Chapter 3
Mechanisms of Action of Fungal Biological Control Agents

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

3.1 Types of Antagonism

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

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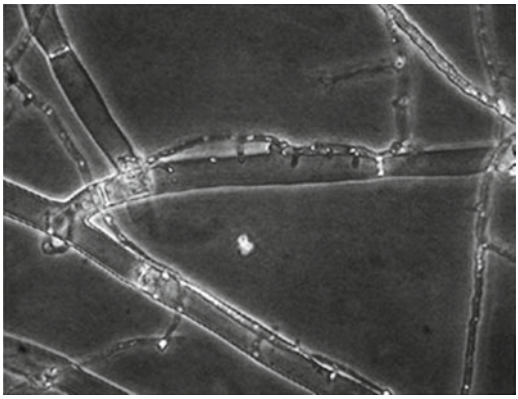

Fig. 3.1 Mycoparasitic activity of *Trichoderma virens* against *Rhizoctonia solani*, causing root rot diseases. Formation of haustoria of BCA within the large hyphae of the pathogen can be visualized using light microscope (Courtesy of Howell 2003 and with kind permission of The American Phytopathological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

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

3.1.1 Mycoparasitism

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

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

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

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

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

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

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

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

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

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

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

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

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

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

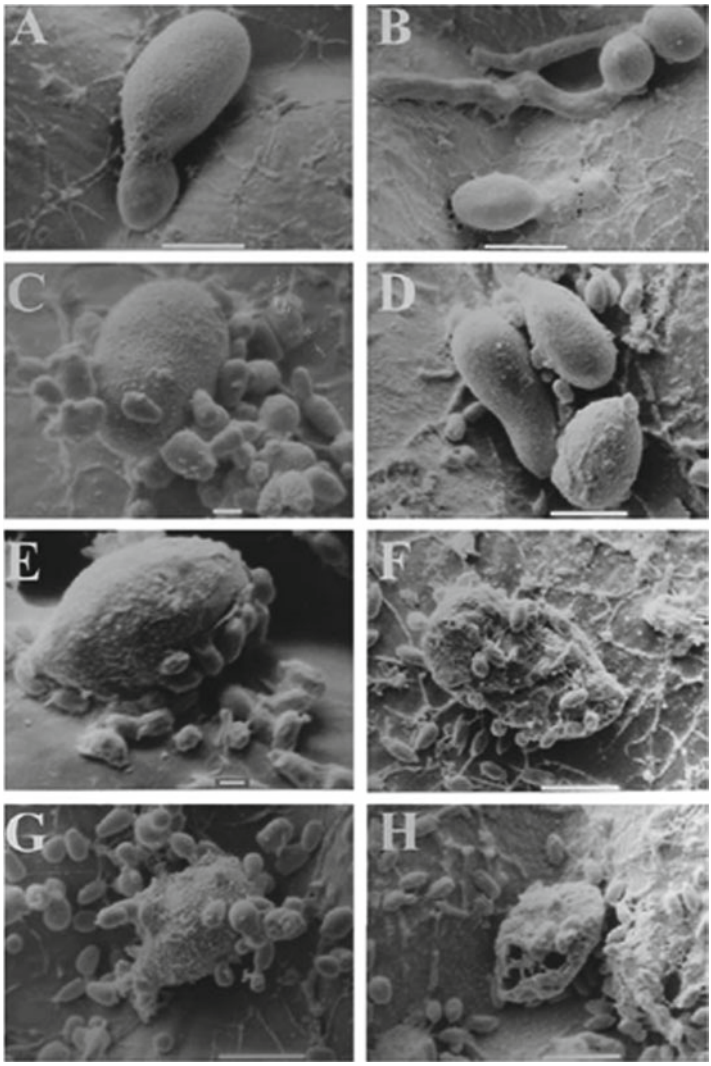

Fig. 3.2 Scanning electron microscopic (SEM) observations of interaction between *Botrytis cinerea* and *Pichia guilliermondii* (**a**) and (**b**): germinating conidia of *B. cinerea* at 6 (**a**) and 24 h (**b**) after application on strawberry leaves; (**c**) and (**d**): cells of *P. guilliermondii*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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.1.2 Antibiosis

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

3.1.2.1 Production of Antifungal Enzymes

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

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

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

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

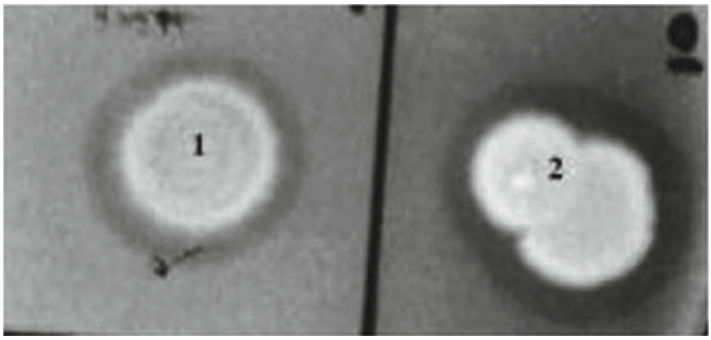

Fig. 3.3 Inhibition of mycelial growth of *Colletotrichum falcatum* by *Trichoderma harzianum* strain T5 in chitin-amended medium (Courtesy of Viswanathan et al. 2003 and with kind permission of Springer Science + Business Media B. V., Heidelberg, Germany)

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.1.2.2 Production of Antibiotics

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

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

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

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

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

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

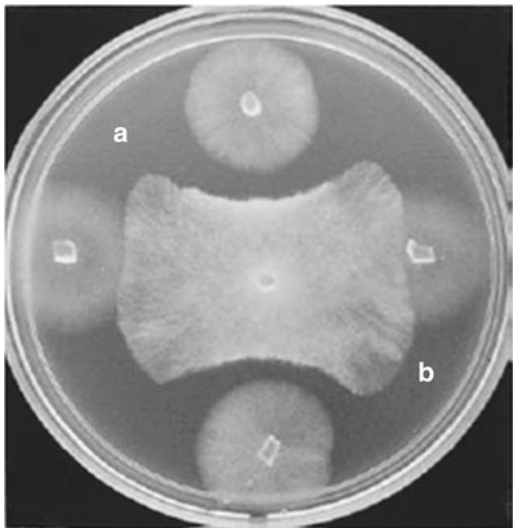

Fig. 3.4 Inhibition of mycelial growth of *Pythium ultimum* by *Trichoderma virens*-produced gliovirin (**a**): parent strain; (**b**): *Gliovirin-deficient* mutant (Courtesy of Howell 2003 and with kind permission of The American Phytopathological Society, MN, USA)

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

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

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

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

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

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

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

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

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

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

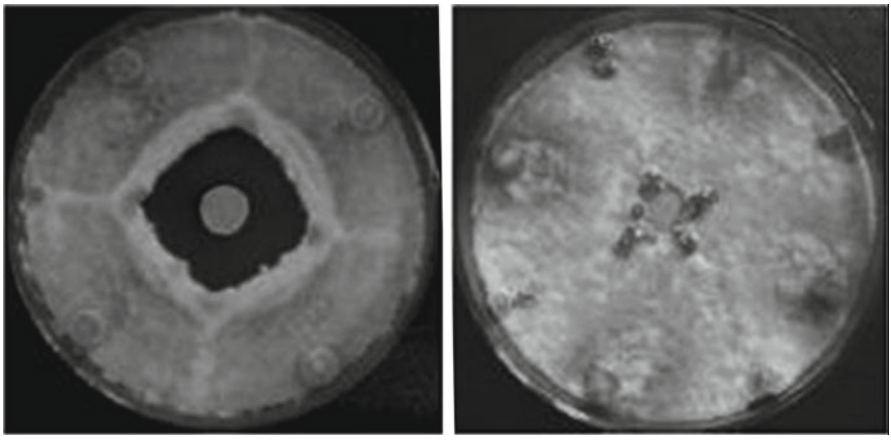

Fig. 3.5 Antifungal activity of the bioactive compound produced by *Penicillium oxalicum* strain PY-1 and isolated by high performance liquid chromatography (HPLC) Inhibition of mycelial growth of *Sclerotinia sclerotiorum* by the compound produced by strain PY-1 (Courtesy of Yang et al. 2008 and with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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.1.3 Competition for Nutrients and Space

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

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

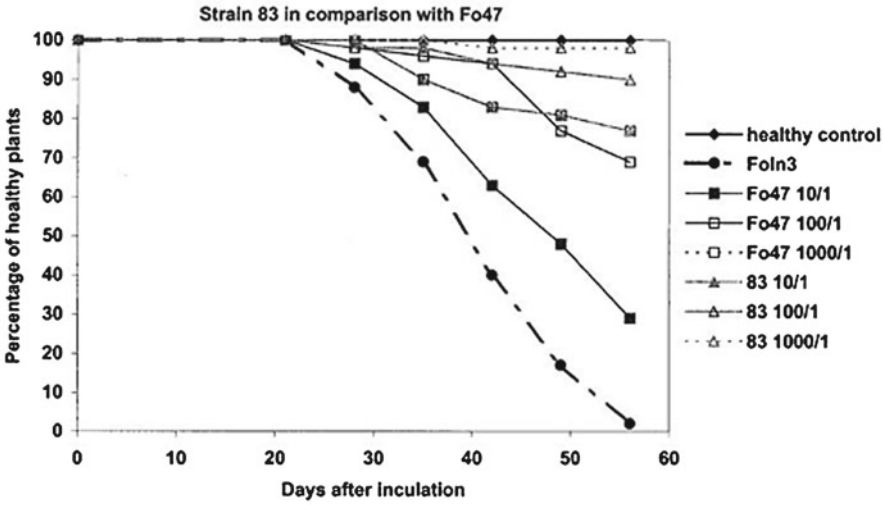

Fig. 3.6 Comparative efficacy of the mutant 83 and wild-type Fo47 (nonpathogenic) strain of *Fusarium oxysporum* in protecting flax plants against Fusarium wilt pathogen *F. oxysporum* f.sp. *lini* at different density ratios (Courtesy of Trouvelot et al. 2002 and with kind permission of The American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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.1.4 Prevention of Colonization of Host Tissues by Pathogens

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

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

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

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

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

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

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

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

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

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

3.1.5 Induction of Resistance in Plants to Diseases

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

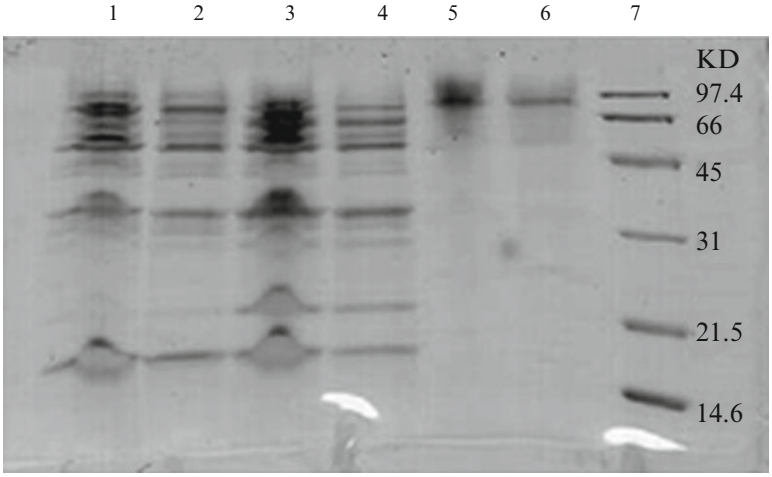

Fig. 3.7 SDS-PAGE analysis of culture filtrates (CFs) of effective and ineffective biocontrol strains of *Trichoderma virens*. Several bands are detectable only in effective strains of *T. virens*. *Lanes 1* and *2*: CF from *T. virens* strain G6 (biocontrol-effective); *Lanes 3* and *4*: CF from *T.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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

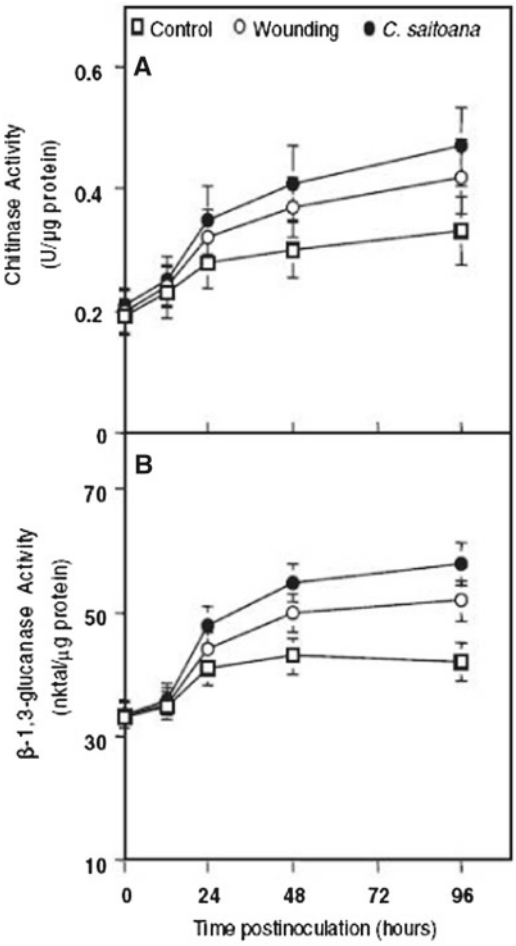

Fig. 3.8 Effect of treatment of wounds of stored apples with *Candida saitoana* on the activities of chitinase and ß-1,3-glucanase at different intervals Treatment with *C. saitoana* (●), sterile water (○) and non-wounded control fruit (□) (Courtesy of El-Ghaouth et al. 2003 and with kind permission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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.1.6 Natural Host Plant Resistance

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

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

3.1.7 Factors Influencing Activities of Biocontrol Agents

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

3.1.7.1 Soilborne Diseases

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

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

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

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

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

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

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

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

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

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

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

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

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

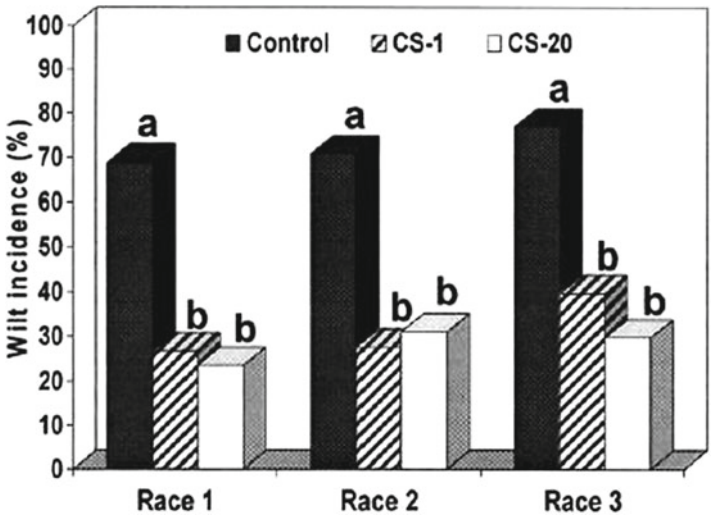

Fig. 3.9 Effect of application of non-pathogenic fungal biocontrol agents *Fusarium oxysporum* CS-20 and *F. solani* CS-1 on the incidence of Fusarium wilt disease of tomato caused by race 1, race 2 and race 3 of *F. oxysporum* f.sp. *lycopersici*. *Bars topped by the same letter* within each race designation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

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

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

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

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

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

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

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

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

3.1.7.2 Aerial Diseases

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

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

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

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

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

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

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

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

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

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

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

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

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

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

3.1.7.3 Postharvest Diseases

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

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

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

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

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

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

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

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

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

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