sp. *niveum* (*Fon*) and *Fusarium oxysporum* f. sp. *melonis* (*Fom*) causing wilt of watermelon and muskmelon, respectively, were used with laboratory-grown inoculum separately. This culture was amended in the soil for 60 days to obtain only chlamydospores. Chlamydospore and conidia-infested soil was subjected to a

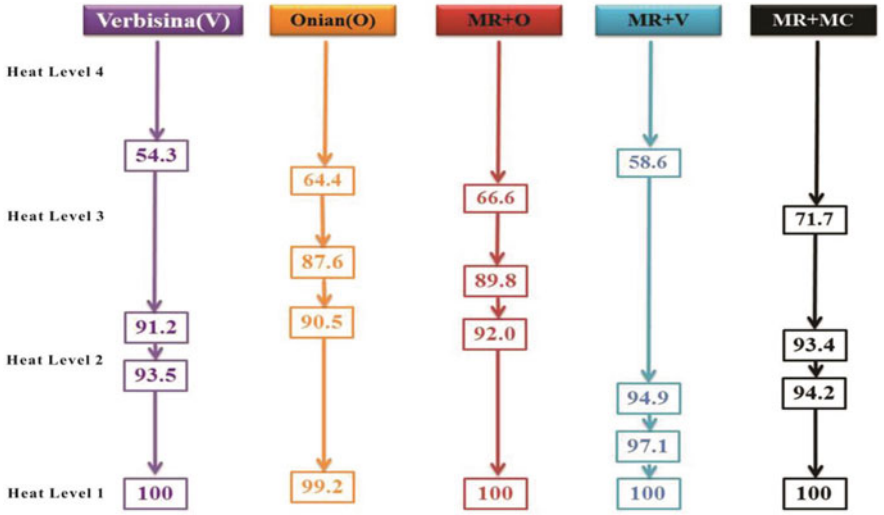

Fig. 16.2 Percent reduction in viable propagules of *Fusarium oxysporum* f. sp. *cumini* after summer exposure and soil solarization at heat level. (1) 56 days summer exposure + polyethylene mulching + summer irrigation. (2) 56 days of summer exposure, three laboratories to infested soil brought in the field on May 25 and four shades. Amendments (0.18%) were mixed separately and irrigation was given on May 27, and mulching was done on May 28. *V Verbesina*, *O* onion residues, *MR* mustard residues, *MC* mustard oil cake

temperature range that caused moderate reduction (0–33%) in amended pathogen inoculum. The declined inoculum density after the heat treatment was estimated on a selective medium. The heat-treated or untreated inoculum was then transferred to Czapek-Dox mineral soil, which was subjected to the action of soil bacteria by adding untreated and solarized soil having soil microorganisms. Fluorescent staining of conidia of *Fon* was done by fluorescein diacetate (FDA). In the soil, seedlings of both the crops dipped in heat-treated or the control suspension were planted in the field to record disease incidence. In another set, watermelon seedlings were inoculated by a pathogen and transplanted in the field to assess their reaction to the pathogen. In FDA test, conidia accepting the staining were lower compared to the control. At all the studied temperatures and exposure duration level, fluorescence staining was lower in SLH treatment compared to the control. Even though a partial reduction in viability of *Fusarium* propagules was estimated in heating treatments, 35–82% reduction in disease incidence was estimated in seedlings with a delay in disease progression. When exposure time (240 min) of SLH (38 °C) was increased, disease incidence reduced significantly on watermelon seedlings.

This study revealed that increased duration of SLH reduced viability of pathogenic propagules, evident by fluorescent staining and reduced disease incidence in the field compared to less duration of exposure at low temperature and in the control treatments. The findings suggest that temperatures close to lethal ranges caused a discernible weakening of *Fusarium* propagules and in reducing their survival, which

was also expressed in late germination of survived, but weakened propagules; reduction in vital fluorescent staining and more decline in the viability due to increased microbial activity ultimately resulted in reduced disease incidence on watermelon seedlings. Increased duration of exposure time caused more reduction in all the target organisms. In the bacteria-treated weakened propagules, lysis was attributed as one of the important reasons for increased microbial activity. It was suggested that after getting the desired weakening effect, a delay in the planting of seedlings will be more beneficial as the survived but weakened propagules during this period will be exposed to the activity of soil microorganism.

16.3.2 Fusarium oxysporum *f. sp.* ciceri

Wilt incited by *Fusarium oxysporum* f. sp. *ciceri* is a serious disease of chickpea (*Cicer arietinum* L.) throughout growing areas in the world. Seed treatment, cultivation of tolerant genotypes, use of biological control agents, composts and cultural practices are recommended to reduce the incidence of wilt. The effect of the prior weakening of propagules achieved by loss of energy was studied against this pathogen. There is loss of endogenous C reserves from the chlamydospores of *Fusarium oxysporum* f. sp. *ciceri* exposed to different heating regimes. The effects of heat stress on germination, the aggressiveness of the pathogen and vulnerability of stressed chlamydospores to microbial colonization in the soil were determined (Arora et al. 1996). Chickpea plants inoculated with spores exposed to continuous lethal heating did not develop disease symptoms; however, those inoculated with freshly harvested untreated spores and those exposed to 45 °C developed disease symptoms. Spores exposed to the short duration of SLH treatment were significantly impaired in causing disease compared to freshly harvested or untreated spores. Intermittent heating cycle-exposed spores could also cause reduced disease severity. In general, spores subjected to moist heat supported large numbers of soil microorganisms than those exposed to dry conditions. A significant difference in colonization was noticed between freshly harvested spores and those previously exposed to unheated or intermittently heated soil for 15 days. Colonized spores failed to cause wilting in chickpea plants. The findings suggest that heat-stressed pathogenic spores lost a significant quantity of organic C. Spores exposed to continuous lethal heating (50 °C) lost a very small amount of C, but spores became non-viable after 6 h. However, short duration of SLH or intermittent heating imposed greater heat stress. Therefore, heat-stressed spores were found to be more vulnerable to microbial antagonism. Short-duration sublethal and intermittent heating increased $^{14}CO_2$ evolutions from the spores during the first 5 days. Other fungal propagules subjected to nutrient stress conditions in soil or exposed to diffusive stress were also reported by Arora et al. (1983, 1985).

16.3.3 F. o. *f. sp.* basilici *and* Sclerotium rolfsii

Reduced doses of fumigant, heating and soil solarization were comparatively evaluated alone or in combination with the survival of *Fusarium oxysporum* f. sp. *basilici* and *S. rolfsii* (Eshel et al. 2000). The aim was to test whether there is any possibility to reduce doses of fumigants and duration of soil solarization by adopting a sequence of applications in a controlled system and under field conditions.

Effect of further stress imposed on propagules treated with SLH by exposure to soil microbial activity was examined, and it was found that combined heating +methyl bromide (MB) treatment exposed to microbial activity increased the mortality to 93–100%. Propagules treated with low doses of MB were incorporated into the moistened soil at different soil depths and fumigated. Extended incubation of propagules in the fumigated soil further decreased the survival of both the pathogens. Combining short solarization and MB or methyl sodium at low doses was more effective than each treatment alone in controlling pathogens. In certain cases, combined treatment was synergistic, but in another inverse, synergism was also observed.

This study demonstrates that combining soil solarization with other management approaches should be more appropriate as this will reduce the period of mulching from 30–40 days to only 8 days and will also improve the effectiveness of the second management approach. A reduced dose of chemical pesticides will also reduce harmful effects without affecting the efficiency of control. Soil incubation of propagules augmented the pathogen control. Assessment of the pathogen population soon after the termination of treatment may not reflect the exact potential of the treatment. Thus, a short period should be given between fumigation and planting in order to further reduce pathogenic propagules due to delayed mortality of weakened propagules. A sublethal dose of a killing agent leads to stress and weakening of survived propagules, making them more vulnerable to other biotic or abiotic agents. Sublethal doses of MB or heating delayed germination of propagules, a typical expression of weakening. Slower germination in the soil would extend the time during which the vulnerable germ tubes are exposed to microbial activity and reduce the chances of their penetration in host tissues. Pre-treatment of pathogen propagules by reduced doses of MB increased pathogen mortality caused by microbial antagonism. It has been experimentally proven that weakened propagules are more vulnerable to enhanced biological control. SLH of sclerotia of *S. rolfsii* caused cracks in the sclerotia, which may facilitate the penetration of MB to the sclerotia and thus elucidate that a sequence of SLH followed by MB treatment is more effective than alternate sequence. Additional benefits of combining solarization with pesticides might include increased retention of volatiles trapped under mulching.

16.3.4 Verticillium dahliae

Crops like tomato, olive, artichokes, *Prunus* sp. and cotton suffer heavily due to wilt caused by *Verticillium dahliae* (Tjamos et al. 1989). Soil solarization has been found effective in reducing losses due to wilt in these crops. It was also observed that there was a concomitant increase in a biocontrol fungi *Talaromyces flavus* during solarization and afterwards. *T. flavus* has shown proven efficacy in controlling wilt on eggplant and tomato. It was hypothesized that the effect of soil solarization lasted only for 1–3 years, but an increased population of *T. flavus* improved the duration of the pathogen control by solarization. The BCA overwinters in plant debris or soil as microsclerotia, and its propagules survive in the soil for many years even without the availability of host plants. Of the many management strategies, soil steaming, soil solarization and fumigation have been attempted to reduce pathogenic propagules of *V. dahliae* and induced diseases. Sclerotia of *V. dahliae* are sensitive to moist soil, which means that dry soil promotes its survival. Increased duration of exposure to heat at 36 °C caused 90% mortality of this pathogen. Therefore, SLH may weaken the surviving propagules of *V. dahliae,* which will be more vulnerable to attack by soil microbes. A study was conducted to assess the detrimental effects of SLH and the use of *T. flavus* on the survival of microsclerotia of *V. dahliae* (Tjamos and Fravel 1995). This information will help improve or augment more efficient control of the pathogen. To obtain microsclerotia of *V. dahliae*, the fungus was grown on Czapek-Dox media shaken for 17 days. Cultures were removed and allowed to stand so that microsclerotia are settled at the bottom. Microsclerotia-containing tubes were placed in a water bath at temperatures similar to those obtained at 30 cm soil depth during solarization, while control tubes were kept at ambient conditions in the water. Tubes containing treated microsclerotia from 1 to 5 days were plated on a medium and incubated at 21 °C to record percent germination. Differences in final germinated microsclerotia and of newly melanized sclerotia were determined.

Interaction of SLH with *T. flavus* and pathogenicity of the *V. dahliae* was studied on eggplant seeds by drenching with ascospores at different intervals after seeding. Therefore, it can be inferred that cumulative effects on the incidence of disease and synergistic interaction of heating regime action of BCA together offered additional improved control by integrated methods. By integrating sublethal doses of solarization coupled with BCA, land can be kept under production without higher doses of soil solarization. Application of *T. flavus* in the soil before polyethylene mulching will facilitate the establishment of *T. flavus* in the soil. More so, the combined effect of both the management approaches may reduce the duration of soil solarization in order to get sufficient control. There are reports that *T. flavus* and many such thermophilic BCAs can survive along with solarization with an increased population (Tjamos et al. 1991). Other than solarization, possibilities can be explored to use sublethal doses of fumigant or other killing agents in combination with *T. flavus,* which may result in equal control. In the case of sublethal doses of the metham sodium, significant reduction in the growth rate of *Verticillium* hyphae was observed (Fravel 1996).

16.3.5 Sclerotium rolfsii

Effect of SLH on sclerotia of *S. rolfsii* was studied by Lifshitz et al. (1983). Laboratory-produced sclerotia were grown on a synthetic medium containing ^{14}C, and harvested sclerotia were mixed to get a homogenous bulk sample. These sclerotia placed in glass tubes in sterilized tap water were incubated in a water bath at 30 or 50 °C for 30 min. Radioactivity of these heat-treated sclerotia was determined with a liquid scintillation count. Heat-treated sclerotia were incubated at 30 or 47 °C for 3 h, removed and again incubated at 30 °C for 10 days. After washing the number of bacteria, fungi and actinomycetes colonizing these sclerotia was determined, while colonies of bacteria and streptomycetes were counted on respective media. These sclerotia were also observed under the scanning electron microscope (SEM). Pathogenicity tests were carried out on seedlings of bean to examine the number of plants with diseased symptoms.

The results revealed a twofold leakage of ^{14}C-labelled sclerotia at 50 °C for 30 min compared with the control sclerotia. Sclerotia exposed to heat treatment recorded a 574-fold and 1420-fold increase in bacteria and actinomycetes, respectively, compared to low fungal colonization in heat-treated or untreated sclerotia. Only 34% of bean plants inoculated with heat-treated sclerotia recorded disease symptoms compared to 60% plants, which were inoculated with untreated sclerotia. In SEM studies, cracks on the surface of the sclerotia were observed with increased heating.

Increased heating resulted in leakage of organic substances by sclerotia, which in turn stimulated the multiplication of soil microorganisms. This resulted in increased colonization of vulnerable sclerotia by bacteria and actinomycetes due to the formation of large cracks. The relatively high population of bacteria around the large cracks indicate that these sites are important for microbial colonization. The reduced potential of treated inoculum, in turn, reduced the incidence of disease. Accumulation of a high population of bacteria around these cracks is an indication that these are vulnerable sites for colonization due to nutrient leakage. This study also established that propagules of a pathogen can be rendered vulnerable to be attacked by other killing agents or their sublethal doses due to weakening effect. This effect can also be used to improve the control of pathogenic propagules at lower soil depths.

In the present study, researchers have agreed that laboratory-produced sclerotia may be physiologically different than those existing in soil, where some alteration in heat sensitivity may be observed. As observed in hot arid soils, sclerotia of *M. phaseolina* withstand soil temperature of more than 50 °C in dry soils without loss of viability. To improve pathogen control under such hostile conditions, the use of irrigation to stimulate germination and then amending soil with appropriate organic amendments is an effective management approach to obtain better pathogen control even at the lower soil depth (Israel et al. 2011). More so, the use of thermophilic BCAs before the application of sublethal doses of heat or after may result in improved control. It is expected that organic amendments will improve microorganisms in soil resulting in increased microbial activity.

16.3.6 Meloidogyne incognita, S. rolfsii *and* Pythium ultimum

Stapleton and Duncan (1998) studied the effects of *Brassica* amendments alone or with SLH regimes against root-knot nematode *M. incognita* and two soilborne plant pathogens, viz. *S. rolfsii* and *P. ultimum* in the control environment. In a naturally infested *M. incognita* and *P. ultimum* field and laboratory-produced inoculum of *S. rolfsii*, different *Brassica* amendments like black mustard (*Brassica nigra*), bok choy (*B. oleracea* var. *chinensis*), broccoli (*B. oleracea* var. *italiensis*), cabbage (*B. oleracea* var. *capitata*), cauliflower (*B. oleracea* var. *compacta*) and radish (*Rhaphanus sativus*) were amended at 2% in the soil. A susceptible tomato cultivar was transplanted in each amended plot with different amendments, and after 6 weeks root galls were estimated on a 0–4 scale. In the case of *S. rolfsii,* surface-disinfested sclerotia were placed on PDA agar plates and germination was recorded, while the effect of *P. ultimum* was assessed by plating on a selective medium. Subsamples for each treatment were pooled for individual amendment after combining results from heat and no-heat treatment. Analysis of variance was performed, and contrasts of interests were analysed for amendments, control, fresh and dry amendments and their relevant interactions. These studies revealed that treatments were not significant for galling of tomato roots by *M. incognita* or for germination of *S. rolfsii*. However, significant differences were estimated for *P. ultimum*. By contrast, gall scoring decreased by 95–100% when an amendment was combined with SLH. Among amendments, bok choy, broccoli and cabbage caused discernible effects on sclerotial germination, but other amendments were not significant. Invariably, SLH reduced germination in all the amendments. In the case of *P. ultimum* also, the increased reduction was observed with SLH.

Earlier, combining cabbage residues with soil solarization resulted in significant control of *P. ultimum* and *S. rolfsii*, and the effect was ascribed to the production of biotoxic volatiles (Gamliel and Stapleton 1993). However, the low concentration of volatiles produced during decomposition without soil heating was possibly due to biological control of weakened propagules (Tjamos and Fravel 1995). In the present study, a laboratory-produced inoculum of *S. rolfsii* was used; therefore these results require further confirmation by using naturally infested soil.

16.3.7 Ralstonia solanacearum

A number of solanaceous crops and bananas suffer heavily due to *R. solanacearum* causing bacterial wilt. Elimination of the primary source of inoculum is one of the most important requirements to reduce losses. Burning of crop residues to reduce or eliminate this inoculum has been attributed to the generation of heat, but this method

resulted in increased air pollution. Efforts were made to exert a weakening effect on the cells of the pathogen (Yamfang et al. 2013). Laboratory-produced inoculum of *R. solanacearum* was exposed to different levels of temperatures in a water bath. Similarly, soil mixed with a suspension of pathogen kept in waterproof polyethylene bags was also submerged in the water bath at different temperatures for a varying period of duration. After the requisite period of exposure, estimation of counts of *R. solanacarum* in heat-treated soil was made on culture medium and compared with the control. There was a drastic reduction in colonies of bacterial pathogens depending on the level of heat and duration of exposure time. Low levels of heat did not cause a reduction in viable colonies. There was a concurrent increase in the rate of bacterial inactivation at 45 °C for 2 h of incubation, but total inactivation was achieved only with an increased duration of heating for 480 min. With increased temperature, amount of time also reduced for inactivation. In dry soils, total inactivation of *R. solanacearum* took place at a higher temperature and increased duration as compared to wet soil. Total carbon was not affected significantly by heat treatments. Increased release of NH_4-N after heat treatment was attributed to the heat-induced breakdown of compounds containing nitrogen from microbial cells. The effect of heat is mainly dependent on the duration of exposure apart from the type of heat used. Sub-detrimental doses of heat treatment of bacterial cells induce blebbing and vesiculation of the outer membrane from the cells accompanied by the release of lipopolysaccharides from the outer membrane (Katsui et al. 1982). This study revealed that drying or wetting at 45–60 °C for 30 min caused a lethal effect on the bacterial pathogen. Wet soil was more effective than dry in the inactivation of cells. Thus, wet soil treatment technology emerged as a cost-effective method in Thailand.

16.3.8 Clostridium perfringens

In another study, germination to outgrowth process of spores from *C. perfringens* was studied at single-cell resolution (Skanou et al. 2018). Significant reduction due to SLH was achieved in the time from completion of germination to the beginning of cell division indicating that heating at sublethal doses of bacteria sensitizes the responsiveness of germination receptors and also facilitates multiple steps during the process of bacterial regrowth.

16.4 Mechanism Involved

More than one mechanism would have possibly operated concurrently or in a sequence in eliminating viable density of pathogenic propagules or making them vulnerable to other management strategies. The weakening effect depends on the

quantity of heat, duration of exposure and the surroundings into which the preheated propagules are introduced (Sztejnberg et al. 1987). However, a requisite level of threshold of heating has to be reached to obtain a detectable weakening effect. In this section, various mechanisms for weakening effects are elucidated.

16.4.1 Direct Heat

The thermal decline of pathogenic propagules at high temperature involves the sustained inactivation of respiratory enzymes. These are direct effects of high temperatures and account for a major share of the reduction in the population of soil microorganisms including pathogens. The effects of SLH achieved by prolonged exposure of pathogenic propagules to natural solar heat in dry soils caused a considerable decline in viable pathogenic propagules. However, variations were recorded due to the presence of inoculum density, soil type, time of exposure and pathogen involved. Direct heat for 90 days reduced viable propagules of *M. phaseolina* at 0–30 cm soil depth by 9.4–18.4% during both the seasons of study (1998–1999) (Mawar and Lodha 2009). This indicates that the effect of direct heat was conspicuous, where subsequent use of *Brassica* amendments and irrigation resulted in an almost equal reduction in pathogenic propagules of *M. phaseolina* and *Foc* (Mawar and Lodha; Israel et al. 2011). This is more important because *M. phaseolina* is more vulnerable to microbial antagonism. After all, fungal propagules were exposed to dry heat for a long duration (Fig. 16.3). The reduction in pathogenic propagules of both the pathogens was low (8.2–12.4%) under shade as direct heat was not involved. Arora et al. (1996) also demonstrated that in the upper layer of solarized soil, pathogen is probably killed or severely weakened by direct heat injury rather than by releasing a substantial amount of C compounds. The possible mechanism of improved control induced by another management approach in heated soil could be by dilution of fungistatic behaviour, stimulating germination of resting structures in moistened soil, which are more prone to increased heat conduction and enhanced microbial antagonism (Katan et al. 1976). In case of *Foc*, the population of bacteria and actinomycetes were invariably greater in amended compared to non-amended soil due to the presence of more residual soil moisture at the lower depth, which encouraged microbial antagonism against remaining but weakened *Foc* chlamydospores. Fungal propagules exposed to SLH are weakened, responding to other physical and biological mechanisms. This elucidates the reason why farmers of the Indian subcontinent are adopting summer ploughing in hot summer days owing to inherited indigenous technical knowledge from ancestors, which was also experimentally proved for the partial control of fungal and nematode pathogens (Champawat and Pathak 1990: Mathur et al. 1987).

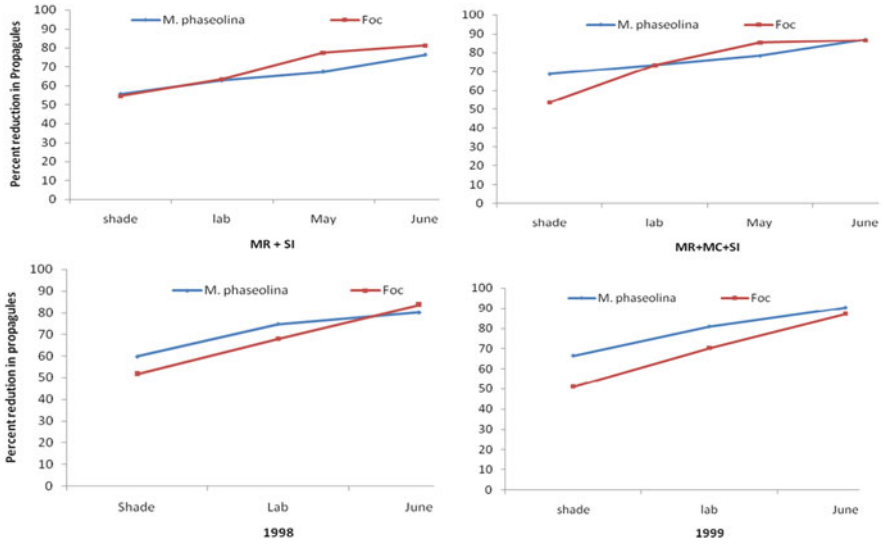

Fig. 16.3 Percent reduction in viable propagules of *Macrophomina phaseolina* and *Fusarium oxysporum* f. sp. *cumini* after summer exposure under the shade, and soil kept in the laboratory was brought in the field, 30 (May) and 56 days (June) of exposure to dry summer heat before the incorporation of amendments and irrigation. *MR* mustard residues, *MC* mustard oil cake, *SI* summer irrigation

16.4.2 Heat Shock Proteins (HSPs)

Experiments conducted in different parts of the world demonstrated that exposing pathogenic propagules at SLH resulted in a weakening effect, thereby reducing capacity and pathogenicity of propagules at the subsequent stage (Freeman and Katan 1988; Lifshitz et al. 1983). These studies established that elevated temperatures lead to the synthesis of a new set of proteins suppressing normal protein synthesis (Plesofsky-Vig and Brambl 1985). The role of HSPs as a mechanism of weakening effect has been reviewed earlier by Katan et al. (1996).

Control of viable propagules of soilborne plant pathogens is frequently achieved by exposure to different levels of heat. Heat treatment or exposure to prolonged dry summer heat leads to changes in cellular metabolism of exposed resting structures. Most important among these changes occur in the proteins, which are often denatured or enzyme activity is reduced or lost. This often arrests the synthesis of normal proteins and stimulates the metabolism to produce HSPs in different amounts. However, the quantity of production depends on the level of heat stress. Lin et al. (1984) reported that the synthesis of HSPs is due to induced thermotolerance. This response allows an organism exposed to moderate or mild stress to withstand more severe form of stress. Under the summer conditions of Georgia, USA, the level of HSPs mRNA in crops was found to increase during daytime and decrease in the

night when the temperature becomes cool (Kimpel and Key 1985). Thus, it can be safely inferred that when resting structures of soilborne plant pathogens are subjected to an extended period of heat stress at near-lethal temperatures, there is a loss of the ability to produce HSPs leading to irreparable weakening effect. Regulatory mechanisms have been developed to check the cellular level of HSPs, to restrict their synthesis during a period when cells of resting structures are subjected to varying levels of heat stress and further to stop their synthesis during a moderate period (Morimoto 1993; Mager and De Kruijff 1995). Once this mechanism is fully understood, it can be used as an effective tool in ascertaining the requisite level of SLH to utilize this knowledge for improved pest control.

In a study by Freeman et al. (1989), radioisotope labelling and heating of conidia were done, and recovery from a heat shock of 40 °C for 60 min was examined after transplanting them to 25 °C. Proteins were pulse-labelled at various periods after initial heat shock and compared to those synthesized at 25 and 40 °C for 60 min each. Simultaneously, parallel fluorescent staining and viability of heated and untreated propagules were determined. Protein extracts were separated, and gel electrophoresis determination of radiolabelled incorporation was done. Vital fluorescent staining with FDA was performed to determine the recovery of germlings from heat shock (Freeman and Katan 1988). Thermotolerance to high temperature (43 or 44 °C) acquired by preheating germlings at SLH was determined by dilution plating, fluorescent vital staining and incorporation of radiolabel in proteins compared to the untreated controls, and the predominant HSPs were estimated. The percentage of intensively stained germlings with FDA, measured soon after the termination of heating, was negatively correlated with temperature levels. The increased synthesis of HSPs was detected as early as 10 min after exposure to heat treatment. Thermotolerance was acquired by preheating germlings at SLH before subsequent exposure to higher temperatures. This study demonstrates that germlings of watermelon wilt pathogen (*Fon*) responded to elevated temperatures by synthesizing HSPs, which were similar to those of *Neurospora crassa* (Kapoor and Lewis 1987). These HSPs of pathogen appear to correspond to the HSPs of the number of families, which are conserved in eukaryotic organisms. HSP 83 in *Fon* is expressed at regular growth temperatures, but synthesis increased with increasing temperatures. At near-lethal temperatures, synthesis of HSPs 95 and 74 continued. Reduced vital staining with FDA was found at high temperatures. Therefore, reduced vital fluorescent staining and synthesis of HSPs can be considered as reliable indicators of heat stress achieved by SLH.

Preheating cells at SLH before exposure to lethal temperatures caused thermotolerance. This was subsequently expressed in reduced mortality, increased fluorescence and enhanced incorporation of radiolabel into protein only, and a slight reduction in pathogenic propagules was achieved by SLH, but exerted a weakening effect on surviving but weakened propagules, which was subsequently reflected in decline in population densities of pathogenic propagules. Conclusively, at this stage, it was difficult to precisely predict that heating of propagules during soil solarization will result in inducing thermotolerance in pathogens or can cause a cumulative

weakening effect. But it was clearly demonstrated that the weakening of propagules resulted in improved pathogen control immediately or after some time.

16.4.3 Melanin Synthesis

Melanin is a black pigment found in all the biological entities. It is multifunctional and provides defence against environmental stresses (Eisenman and Casadevall 2012). The biosynthetic pathway of melanin synthesis was first discovered in *V. dahliae* (Bell et al. 1976). It is considered important for the survival and longevity of fungal propagules (Wheeler and Bell 1988). Effect of SLH on the formation of melanized microsclerotia indicated that germinated microsclerotia in the non-heated control formed numerous round and melanized microsclerotia. By contrast, only 40% of colonies arising from microsclerotia heated for 1 day compared to control, 3 days delay in melanin deposition in colonies arising from microsclerotia heated for a long duration. In soil, melanization of newly formed microsclerotia was noticed (Tjamos and Fravel 1995). A highly significant and synergistic interaction between heating and the presence of BCA *T. flavus* was estimated in the formation of melanization of newly formed microsclerotia. SLH is known to inactivate certain key enzymes involved in the production of melanin, which is responsible for defence against microbial attack and ultraviolet radiation, and its interruption may have a detrimental effect on the survival of pathogenic propagules. It is a fact that melanin functions in the defence of microsclerotia from microbial attack (Hawke and Lazarovits 1994). Therefore, a reduction in the rate of melanin deposition can be a possible mechanism, which resulted in increased susceptibility to the attack of BCAs. In these experiments, SLH was adequate in reducing the viability, and the remaining weakened microsclerotia caused reduced melanin deposition and pathogenicity, thus increasing susceptibility to *T. flavus.*

Pathogens like *Verticillium*, *Sclerorium*, *M. phaseolina*, *Sclerotinia,* etc. have melanin in their resting structures. The possibility of reduced melanin synthesis cannot be ruled out due to the weakening effect. The cumulative effect of these factors resulted in the final reduction in viable counts of sclerotia. Population density, as measured soon after treatment, does not always reflect the full effect of the treatment, because it does not take into account the possible further decline of the pathogen population and consequent disease control generated by weakening. The presence of greater residual soil moisture in amended compared to non-amended soil might have further accelerated bacteria antagonism against weakened sclerotia at lower soil depth may be due to reduced melanin.

16.4.4 Leakage of Organic Substances

Increased heating resulted in leakage of organic substances by sclerotia, which in turn stimulated the multiplication of soil microorganisms. This resulted in increased colonization of vulnerable sclerotia by bacteria and actinomycetes due to the formation of large cracks. The reduced potential of treated inoculum reduced the incidence of disease. Accumulation of a high population of bacteria around these cracks is an indication that cracks are vulnerable sites for colonization also due to nutrient leakage. This study thus established that propagules of a pathogen can be rendered vulnerable to be attacked by other killing agents due to weakening effect or their sublethal doses. Dried and re-moistened *S. rolfsii* sclerotia leak sugar and amino acids, and these substances stimulate the microbial breakdown of the sclerotia (Smith 1972). This weakening effect can be used to improve the control of pathogenic propagules even at lower soil depths.

Researchers have agreed that laboratory-produced sclerotia may be physiologically different than those existing in the soil. One alternative is that such laboratory-produced inoculum should be allowed to stabilize in the field conditions for some period so that only resistant propagules may survive. In hot arid soils, many of such sclerotia of *M. phaseolina* withstand soil temperature of more than 50 °C in dry soils without complete loss of viability. To improve pathogen control, the use of irrigation to stimulate germination and then amending soil with organic amendments is another effective management approach to obtain better pathogen control at lower soil depth. More so, the use of thermotolerant BCA(s) before the application of sublethal doses of heat or after may result in improved control. It is expected that the use of organic amendments will enhance microorganisms in soil resulting in increased microbial population and activity.

16.4.5 Crack Formation

Cracking of any living structure of the living organism leads to increased microbial antagonism. This, in turn, makes a pathogen or host more vulnerable often reaching ‘a point of no return’. This aspect is frequently witnessed in human beings also when an injury to any organ accelerated infection, making the body more vulnerable. One such aspect is burning where the human body is kept under isolation to avoid infection from microorganisms. Often antiseptic creams, injections and antibiotics are administered to prevent such infections from external microbial sources. Incidentally, such devices are not applicable to keep cracked pathogenic structures in isolation, which ultimately become more prone to microbial antagonism either by parasitism, competition or antibiosis by the surrounding microflora. This aspect of antagonism has been investigated in various studies discussed earlier. Cracking is often aggregated by loss of melanin synthesis, leakage of nutrients, loss of HSPs and use of other management strategies. In SEM studies, more cracks on the surface of

the sclerotia were observed in case of increased heating, resulting in colonization by bacteria compared to 56% in untreated ones (Lifshitz et al. 1983). Large cracks were an important site for bacterial colonization.

16.4.6 Delayed Germination and Mortality

Under unfavourable conditions, spore remains exogenously dormant and its germination is delayed. Germination and viability of heat-treated spores incubated in soil ranged between 66% and 79%, while germination of untreated spores was higher (84–97%). Spores exposed to continuous heating at 45 and 50 °C showed significant differences in germination. However, when spores were subjected to a short duration of SLH, significant difference in germination was observed. In general, germination reduction was greatest in spores exposed to continuous lethal heating, followed by intermittent and short-duration SLH regimes.

Treating conidia of pathogen(s) at 38 and 40 °C resulted in delayed germination of pathogenic propagules with reduced germ tube length. It was observed that conidia of *Fon* were more affected compared to chlamydospores. However, increased heating (40 and 42 °C) reduced the viability of chlamydospores by 6–17%. At 40 °C, 36% decline in survival of the conidia of *Fon* was estimated (Freeman and Katan 1988). Sublethal doses of MB or heating delayed germination of propagules, a typical expression of weakening (Eshel et al. 2000). Slower germination in the soil would extend the time during which the vulnerable germ tubes are exposed to microbial activity and reduce the chances of their penetration in the host tissues.

Delayed germination was observed in sublethally heated spores of *Fusarium oxysporum* f. sp. *ciceri* (Arora et al. 1996). This indicates that heat damage is cumulative and becomes more severe in making spores non-viable. More susceptibility to parasitism was recorded by soil microorganisms by SLH, and these spores exhibited a partial loss of pathogenicity and viability. Initially greater loss of $^{14}CO_2$ could be related to the increased metabolic activity of the spores before germination, but in the present study heat stress germination of spores was completely arrested. The greater initial burst of $^{14}CO_2$ seems to be due to the respiration of heat-stressed spores. Hyakumachi and Lockwood (1989) demonstrated early $^{14}CO_2$ evolution from fungal spores subjected to nutrient stress. The weakening of the viability of spores and reduced pathogenicity can be attributed to the enhanced loss of readily metabolized C compounds (Arora et al. 1996). Exposure of fungal propagules to near-lethal heating can alter the permeability and fluidity of the cell membrane (Plesofsky-Vig and Brambl 1985) and cause morphological deformities and cracks on the surface of fungal cell walls. This study inferred that in upper layers of solarized soil pathogens are probably killed or severely weakened by direct heat injury rather than by releasing substantial amounts of C compounds. However, at lower soil depths, the weakening of propagules occurs by the synergistic effect of

heat stress coupled with microbial nutrient stress and may be due to the availability of more soil moisture.

Exposure of *S. rolfsii* sclerotia and *F.o.* f. sp. *basilica* to MB or heating reduced rate and delay in germination (Eshel et al. 2000). In a subsequent experiment, MB (37.5 g m^{-3}) and 2 h heating at 45 °C, exposure to MB delayed germination initially, but after 48 h germination was equal to control treatment. However, combining both the strategies in both application sequences delayed germination, and by synergistic action, final germination was also affected. The sequence having heating followed by fumigation was found more effective than the opposite sequence in the case of both the pathogens. This study revealed that the synergistic effect in mortality was more conspicuous in sequence having heating and MB.

In *V. dahliae* also, all microsclerotia, which were not heated, germinated after 1–2 days and by 100% within 48 h. But at the varying durations of heating, 90% of the microsclerotia germinated 5 days after planting, although increased duration of heating did not increased germination (Tjamos and Fravel 1995).

A study was carried out to assess and quantify the reduction in viability and weakening of propagules of *Fon* after heat treatment by using flow cytometric, physiological and microscopic studies and to characterize and quantify the delayed mortality of heated propagules (Assaraf et al. 2002). One of the hypotheses includes that when fluorochromes are combined with flow cytometry, it provides a strong tool for studying microbial physiology and can also differentiate between cells that are different physiologically or phenotypically. Important physiological process apoptosis is known as programmed cell death (PCD), by which cells are eliminated during development and morphogenesis. Cells undergoing apoptosis show biochemical and morphological events including membrane blebbing, aggregation of chromatin at the nuclear membrane, formation of membrane-bound vesicles, and mitochondria becoming leaky due to poor formation involving proteins of bcl-2 family. Laboratory-produced conidia of the pathogen were suspended in phosphate buffer, which was filtered and centrifuged. After that population of *Fusarium* was determined.

Studies on flow cytometry were carried out for viability assessment using FDA, acridine orange (AO) and propidium iodide (PI). Heated conidia at varying temperatures were centrifuged, and conidial fluorescence was analysed in a flow cytometer. Conidial staining with AO and PI was carried out and excitation of FDA was monitored. The activity of specific vacuolar enzymes was done by FUN-1 staining, which differentiates stains between living and dead cells. When the germination percentage was determined, it was considered when the germ tube was 6–10 μm long. Germinating conidia were stained for light microscopic studies and prepared for SEM.

Apoptosis studies were done with the help of the Annexin V-FITC apoptosis detection probe. This is based on the translocation of membrane phosphatidylserine (PS) from the cytoplasmic to the extracellular side of the cell membrane. Detection was carried out by using a fluorescent microscope and a flow cytometer. Cell-cycle measurements using the AO and PI DNA durability assays by flow cytometer were also carried out for heated or germinating conidia. These were analysed by flow

cytometer. DNA fragmentation detection of heated conidia was done by adopting standard procedures. The results revealed that heating the conidia at 45 and 47 °C for 60 min resulted in pronounced mortality in the population up to 94%. The survival of germinating conidia was not significantly affected by heating at 36 and 42 °C compared to the non-heated propagules indicating a weakening of the heat-stressed propagules. With increase in heating (45 °C), the fluorescence level also increased. In flow cytometer studies, exposure of conidia to 45 and 47 °C for 60 min revealed a decrease in their vital fluorescence compared to the control. Further, heat-treated conidia showed increased AO fluorescence, which indicated reduced membrane integrity. Heating conidia at 45 °C for 60 min and staining with PI and assessed with flow cytometer resulted in 29% non-viable conidia compared to only 2.9% in the control. In FUN-1 staining, non-heated conidia appeared diffuse green, and after incubation for 40 min bright red, round intravacuolar structures were visible. However, exposure to a lethal heat treatment killed conidia containing no inclusions and fluorescent orange green. At SLH regime, reduction in the viability was 17%, while exposure of conidia to heating (45 and 47 °C) for 60 min resulted in decreased germination. There was a further decrease between 13 and 24 h. Dilution plate counts required 5 days for colony formation, and germination percentage decreased further after incubation for 13 or 24 h. These propagules suffered from a detrimental effect of heat that is manifested only at a later stage. Most of the heated conidia though germinated, but ceased to grow, did not form colonies and eventually died suggesting that the damage was irreversible. This explains that SLH causes delayed germination, which finally resulted in delayed mortality after prescribed incubation.

16.4.7 Nutrient Leakage

Studies conducted by Arora et al. (1996) showed that spores exposed to heat treatments of varying intensities lost a variable amount of $^{14}CO_2$. On continuous exposure to lethal heating (45 °C), $^{14}CO_2$ evolution rapidly increased within 24 h, but then a sharp decline was estimated in the next 36 h. However, after 60 h these spores became non-viable and produced no $^{14}CO_2$.

At SLH (40–45 °C) also, evolution increased within 24 h, but from 5 to 15 days $^{14}CO_2$ evolved again.. Residual ^{14}C loss at lethal heating was 1.8% at –KpA and 1.5% at –20 KpA in 25 h, but subsequently, loss of ^{14}C was not estimated after 60 h. In all the treatments, $^{14}CO_2$ evolution and residual ^{14}C loss were always greater in heated compared to untreated soil. In general, ^{14}C loss from the spores was more during the initial 5 days, and cumulative total C loss differed greatly under varying heating regimes. Thus, the maximum cumulative loss was estimated when spores were exposed to intermittent heating followed by short or SLH and continuous duration of lethal heating.

16.4.8 Microbial Antagonism

Among various mechanisms, microbial antagonism by soil fungi, bacteria and actinomycetes is the most important and studied mechanism during and after discernible weakening effect on pathogenic propagules. Various studies have demonstrated this phenomenon of increased weakening often resulted in the death of pathogenic propagules like sclerotia, chlamydospores and conidia of soilborne plant pathogens. In any management strategy, where discernible weakening effect is achieved, pathogenic propagules are vulnerable to enhanced microbial antagonism may be due to reduced melanin synthesis, cracking, dehydration or any such factor, which reduces inoculum potential of infection-causing propagules of soilborne plant pathogens leading to delayed germination and mortality, besides the loss of aggressiveness in causing disease. Culture-produced sclerotia or chlamydospores are often physiologically different from those resident propagules in the soil under the natural environment. This difference may alter the heat-sensitive nature of pathogenic propagules. In various studies mentioned above, a manyfold increase in bacteria and fungi has been observed after weakening (Lifshitz et al. 1983). The use of organic amendments, which enhances the microbial population, is one such option where the weakening effect can be accelerated due to an increased population of microorganisms.

Use of *Brassica* amendments following the weakening of sclerotia of *M. phaseolina* and chlamydospores of *Foc* increased population of BCAs like *B. firmus* (Lodha et al. 2013) and *A. versicolor* (Israel and Lodha 2005). These BCAs accelerated antagonism besides direct action of biotoxic volatiles evolved due to *Brassica* residues in heated soil. Incidentally, *B. firmus* is a specific BCA against *M. phaseolina*. Studies have shown that both the BCAs are thermophilic antagonists to heat-tolerant structures of pathogenic propagules. Their action becomes more aggressive on weakened resting structures of *M. phaseolina* or *Foc*.

Lifshitz et al. (1983) studied microbial antagonism against laboratory-produced sclerotia of *Fon*. Heat-treated sclerotia were incubated at 30 or 47 °C for 3 h, removed and again incubated at 30 °C for 10 days. After washing, the number of bacteria, fungi and actinomycetes colonizing these sclerotia was determined by the dilution plate count method, while colonies of bacteria and streptomycetes were counted on soil extract medium and fungi on Martin rose Bengal agar medium. These sclerotia were also observed under SEM. Pathogenicity tests were carried on seedlings of bean to examine number of plants with diseased symptoms. The results revealed that a twofold leakage of ^{14}C-labelled sclerotia at 50 °C for 30 min was observed compared with the sclerotia control. Sclerotia exposed to heat treatment recorded a 574-fold and 1420-fold increase in bacteria and actinomycetes, respectively, compared to low fungal colonization in heat-treated or untreated sclerotia after cracking. Only 34% of bean plants inoculated with heat-treated sclerotia recorded disease symptoms compared to 60% plants which were inoculated with untreated sclerotia. When heat-treated and untreated conidia were exposed to soil

bacteria, the viability of conidia was reduced by 22% compared to no change in the control (Freeman and Katan 1988).

16.5 Conclusion and Future Perspective

It is a well-established fact that stubborn soilborne plant pathogens are difficult to control by adopting a single management strategy. Resistant sources are not adequately available in host crops against most of these pathogens. Therefore, integration of more than one management approach in a particular sequence is required to eliminate or to bring down the viable pathogenic population at a level below the economic threshold limit to the desired soil depth. The prior weakening of resting structures by use of sublethal doses of killing agents offers a promising option before application of another important management approach to obtain improved control of soilborne pathogens. Once the most appropriate weakening tool is identified for a particular pathogen or complex of pathogens, soil type, inoculum density and other socio-economic consideration of the region, then it is possible to determine the optimum concentration, duration and other climatic features of the region to be utilized for exerting weakening effect. After that use of another management approach will be more successful either at the same or maybe at reduced doses or duration. In order to use this as an important tool, more research efforts are needed to ascertain feasibility, time of application and duration of a sublethal killing agent. The use of this killing agent will also depend on bio-ecological factors including the presence of beneficial microorganisms in a particular soil type, inoculum density of propagules at different soil depths and type of exposure. The involvement of other abiotic factors such as pH, soil moisture and texture and interaction between them in inducing greater competitive stress on fungal propagules merits further investigations. Sublethal heating is a well-understood tool for exerting a weakening effect and has been investigated in detail. However, this requires adequate soil temperatures in the areas of operation, particularly during crop free periods. There is an increase of approximately 10 °C from ambient temperatures at 0–5 cm soil depth. At lower soil depth, temperatures do not reach to get desired weakening effect. Studies should be initiated for determining the effect of summer ploughing also so that soil from lower depth is turned at the topsoil layer to get a discernible weakening effect on propagules infesting lower soil depth.

In order to ascertain a reliable weakening effect, there are specific areas of intensive research investigations. These may vary with sclerotia, chlamydospores, oospores and other resting structures of soilborne plant pathogens, which otherwise can survive for several years without a host in the soil often making the change of crop sequences unsuccessful or less remunerative. In addition, the wide host range of some pathogens is yet another disadvantage to reduce inoculum density by crop rotation or any other cultural methods of control. However, to work out the most authentic and reproducible assessment, it is essential to ascertain actual change to determine the desired weakening effect and to find out whether this effect is

short-lived or has reached an irreversible recovery or to say 'point of no return'. Another field of research is to estimate that weakening effect in the case of a particular pathogen or soil type is the result of combined effects of several mechanisms like heat shock proteins, dehydration, cracking and increased microbial antagonism. It is also an area of interesting effort to find out whether the effect of application of more than one approach of the sublethal killing agent will remain the same, additive or maybe synergistic. For example, SLH and *Brassica* amendments result in a synergistic effect due to increased heating allowing the release of more biotoxic volatiles from decomposing residues and finally to increased microbial antagonism. The most important aspect for consideration of research input is the sequence of use of sublethal killing agents to improve the reduction or elimination of pathogenic propagules at desired soil depths. Temperature and duration of exposure for inactivation of thermotolerant propagules need concerted efforts in those subtropical and tropical regions where high soil temperatures are a regular phenomenon. Apart from this, data are required to be generated in natural pathogen-infested soil, also, to the use of laboratory-produced inoculum to estimate the effect on disease incidence with or without applying another management strategy. These investigations will result in the successful implementation of the integrated management of soilborne plant pathogens.

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