Chapter II

REVIEW OF LITERATURE
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Plant diseases have been with us since agriculture began. The population explosion has necessitated increasing food production with available land devoted to crop production. Intensification of mono-cropping has resulted in increasing disease pressure although application of synthetic fertilizers and pesticides has given higher yields, environmental balances have been disturbed and considerable crop damage by insects and pathogens continued to occur in varying degree of severity. Plant disease causes 13-20% of losses in crop production world wide. Control of plant diseases by chemicals can be spectacular but this is relatively a short term measure creating accumulation harmful chemical residues over time causing serious ecological problems.

In the recent years, increasing use of potentially hazardous pesticides and fungicides in agriculture has resulted in growing concern of environmentalists and public health authorities. More over, use of such chemicals entails a substantial cost to the nation and developing country like India can’t afford it. Biological methods on the other hand can be economical, long lasting and free from residual affects. The main purpose of the biological control of a plant disease is to suppress the inoculum load of the target pathogen to a level, which would not cause potential economic loss in a crop.

Chemical compounds have been used to control plant diseases (chemical control), but abuse in their employment has favored the development of pathogens resistant to fungicides. Unfortunately, the more specific the effect of a chemical on an organism, the greater the probability of decreasing the effect through genetic shifts in the population, whereas fungicides of broad spectrum produce undesirable consequences on non-target organisms (Jamos et al. 1992). Use of microorganisms
that antagonize plant pathogens (biological control) is risk-free when it results in enhancement of resident antagonists. Moreover, the combination of such biological control agents (BCAs) with reduced levels of fungicide (integrated control) promotes a degree of disease suppression similar to that achieved with full fungicide treatment (Monte, 2001).

The success of *Trichoderma* strains as BCAs is due to their high reproductive capacity, ability to survive under very unfavorable conditions, efficiency in the utilization of nutrients, capacity to modify the rhizosphere, strong aggressiveness against phytopathogenic fungi, and efficiency in promoting plant growth and defense mechanisms. These properties have made *Trichoderma* a ubiquitous genus present in any habitat and at high population densities (Chet *et al.* 1997). *Trichoderma* BCAs control ascomycetous, deuteromycetous and basidiomycetous fungi, which are mainly soil-borne but also airborne pathogens (Monte, 2000). Excellent results of integrated control have been attained with strains of *T. virens* and metalaxyl against *Pythium ultimum* infecting cotton (Chet *et al.* 1997), *T. harzianum* and captan against *Verticillium dahliae* infecting potato (Chet and Inbar, 1994), and *T. virens* and thiram against *Rhizoctonia solani* infecting tobacco, and others (Chet *et al.* 1997).

*Trichoderma* species are ubiquitous in the environment, especially in soils. They have been used or encountered in many human activities, including commercial applications in production of enzymes and biological control of plant disease (Samuels, 1996). *Trichoderma* species are widely distributed all over the world (Domsch *et al.* 1980), and found in all soils including forest humus layer (Wardle *et al.* 1993) as well as in agricultural orchard soils (Roiger *et al.* 1991) and natural habitats, especially in those containing or consisting of organic matter (Henis and Papavizas, 1983; Papavizas, 1985).
The physical parameters like pH, temperature, aeration and major nutrients carbon and nitrogen sources are important factors in fungal growth. Although fungi need nitrogen to produce cells and synthesis of biomolecules, they are not able to assimilate the nitrogen directly from atmosphere, hence they depend on the nitrogen compounds.

The pH and temperature are two keys parameter which manipulate the growth, sporulation and saprophytic ability as well as production of volatile and non-volatile metabolites, involved in nutrition, competition, mycoparasitism, and extra cellular enzymes that disintegrate cell wall of fungi. It has been demonstrated that *Trichoderma* strains are active under a wider range of pH (Kredics *et al.* 2003). The optimum temperature for growth differs among the *Trichoderma* isolates; although most *Trichoderma* strains are mesophilic (Kredics *et al.* 2003).

Variation in the biomass production of *Trichoderma* species and isolates was observed at different pH levels by various researchers. The *Trichoderma* isolates from rice cultivated soils showed their preference of pH 5.5 and 6.5 for optimal growth (Sarojini *et al.* 2012). Singh and Vijay Kumar (2011) screened five isolates of *T. harzianum* for their ability to grow at different pH and found maximum at pH 5 and minimum with pH 11. Increase and decrease in the pH of the culture medium from optimum, drastically reduced the radial growth of the isolates. Shafique *et al.* (2009) reported that *T. reesei* showed maximum growth at acidic pH ranged from 4 to 6. Among all *Trichoderma* spp., the maximum biomass produced by *T. reesei* was attributed to its capacity to utilize nutrients better than other species at varied pH levels. Rousk *et al.* (2009) reported that acidic pH favored fungal growth than alkaline pH. Behzad Hajieghrari (2008) reported that *Trichoderma* isolates mycelial growth was statistically differed in respect of the tested pH. Mycelial growth of *T.*
hamatum T612, *T. harzianum* T447 and *T. virens* T523 was the highest at pH 5 and mycelial growth of *T. harzianum* T969 was maximum at pH 7 whereas *T. hamatum* T614 has the highest mycelial growth at pH 8.

Miguel *et al*. (2007) found strain variation among the isolates within the *Trichoderma* spp. Growth of *Trichoderma* is more efficient in acidic than alkaline soils and they modify the rhizosphere soil by acidifying the soil (Benitez, 2004), which explains the reason for isolates which prefer acidic pH. Most fungi do not grow at very low pH conditions (Bitton and Boylan, 1985). Jackson *et al*. (1991b) reported that *T. harzianum* isolate showed optimum mycelial growth between pH 4.8 to 6.8 whereas Kredics *et al*. (2004) reported that they grow in wide range of pH 2.0 to 6.0, with maximum growth at 4.0. At lower pH, not even minimal growth of mycelia was recorded by Dursun *et al*. (2002). Isolates from forest soils preferred to grow at pH 7.5. This clearly indicates that the isolates obtained from rice fields have adopted themselves to different pH levels as the cultivation practices are known to affect the pH levels of agricultural soils (Sarojini *et al*. 2012). Yamanaka (2003) reported that the pH suitable for the *in vitro* growth of the fungi was correlated with the pH of forest soil from where these fungi occur and their non-adaptability to varied pH levels. This investigation clearly indicates that the isolates source plays an important role. The adaptation of *Trichoderma* isolates with varied pH is clearly indicated that isolates from intense agricultural soils are tolerant to varied pH conditions. In contrast, isolates obtained from virgin soils where there is no intervention of agricultural practices are not tolerant to varied pH levels.

Singh and Vijay Kumar (2011), reported the maximum growth of *Trichoderma* isolates at 25°C whereas very slow growth at 35°C and no growth at 40°C. Varsha Gangta *et al*. (2009) reported that *T. hamatum* and *T. harzianum* were
grown at eight different temperatures viz., 0, 5, 10, 15, 20, 25, 30 and 35°C for ten days. Both the isolates grew well at all the temperatures tested. However, maximum dry weight of mycelium was recorded at 25°C giving 421.2 mg weight in *T. harzianum* and 380.0 mg weight in *T. hamatum*. There was a significant decrease in growth of the test fungus on either side of the optimum temperature (25°C). Behzad *et al.* (2008) reported that *Trichoderma* isolates mycelial growth was statistically differed in respect of the tested temperatures. The isolates *T. hamatum* T612, *T. harzianum* T447, *T. harzianum* T969 and *T. hamatum* T614 showed the highest mycelial growth at 25°C whereas isolate *T. virens* T523 showed maximum at 30°C. Growth of *Trichoderma* is directly dependent on the temperature of the environment and temperatures between 20°C and 37°C were good for both growth and sporulation of *T. viride* (Jaiswal *et al.* 2003). A broad range of temperature tolerance for growth and sporulation of *Trichoderma* is very interesting feature for suitability of the antagonists as biocontrol agents.

**Effect of nutrients on growth of Trichoderma**

The knowledge of nutritional requirements is essential in the cultivation of microorganisms using any cultural technique. The carbohydrates, proteins, lipids, nucleic acids are made up of macro elements like carbon, hydrogen, nitrogen, sulphur, phosphorus and these are involved in mechanisms like host pathogen interaction and self defense mechanisms. Carbon is the major component and the molecules of carbon also contribute to oxygen and hydrogen. Knowledge on these factors may lead to a better understanding of the population dynamics of *Trichoderma* in soil and other habitats. Also, studies on the factors affecting growth and sporulation of *Trichoderma* *in vitro* can be used in the technology of large scale production of biomass for biocontrol use. Although, there is extensive literature on the enzymes and biological
control potential of *Trichoderma*, studies on local isolates and their ecological requirements in relation to their growth and sporulation is required. Krystofova *et al.* (1998) have studied the effect of phosphoinositides and inositol phosphate on growth and photoconidiation in *T. viride*. Danielson and Davey (1973a, b), reported the ability of *T. viride* to use a variety of carbon as well as organic and inorganic nitrogen compounds as sole source of carbon or nitrogen. Papavizas (1985) has stated that different species of *Trichoderma* have their own ecological preferences. These observations are interesting for the ecological behavior of this antagonistic fungus. Maximum growth of *T. viride* was recorded when peptone was used in the medium. This superior growth in peptone among nitrogen compounds may be attributed to its being a complex mixture of peptides and amino acids containing some water-soluble vitamins (Cochrane, 1958). Other workers have also reported that fungi are favored by peptone as a source of carbon in the medium (Jaiswal *et al.* 2003). Among amino acids aspartic acid and asparagine were good for the growth and sporulation of *T. viride* (Chattopadhyay and Nandi, 1981).

It was remarkable to note that *T. viride* preferred ammonium forms of nitrogen. This may be important for *Trichoderma* spp. as biocontrol application in relation to agricultural practices. Ammonium fertilizers are commonly used in agronomic practices. Therefore, information on these factors affecting the population dynamics of this biocontrol agent will be useful if the system is to be used for biocontrol of fungal pathogens where *Trichoderma* is a component. It has been observed by Nicholas (1965) that ammonium ions can diffuse more quickly into the cells and then is utilized quickly. Other explanation for better growth of *T. viride* with ammonium forms of nitrogen may be because of the fact that uptake of ammonium nitrogen reduces the pH of the surrounding (MacNish, 1988). In addition, *T. viride*
grows better when the pH of the medium is lowered; this situation provides *T. viride* a better opportunity to grow. This lowering of pH due to quick and efficient ammonium uptake (Nicholas, 1965) may have contributed to a better growth and sporulation of *T. viride* with ammonium forms of nitrogen. Simon and Sivasithamparam (1988b) have proposed that application of ammonium sulphate results into an increased activity of *Trichoderma* spp. with a consequent increase in suppression of *Gaeumannomyces graminis* var. *tritici*. When ammonium treated soil was limed to increase the pH, a reduction in the activity of *Trichoderma* occurred. Therefore, it seems obvious that efficient utilization of ammonium forms of nitrogen is related to pH changes in the medium, which may be a reason for an increased growth and sporulation of *T. viride*. It has also been observed that elevation of soil pH results into increased severity of several diseases (Simon and Sivasithamparam 1988b, 1990). This may be correlated to a decreased activity of antagonists like *Trichoderma*, which form some times up to 70% of the total number of fungi in certain soils (Simon and Sivasithamparam, 1988a, c).

Monga (2001) studied the nutritional requirements of biocontrol agents and reported that nitrogen source was essential for the spore germination. Effect of carbon sources on sporulation and growth was also showed that sporulation of *T. viride* was poor on all carbon sources while the sporulation of *G. virens* was excellent on all carbon sources except maltose. *T. koningii* and *T. harzianum* showed excellent sporulation on sucrose and glucose respectively. Fructose was best carbon source for the biomass production of *T. viride* and *G. virens*. However, *T. koningii* and *T. harzianum* produced maximum biomass on maltose and glucose respectively. The antifungal metabolites produced in culture filtrates of bioagents raised on different carbon and nitrogen sources when tested against root rot pathogens showed varying
inhibitions. Jayaswal et al. (2003) studied the influence of physiological and environmental factors on an antagonistic strain of *T. viride* RSR7 to optimize its biocontrol potential. The growth and sporulation of *T. viride* was greatly influenced by various carbon and nitrogen sources, and the environmental factors such as pH and temperature. The best growth and sporulation of *T. viride* was observed when sucrose, peptone and trehalose were supplemented in the medium as sole carbon sources. Rhamnose, pyruvic acid and sorbitol also supported a good growth. Growth and sporulation was also affected by various nitrogen sources. Growth and sporulation both were favoured by ammonium forms of nitrogen compared to nitrite or nitrate forms. Urea did not support either growth or sporulation. Among amino acids, glutamic acid, asparagine, leucine, aspartic acid, and alanine supported good growth as well as sporulation. *T. viride* was able to utilize large number of amino acids as sole nitrogen source. Proline was good for growth, but not for sporulation. Syahiddin Dahlan Said (2007) reported that spore production of biocontrol agent *T. harzianum* UPM 29 was significantly affected by glucose concentration and CN ratio of the culture media. Biomass increased with increase in initial glucose concentration in the range of 10 to 50 g/l. The same correlation was also observed for the maximum spore concentration reached in the range of 10 to 30 g/l. The CN ratio however did not significantly affect the spore viability, but increasing glucose concentration from 10 to 50 g/l increased spore viability. Jitendra Mehta et al. (2012) studied the effect of different carbon and nitrogen sources on *in vitro* mycelial growth and sporulation of *T. viride* and *B. bassiana*. *T. viride* and *B. bassiana* showed higher growth potentials on almost all the nutrient sources studied. Among the nitrogen sources (sodium nitrate, potassium nitrate and ammonium sulphate) tested, *T. viride* showed the high biomass product in ammonium sulphate whereas *B. bassiana* showed maximum
production of biomass in potassium nitrate. Among the carbon sources used (fructose, lactose and dextrose), *T. viride* showed maximum production of biomass on dextrose whereas *B. bassiana* on fructose.

**Effect of cultural conditions on production of extracellular hydrolytic enzymes**

Selection of biocontrol agents as well as understanding the mechanisms involved in the antagonistic effect of *Trichoderma* spp. on plant pathogens are important in designing effective and safe biocontrol strategies. Different isolates of *Trichoderma* have various strategies for fungal antagonism and indirect effects on plant health also vary. Therefore, one of the most interesting aspects of biology is the study of the mechanisms employed by biocontrol agents to affect disease control. Possible mechanisms of antagonism employed by *Trichoderma* spp. includes nutrient and niche competitions, antibiosis by producing volatile components and non-volatile antibiotics (Harman and Hadar, 1983; Dennis and Webster, 1971b,c) that are inhibitory against a range of soil borne fungi, as well as parasitism (Dennis and Webster, 1971a). Also synergism between different forms of action modes occurs in the natural condition in biocontrol of fungal pathogens. It is widely known that environmental parameters such as abiotic (soil type, soil temperature, soil pH, water potential and such like) and biotic (plant species and variety, microbial activity of the soil) factors as well as other factors such as method and timing of applications may have influence on the biological control efficacy of *Trichoderma* isolates.

A wide range of prokaryotic and eukaryotic microorganisms have the potential to produce cell-wall-degrading enzymes when chitin or isolated fungal cell wall material are present in the growth medium (Shahai and Manocha, 1993; Pitson et al. 1993). The direct mycoparasitic activity of *Trichoderma* species has been proposed as one of the major mechanisms for their antagonistic activity against phytopathogenic
fungi (Baker and Paulitz, 1996; Chet, 1987; Chet, 1990). *Trichoderma* spp. attach to the host hyphae by coiling, hooks or appressorium-like structures and penetrate the host cell walls by secreting hydrolytic enzymes such as a basic proteinase (Geremia, 1990), β-1,3-glucanase and chitinase (Elad et al. 1993).

Production of extracellular β-1,3-glucanases, chitinases and proteinase increases significantly when *Trichoderma* spp. are grown in media supplemented with either autoclaved mycelium or isolated purified host fungal cell walls (Carsolio et al. 1994; De la Cruz et al. 1995). These observations, together with the fact that chitin, β -1,3-glucan and proteins are the main structural components of most fungal cell walls (Peberdy, 1990), are the basis for the suggestion that hydrolytic enzymes produced by some *Trichoderma* spp. play an important role in destruction of plant pathogens (Chet, and Baker 1981). *Trichoderma* spp. (especially *T. harzianum* and *T. viride*) exhibit considerable variability among strains with respect to their biocontrol activity and host range (Sivan and Chet, 1992). El Katatny et al. (2000) screened twenty-four *Trichoderma* isolates and found a strain identified as *T. harzianum* Rifai to be the best producer of both chitinase and β -1,3-glucanase.

The physiological age of the mycelium used for inoculation was an important factor for enzyme production. Chitinase and β -1,3-glucanase production by *T. harzianum* was highest by using 3-day-old mycelium as inoculum (El Katatny et al. 2000). Ulhoa and Peberdy (1991) found in their study on chitinase production using washed mycelium of another *T. harzianum* strain that both mycelium harvested at 18 h (exponential phase) and at 24 h (early stationary phase) showed similar production of extracellular chitinase. El Katatny et al. (2000) showed the enzyme production using agar discs was higher than using spore suspensions of *T. harzianum*, which was
in agreement with the results from Seyedi-Rashti (1994) on the xylanase production by *T. harzianum*.

Most of the chitinolytic enzyme systems reported in the literature is inducible (Ulhoa and Peberdy, 1991; Gupta *et al.* 1995; Monreal and Rees, 1969). Monreal and Reese (1969) suggested that the most probable inducers of chitinase in *Serratia marcescens* are soluble oligomers derived from chitin, but not the monomer (GlcNAc). Ulhoa and Peberdy (1991) suggested that products of chitin degradation also regulate chitinase synthesis in *T. harzianum* 39.1. The findings of El Katatny *et al.* (2000) were in agreement with these findings and found high chitinase activity only in cultures supplied with chitin but not with other polymers such as cellulose and chitosan, which is further indicative of induction. Previously, in *T. harzianum* 39.1 neither chitobiose nor N-acetylglucosamine promoted enzyme production (Ulhoa and Peberdy, 1991). El Katatny *et al.* (2000) found that chitinase activity increased with increasing chitin concentration up to 1%.

Ulhoa and Peberdy (1991) suggested that chitinase production was substrate concentration dependent and above 0.5 % (w/v) chitin there was no further promotion of synthesis. Elad *et al.* (1982) reported that chitinase secretion into the growth medium by *T. harzianum* was increased up to 1% chitin. Chitinase production decreased with glucose or GlcNAc (0.5 %) addition along with 0.5 % chitin (El Katatny *et al.* 2000). This is consistent with the results obtained by Mahadevan and Crawford (1997), who found that various hexoses and pentoses repressed enzyme production by *Streptomyces lydicus* WYEC108. The same results were obtained for β-1,3-glucanase production when glucose (0.5 %) was added along with 0.5 % laminarin, which decreased the activity to nearly 50% when compared to laminarin.
alone. Production of β-1,3-glucanase under otherwise inducing conditions was inhibited by the addition of glucose (Vazquez-Garciduenas et al. 1998). Results of El Katatny et al. (2000) indicated the highest production of β-1,3-glucanase in the presence of laminarin (β-1,3-glucan), pustulan (β-1,6-glucan) and pullulan (1,6-glucan) in descending order of efficacy, suggesting that the induction patterns of the enzyme may vary in response to the glucan structure and that β-1,3-glucanase induction depends on the type of linkage (Vazquez-Garciduenas et al. 1998). Elad et al. (1982) reported that T. harzianum secreted β-1,3-glucanase when grown on laminarin or starch (as a component of wheat bran) and not on glucose, concluding that the enzyme is produced inductively. El Katatny et al. (2000) reported significant variation in activities of enzyme was observed in presence of cellobiose, lactose or xylose. Enzyme production increased up to 0.75 % of laminarin concentration, but decreased at higher concentrations. β-1,3-glucanase, in contrast to chitinase, only partially repressed in the presence of sucrose or glucose, suggesting that a certain activity of this enzyme is produced constitutively. A low level of β-1,3-glucanase and no chitinase was measured when the mycoparasite was deprived of a carbon source and its growth was low. Although the exact reasons for this remain to be established, it seems probable that in conditions of carbon starvation and poor growth the fungus actively secrets some levels of hydrolytic enzymes. This type of control has been demonstrated in other fungi such as Neurospora crassa (Del Rey et al. 1979) and S. glucanicum (Rapp, 1989). Alternatively, the production of β-1,3-glucanase in carbon-deprived media could be related to metabolism, such as mobilization of wall glucans (Del Rey et al. 1979) and to some morphogenetic functions and changes (Santos et al. 1977). No production or low level of chitinase activity in deprived carbon source or
glucose medium confirms that chitinase enzyme is produced inducibly not constitutively.

Chitinase and β-1,3-glucanase production was favored by acidic pH (pH 6.0 and 5.5, respectively). Acidic pH was also reported to be an important growth parameter in the production of chitinases and β-1,3-glucanases in mycoparasite *T. harzianum* (Elad *et al.* 1982) and the thermophilic *Streptomyces* spp. (Tweddell *et al.* 1994) respectively. Ulhoa and Peberdy (1991) found that the production of chitinase was markedly affected by pH and the optimum was at pH 6.0. Acidic pH was also reported to be an important growth parameter in production of chitinase and β-1,3-glucanase in *T. harzianum* (Someshwar Bhagat and Sitansu Pan, 2008). The pH with an optimum temperature is required for production of enzymes (Jijakli and Lepoivre, 1998; Someshwar Bhagat and Sitansu Pan, 2008).

Levels of enzymes produced by *T. harzianum* decreased within six days of incubation with fresh mycelium; however, there was a continuous increase in enzymes production with dried mycelium. This effect might be a result of some metabolites produced by the phytopathogen against the antagonist (El Katatny *et al.* 2000). Crude culture filtrates of *T. harzianum* possessing β-1,3-glucanase and chitinase activities had the ability to release reducing sugars (glucose, GlcNAc) from dried or fresh mycelium of the phytopathogenic fungus *S. rolfsii*. These results are in agreement with those of Tweddell *et al.* (1994), who demonstrated that lytic enzymes of *Stachybotrys elegans* degrade *R. solani* mycelium. The culture filtrates of *T. harzianum* showed lytic activity on purified cell walls of *Saccharomyces cerevisiae* and *Botrytis cinerea* (De la Cruz *et al.* 1995). Culture filtrates have been used by Calistru *et al.* (1997) to demonstrate the possible presence and role of fungal metabolites in the process of antagonist behavior of *Trichoderma* spp. Antifungal
potential of culture filtrates of two *Trichoderma* spp. on wood decay fungi and filtrates of *T. harzianum* were found to be suppressive of the white-rot pathogen, *S. cepivorum* (Papavizas *et al.* 1982). Calistru *et al.* (1997) suggested that *Trichoderma* culture filtrates had only marginally curtailed pathogen growth, in contrast with, metabolites of *Gliocladium virens*, which completely inhibited the growth of *S. cepivorum* (Jackson *et al.* 1991b). The macroscopic and microscopic observations of plates with the pathogens cultured with filtrates of *Trichoderma* spp. suggested that *Trichoderma* spp. were able to induce morphological alterations in both *Aspergillus flavus* and *Fusarium moniliforme* (Calistru *et al.* 1997). Inhibition of *S. rolfsii* depended on the carbon source used and correlated with the level of chitinase activity, rather than β-1,3-glucanase activity. However, there was still some inhibition observed at low levels of both chitinase and -1,3-glucanase. However, strong synergism was observed with chitobiosidase, β-1,3-glucanase and also with mycotoxic metabolites (Lorito *et al.* 1993).

**Mechanism of Biocontrol**

Biological control involving *Trichoderma* spp. operates by way of mycoparasitism, antibiosis and competition. Mechanism of mycoparasitism includes interactions like coiling of hyphae around the pathogen and penetration by haustoria and lyses of the cell organells. *Trichoderma* spp. secret some cell wall degrading enzymes like chitinases and also secret some volatile and non volatile compounds which enter into the cell in the form of signal and triggers the secondary messengers and it alter the metabolic pathway of the pathogen. Many of biocontrol agents produce volatile or non-volatile compounds in to the medium which are inhibitory to the pathogen.
Some hydrolytic enzymes secreted by *Trichoderma* spp. involved in antagonism against some filamentous fungi. Among these enzymes, β-1,6-glucanases were involved in the degradation of polymers maintaining the fungal cell wall structure and lytic enzymes are β-1,3-glucanase, 42-kda endochitinase, cellulase, chitinase, proteinase, beta-xylosidase, endo-1,4-beta d-glucanases etc.

**Modes of action by biocontrol agents**

No single mode of action for *Trichoderma* spp. against fungal plant parasites function alone. There are several mechanisms of action suggested for *Trichoderma* spp., mycoparasitism, antibiosis, competition for nutrients or space, tolerance to stress through enhanced root and plant development, induced resistance, solubilization and sequestration of inorganic nutrients, and inactivation of the pathogens enzymes (Samuels, 1996). The first three were the ones by which these fungi have always been considered to function (Harman, 2000).

**Fungistasis:** Fungistasis is a natural mechanism of inhibition of growth of fungi imposed by soil microbes and overcome by the nutrient-rich rhizosphere of a host plant, allowing soilborne pathogens to infect the plant. (Papavizas and Lumsden, 1980). Good antagonists are usually able to overcome the fungistatic effect of soil that results from the presence of metabolites produced by other species, including plants, and to survive under very extreme competitive conditions. *Trichoderma* strains grow rapidly when inoculated in the soil, because they are naturally resistant to many toxic compounds, including herbicides, fungicides and pesticides such as DDT, and phenolic compounds (Chet *et al*. 1997), and because the strains recover very rapidly after the addition of sublethal doses of some of these compounds. Resistance to toxic compounds may be associated with the presence in *Trichoderma* strains of ABC transport systems (Harman *et al*. 2004). Because of this reason, preparations of
Trichoderma strains are very efficient in controlling several phytopathogens, such as R. solani, P. ultimum or S. rolfsii, when alternated with methyl bromide, benomyl, captan or other chemicals (Vyas and Vyas, 1995). Denielson and Davey (1973b) found that the sensitivity to fungistasis was higher in neutral or alkaline than in acid soils.

**Antibiosis:** Antibiosis occurs during interactions involving low-molecular-weight diffusible compounds or antibiotics produced by *Trichoderma* strains that inhibit the growth of other microorganisms. Most *Trichoderma* strains produce volatile and non-volatile toxic metabolites that impede colonization by antagonized microorganisms. The importance of antibiotics for biocontrol activity was demonstrated in several studies. Dennis and Webster (1971a) found that many *Trichoderma* strains produced volatile and nonvolatile antibiotics. In 1983, Howell isolated and described a new antibiotic, gliovirin, from *T. virens* that was strongly inhibitory to *Pythium ultimum* and a *Phytophthora* species, but not to *R. solani*. Lumsden et al. (1992) found that suppressive activity of *T. virens* to damping-off of zinnias, incited by both *R. solani* and *P. ultimum*, was correlated with production of the antibiotic gliotoxin by the biocontrol agent. *Trichoderma* spp. produces 43 substances that have antibiotic activity which do not include enzymes, of these, alkyl pyrones, isonitriles, polyketides, peptaibols, dikeyopiperazines, sesquiterpenes and steroids have been associated with biocontrol activity of some species and strains of *Trichoderma* (Howell, 1998). Most *Trichoderma* strains produce volatile and non-volatile toxic metabolites, among these metabolites, the production of harzianic acid, alamethicins, tricholin, peptaibols, antibiotics, 6-pentyl-α-pyrone, massoilactone, viridin, gliovirin, glisoprenins, heptelidic acid and others have been described (Vey et al. 2001).
The combination of hydrolytic enzymes and antibiotics results in a higher level of antagonism than that obtained by either mechanism alone (Howell, 1998; Monte, 2001). Synergetic effects between an endochitinase from *T. harzianum* and gliotoxin, and between hydrolytic enzymes and peptaibols on conidial germination of *B. cinerea* is well known (Howell, 2003). A mutant from strain 2413 that had higher levels of extracellular enzymes and of pyrone performed better than the wild-type in *in-vitro* confrontation experiments against *R. solani* and in assays of grape protection against *B. cinerea*, both under repression (only pyrones were produced) and derepression conditions (enzymes and pyrones were produced) (Rey *et al.* 2001). Similarly, in transformants of strain 2413 that over expressed chitinase, biocontrol activity correlated with chitinase production, except for one transformant which was unable to completely overgrow *R. solani* and did not produce pyrone, so that synergism did not occur (Limon *et al.* 2004). Sequential roles of antibiosis and hydrolytic enzymes during fungal interactions have also been described (Howell, 2003). When combinations of antibiotics and several kinds of hydrolytic enzymes were applied to propagules of *B. cinerea* and *F. oxysporum*, synergism occurred, but it was lower when the enzymes were added after the antibiotics, indicating that cell-wall degradation was needed to establish the interaction (Howell, 2003).

**Competition and rhizosphere competence:** Competition for space or nutrients is considered one of the classical mechanisms of biocontrol by *Trichoderma* spp. (Elad *et al.* 1999). The competition primarily carbon, nitrogen and iron is one of the methods of the biological control of soil borne plant pathogens (Scher *et al.* 1984). *Trichoderma* species are generally considered to be aggressive competitors and the ability of *Trichoderma* to compete was varied with species (Wardle *et al.* 1993). Competition through rhizosphere competence is a mechanism that has gained
importance (Howell, 2003). It is an important mechanism because a biocontrol agent cannot compete for space and nutrients if it is unable to grow in the rhizosphere. Lo et al. (1996) found that a strain of *T. harzianum* (T-22) was strongly rhizosphere competent and able to control several plant pathogenic fungi including *R. solani* and it reduced the initial disease severity by as much as 71% on a variety of crops.

Starvation is the most common cause of death for microorganisms, so that competition for limiting nutrients results in biological control of fungal phytopathogens (Chet et al. 1997). For instance, in most filamentous fungi, iron uptake is essential for viability (Eisendle et al. 2004), and under iron starvation, most fungi excrete low-molecular-weight ferric-iron-specific chelators, termed siderophores, to mobilize environmental iron (Eisendle et al. 2004). Subsequently, iron from the ferrisiderophore complexes is recovered via specific uptake mechanisms. In *Aspergillus fumigatus* and *Aspergillus nidulans*, siderophore biosynthesis is negatively regulated by carbon source (Eisendle et al. 2004). In *Ustilago maydis*, gene products related to iron uptake affect the development of plant disease (McIntyre et al. 2004). Some *Trichoderma* biocontrol agents produce highly efficient siderophores that chelate iron and stop the growth of other fungi (Chet and Inbar, 1994). For this reason, soil composition influences the biocontrol effectiveness of *Pythium* by *Trichoderma* according to iron availability. In addition, *T. harzianum* T35 controls *Fusarium oxysporum* by competing for both rhizosphere colonization and nutrients, with biocontrol becoming more effective as the nutrient concentration decreases (Tjamos et al. 1992). The advantage of using *Trichoderma* to control *Botrytis cinerea* is the coordination of several mechanisms at the same time, thus making it practically impossible for resistant strains to appear. Among these mechanisms, the most important is nutrient competition, since *B. cinerea* is
particularly sensitive to the lack of nutrients. *Trichoderma* has a superior capacity to mobilize and take up soil nutrients compared to other organisms. The efficient use of available nutrients is based on the ability of *Trichoderma* to obtain ATP from the metabolism of different sugars, such as those derived from polymers wide-spread in fungal environments: cellulose, glucan and chitin among others, all of them rendering glucose (Chet *et al*. 1997).

The soil environment influences spore germination, chlamydospore formation and the production of secondary metabolites, such as siderophores (Eisendle *et al*. 2004), antibiotics (Chet *et al*. 1997) and enzymes (Arst and Penalva, 2003). There are abundant data in the literature describing rhizosphere modifications by BCAs that impede colonization by pathogens; for instance, antibiotics and toxic metabolites produced by entamopathogenic, mycoparasitic or mycoherbicide fungi (Vey *et al*. 2001). Environmental pH is one of the major factors affecting the activity of both *Trichoderma* and pathogenicity factors secreted by different microorganisms. Some antibiotics are degraded at high pH and air drying and low pH may induce enzyme degradation by acidic proteases (Delgado-Jarana *et al*. 2000; Delgado-Jarana *et al*. 2002). The growth of many fungi is inhibited by weak acids, such as sorbic acid, due to a rapid decline in cytosolic and vacuolar pH (Arst and Penalva, 2003). Therefore, the ability to thrive over a wide range of external pH conditions is an important component of the complex set of characteristics of *Trichoderma*, that made to best adapted to acidic soil, encounters during its interaction with other organisms. One of the mechanisms of *Trichoderma* strains for achieving colonization and pathogen control in a dynamic pH environment is an appropriate response to each given pH condition. Some strains of *T. harzianum* control external pH strictly, ensuring optimal values for their own secreted enzymes (McIntyre *et al*. 2004). Different extracellular
proteins are synthesized at different pHs. In addition, at the transcriptional level, several proteases, glucanases, cell-wall proteins and a glucose transporter are pH-controlled, which suggests a pH-dependent transcriptionally controlled response of different enzymes. External pH is also important to pathogens because their pathogenicity factors are produced only within a very narrow range of pHs (Prusky and Yakoby, 2003), so that pH modification determines the pathogen's ability to successfully colonize and invade the targeted host. *Trichoderma* strains able to modify external pH and to adapt their own metabolism to the surrounding growth conditions would consequently reduce the virulence of phytopathogens because most pathogenicity factors could not be synthesized.

**Induced resistance:** *Trichoderma* strains are always associated with plant roots and root ecosystems. Some authors have defined *Trichoderma* strains as plant symbiont opportunistic avirulent organisms, able to colonize plant roots by mechanisms similar to those of mycorrhizal fungi and to produce compounds that stimulate growth and plant defense mechanisms (Harman *et al.* 2004). Some *Trichoderma* strains establish long-lasting colonization of plant roots and penetrate into the epidermis. There, they produce or release compounds that induce localized or systemic plant resistance responses (Harman *et al.* 2004). Plants react against fungal invasion by synthesizing and accumulating phytoalexins, flavonoids and terpenoids, phenolic derivatives, aglycones and other antimicrobial compounds. *Trichoderma* strains are generally more resistant to these compounds than most fungi. This resistance, considered an essential requirement for plant colonization, has been associated with the presence of ABC transport systems in *Trichoderma* strains (Harman *et al.* 2004). Induction of resistance in host plant by treatment with *Trichoderma* species is another biological control mechanism (Howell, 2003). Some strains of fungi in the genus *Trichoderma*
colonize and penetrate plant root tissues and initiate a series of morphological and biochemical changes in the plant, which finally leads to induced systemic resistance (ISR) in the entire plant (De Meyer et al. 1998). Yedidia et al. (1999) showed that inoculating roots of 7-day-old cucumber seedlings in a hydroponic system with T. harzianum (T-203) spores initiated plant defense responses in both roots and leaves of treated plants. They also reported that hyphae of the biocontrol fungus penetrated the epidermis and upper cortex of the cucumber root. The plant response was marked by an increase in peroxidase activity (often associated with the production of fungitoxic compounds), an increase in chitinase activity, and the deposition of callose-enriched wall appositions on the inner surface of cell walls (Howell, 2003). Yedidia et al. (2000) showed that inoculation of cucumber roots with T. harzianum (T-203) induced an array of pathogenesis-related proteins, including a number of hydrolytic enzymes which were similar to plants treated with a chemical inducer (2,6-dichloroisonicotinic acid) of disease resistance displayed defense responses.

**Solubilization and sequestration of inorganic plant nutrients:** There are very few reports on Trichoderma strains that produce growth factors which have been detected and identified in the laboratory (auxins, cytokinins and ethylene) (Arora et al. 2002). Many filamentous fungi that produce phytohormones, such as indole acetic acid (IAA) and ethylene, whose metabolic pathways have been identified (Arora et al. 2002; Osiewacz, 2002). Trichoderma strains that produce cytokinin-like molecules, e.g. zeatin and gibberellin (GA3 or GA3-related), have been recently detected. The controlled production of these compounds could improve biofertilization (Osiewacz, 2002). Plant nutrients undergo sometimes transitions in soil from soluble to insoluble forms that influence their accessibility and absorption by roots. These transitions may be influenced by microorganisms (Altomare et al. 1999). Together with the synthesis
or stimulation of phytohormone production, most *Trichoderma* strains acidify their surrounding environment by secreting organic acids, such as gluconic, citric or fumaric acid (Gomez-Alarcon *et al.* 1994). These organic acids result from the metabolism of other carbon sources, mainly glucose, and in turn, are able to solubilize phosphates, micronutrients and mineral cations including iron, manganese and magnesium (Harman *et al.* 2004). Therefore, the addition of *Trichoderma* to soils where these cations are scarce results in biofertilization by metal solubilization and an increase in crop productivity. *In vitro*, strain of *T. harzianum* produces a large number of chemicals to solubilize rock phosphate, Zn, Mn4+, Fe3+, and Cu2+ and increase iron availability and enhance iron uptake (Altomare *et al.* 1999).
Coiling of *Trichoderma* around a pathogen (Plant Biocontrol by *Trichoderma spp.* Ilan Chet, Ada Viterbo and Yariv Brotman)
**Mechanism Of Disease Resistance & Control By Trichoderma**

Trichoderma colonizes the root surface and establishes an interaction zone in the root cortex. Trichoderma then release enzymes and other bioactive molecules and cell wall fragments which elicit a resistance response in the plant. The plant produces cell wall deposits which cause the Trichoderma to become avirulent.

**Mycoparasitism By Trichoderma**

Trichoderma mycelium also outcompetes pathogen growth, and colonizes plant rhizosphere.
**Mycoparasitism**: Mycoparasitism is a complex process including several steps. The initial interaction shows that the hypha of the mycoparasites grows directly towards its host (Chet *et al.* 1981). When the mycoparasite reaches the host, its hypha coils it or attaches to it by forming a hook-like structure. Following these interactions hypha sometimes penetrates the host mycelium, apparently, by partially degrading its cell wall (Elad *et al.* 1983). The control of *Rhizoctonia solani* and *Pythium ultimum* and by *Trichoderma* species, including *T. harzianum*, may be affected through direct penetration of host hyphae (Dennis and Webster, 1971b; Benhamou and Chet, 1993). They grow toward hyphae of other fungi, coil about them in a lectin mediated reaction, and degrade cell walls of the target fungi by the activity of enzymes, which may be associated with physical penetration of the cell wall (Chet, 1987). The pattern of induction differs from one *Trichoderma* strain to another. It is believed that fungi secrete exochitinases constitutively at low levels. When chitinases degrade fungal cell walls, they release oligomers that induce exochitinases, and attack begins. Mycoparasitism involves morphological changes, such as coiling and formation of appressorium-like structures, which serve to penetrate the host and contain high concentrations of osmotic solutes such as glycerol (McIntyre *et al.* 2004). *Trichoderma* attaches to the pathogen with cell-wall carbohydrates that bind to pathogen lectins. Once *Trichoderma* is attached, it coils around the pathogen and forms the appresoria. The following step consists of the production of cell-wall-degrading enzymes (CWDEs) and peptaibols (Howell, 2003), which facilitate both the entry of *Trichoderma* hypha into the lumen of the parasitized fungus and the assimilation of the cell-wall content. The significance of lytic enzymes was reviewed by Viterbo *et al.* (2002).
Production of extracellular hydrolytic enzymes: Mycoparasitism is considered an important mechanism of biological control and probably depends on the production of lytic enzymes including β-1,3-gluconase and proteases (Haran et al. 1996). Several chitinolytic enzymes have been reported in *T. harzianum* including endochitinases, exochitinases and 1, 4-β-N-acetylglucosaminidases which are induced during growth in liquid medium containing chitin as carbon source. Enzymes such as chitinases and/or glucanases produced by the biocontrol agent are responsible for suppression of the plant pathogen. These enzymes function by breaking down the polysaccharides, chitin, and β-glucans that are responsible for the rigidity of fungal cell walls, thereby destroying cell wall integrity (Howell, 2003).

*Trichoderma* spp. secret some cell wall degrading enzymes like chitinases and also secret some volatile and non-volatile compounds which will enter into the cell in the form of signal and triggers the secondary messengers and it will alter the metabolic pathway of the pathogen. The direct mycoparasitic activity of *Trichoderma* species has been proposed as one of the major mechanisms for their antagonistic activity against phytopathogenic fungi (Baker and Paullitz, 1996; Chet, 1987; Chet, 1990). *Trichoderma* spp. attach to the host hyphae by coiling, hooks or apressorium-like structures and penetrate the host cell walls by secreting hydrolytic enzymes such as a basic proteinase (Geremia et al. 1993), β-1,3-glucanase and chitinase (Elad et al. 1993). Production of extracellular β-1,3-glucanases, chitinases and proteinase increases significantly when *Trichoderma* spp. are grown in media supplemented with either autoclaved mycelium or isolated purified host fungal cell walls (Carsolio et al. 1994; De la Cruz et al. 1995). The degraded cell wall fragments of target fungi are highly potent inducers of enzymes, induction, an enhancement in *Trichoderma* growth (Harman, 2006). These observations, together with the fact that chitin, β-1,3-glucan
and proteins are the main structural components of most fungal cell walls (Peberdy, 1990), are the basis for the suggestion that hydrolytic enzymes produced by some Trichoderma spp. play an important role in destruction of plant pathogens (Chet and Baker 1981). The chitinolytic system of Trichoderma comprises many enzymes and the list of its components is rapidly being updated as new enzymes and genes are reported. Chitinases are divided into 1,4-acetylglucosaminidases (GlcNAcases), endochitinases and exochitinases. T. virens transformants over expressing Chit42 showed significantly enhanced biocontrol activity compared with the wild-type when assayed against R. solani in cotton seedlings (Howell, 2003). It has been shown that β-1,3-glucanases inhibit spore germination or the growth of pathogens in synergistic cooperation with chitinases (Benitez et al. 1998; El-Katatny et al. 2001) and antibiotics. The hydrolase β-1,3-glucanase has been purified from strain 2413, and their genes isolated and over expressed, which resulted in increased biocontrol activity of the transformant strains (Ait-Lashen et al. 2001).
Penetrate the host cell walls by secreting lytic enzymes

a. Chitinases
b. Proteases
c. Glucanases
Synergism among lytic enzymes and between enzymes and antibiotics suggests formulations to test mixtures of *Trichoderma* transformants that produce different enzymes, in order to improve the antagonistic effects of BCAs on phytopathogenic fungi. For instance, in experiments carried out using cellophane, which show the effect of enzymes and secondary metabolites secreted by BCAs, *T. harzianum* wild-type inhibited the growth rate of *B. cinerea* by 30% and transformants expressing either a 1,3-glucanase, a chitinase, or a -1,6-glucanase inhibited the growth rate of *B. cinerea* by 60%. Transformants were differently combined in order to test synergism among the enzymes secreted against several phytopathogens. The combination that overproduced chitinase and 1,3-glucanase was more effective than the individual transformants in inhibiting *Rhizoctonia meloni*, whereas using other combinations the inhibition was not improved (Ait-Lashen *et al.* 2001).

Research on the mechanisms responsible for the biocontrol exerted by *Trichoderma* spp. on phytopathogenic fungi have led to a better understanding of such mechanisms, as well as to the isolation of several genes encoding either enzymes and structural or regulatory proteins, or components of signaling pathways that are involved in processes such as the specific recognition of hosts by *Trichoderma* strains. These tools will allow the isolation of improved strains and thus of more efficient formulations to control fungal pathogens in pre- and post-harvest periods.

**Dual culture**

The dual culture technique, also known as biculture, cross culture or paired culture has been extensively used for preliminary screening of large populations of fungal biocontrol agents. This method was followed to study the antagonistic property of *Trichoderma* isolates. In this technique, the pathogen and the biocontrol
agent were allowed to interact in a Petri dish under optimum conditions for both the pathogen and biocontrol agent. The inhibition is recorded either in the form of the inhibition zone produced or the overgrowth of the pathogen by the biocontrol agent.

Nagamani et al. (2013) evaluated 12 Trichoderma isolates against S. rolfsii by dual culture and reported the maximum inhibition with TRI6, which was significantly superior over other isolates to an extent of 82.1% inhibition followed by TRI5 (74.1%) and lowest % inhibition was recorded with TRI3 with 25.4% inhibition. Six out of 16 isolates of Trichoderma species suppress the development of A. alternata mycelia more than 55%. Many of them are tolerant and are compatible with more fungicide active ingredients (Umamaheswari et al. 2009).

The antagonistic activity of Trichoderma isolates was reported by Singh and Singh (2003), Tripathi and Khare (2005) and Srivastava et al. (2009). Faheem Amin et al. (2010) tested six isolates of Trichoderma spp. for their ability to inhibit soil borne pathogens of different vegetables viz., Rhizoctonia solani (isolates from tomato), Sclerotium rolfsii (causing collar rot of tomato) and Sclerotinia sclerotiorum (causing web blight of beans) under in vitro conditions. The isolate T. viride (Tv-2) highly inhibited the mycelial growth (71.41% over control) in case of R. solani followed by T. viride (Tv-1) and T. harzianum (Th-1) showing 65.71 and 60.51 per cent inhibition over control, respectively. Similarly in case of S. rolfsii and Sclerotinia sclerotiorum, T. viride (Tv-1) proved to be best over all isolates in inhibiting mycelial growth of test pathogens. Further, all Trichoderma isolates significantly inhibited the production of sclerotia in test pathogens. Inhibition of Sclerotium growth by Trichoderma isolates was reported (Siva Raju et al. 2009; Sitansu Pan and Someshwar Bhagat, 2008). Siva Raju et al. (2009) reported that inhibition of S. rolfsii (infecting tobacco) growth by 10 isolates of Trichoderma was ranged from 50.66 to
70.66%. Such an identification of an effective isolates to suit the purpose of locality is important (Harman, 2000). Sitansu Pan and Someshwar Bhagat (2008) reported the inhibition of *Pythium* and *Sclerotium* growth by 10 isolates of *Trichoderma* by 57.8 to 62.6% respectively. Prasad et al. (2003) reported that *T. harzianum* was found superior than *T. viride* in reducing colony diameter of *S. rolfsii*. Inhibition of fungal growth by production of nonvolatile substances by *T. harzianum* was reported by Bunker and Mathur (2001). Siva Raju et al. (2009) reported the inhibition of *S. rolfsii* growth by production of nonvolatile compounds by the 10 isolates of *Tichoderma* varied from 62 to 92.35%. Sitansu Pan and Someshwar Bhat (2008) showed the inhibition of mycelial growth of *Sclerotium* and *Pythium* by production of nonvolatile compounds varying from 57.8 to 62.65% respectively. They also reported the inhibition of mycelial growth of *Sclerotium* and *Pythium* by production of volatiles by *T. viride*. Ghildyal and Pandey (2008) reported that three species of *Trichoderma* produced diffusible and volatile metabolites and showed maximum inhibition of *A. alternata* growth among the investigated pathogens. Morphological abnormalities in the pathogens’s structure were confirmed by Ghildyal and Pandey (2008).

The variation in antagonistic potential of different aggregates of *Trichoderma* against different pathogens has been reported (Bose et al. 2005; Sitansu Pan and Someshwar Bhat, 2007). Similarly Bell et al. (1982) reported significant differences in antagonistic potential of 77 isolates of *T. harzianum* against six fungal pathogens. Selective activity of both volatile and nonvolatile compounds by *Trichoderma* isolates against pathogens is well known (Dennis and Webster, 1971a, 1971b; Upadhyay and Mukhopadhyay, 1983). Upadhyay and Mukhopadhyay (1983) have claimed that the culture filtrate of *Trichoderma* spp. produces volatile and nonvolatile antibiotics effective against *S. rolfsii* and *R. solani*. Dubey (2000) reported that *T. virens*
inhibited 59.85% mycelial growth and 70% sclerotial production followed by *T. viride* and *T. harzianum*. Production of nonvolatile substances by *Trichoderma* spp. was considered as more advantageous than the volatile substances as they diffuse through the air filled pores in soil and actual contact between pathogen and antagonists might not be necessary for inhibition of the pathogen. Kumar and Dubey (2001) reported inhibition of growth of various soil borne pathogens by producing volatile and nonvolatile compounds by *Trichoderma* spp. This study revealed that different strains have different capacities as biological weapons in inhibiting the pathogen. Prokkola (1992) and Harman (2000) observed variation in mechanisms by which *Trichoderma* spp. exerts biocontrol activity and thus they also showed strain variation in controlling the pathogen. Siva Raju *et al.* (2009) reported HCN and siderophore production varied among the *Trichoderma* isolates. Isolates from tobacco host (EG, SM and DH) showed maximum production of siderophore whereas the isolate KP showed no production. Siderophores are the iron chelating agents, which deprive the surrounding pathogens of iron and thus concentrated iron is made available to the plant. In addition to competition for limited carbon sources in the rhizosphere, antagonism can be mainly attributed to the production of antibiotics, siderophores and cyanides (Kloepper *et al.*, 1980). Siva Raju *et al.* (2009) reported the production of IAA varied among the *Trichoderma* isolates. Glick *et al.* (1998) defined the role of microbial production of IAA and the response of host plant. With this system, microbe produced IAA may stimulate plant cell proliferation or elongation and result in plant production of ACC (1-aminocyclopropane-1-carboxylate). ACC is a precursor of ethylene and inhibits root elongation of the seedlings. The plant produced ACC is taken up by the microbial strains and is hydrolyzed by ACC deaminase, thereby lowering the pool of ACC and reducing the level of ethylene. The
net biological effects of this system are increased root elongation of the plant and nitrogen source for the microbes.

**Compatibility of biocontrol agents with fungicides**

*Trichoderma spp.* can thrive in diverse environmental conditions as aggressive colonizers of soil and the roots of plants and act as natural bioagent to protect plants from infection by soil-borne fungal pathogens. Laboratory experiments have to be conducted to test the possibility of combining fungicides and botanicals with *Trichoderma spp.* to work out their compatibility to devise a suitable integrated management of soil borne plant diseases. There is a need to study the compatibility of *Trichoderma* and chemicals in managing soil borne diseases of various crops under greenhouse and field conditions. Long term goal is to develop an integrated disease management strategy by combing *Trichoderma* and chemicals so as to prevent pathogen from gaining resistance as well as in building up of *Trichoderma* population levels in the soil that will be effective on a long term basis.

Deepti (2013) isolated 40 antagonists from groundnut rhizosphere and root endophytes and tested *in vitro*, and identified potential biocontrol agents GRE-9 (100%) and GRB-16 (85%) which are predominantly aggressive in inhibiting the mycelial growth of the pathogen in dual culture against *S. rolfsii*. Among the fungicides tested, mancozeb was most compatible with all antagonists. The fungal isolate GRHF4 was more compatible with mancozeb followed by copper oxychloride. Among all the fungicides tested mancozeb was found to be more compatible. Ashwani Tapwal *et al.* (2012) tested five fungicides viz., dithane M-45, ridomil, captaf, blue copper, bavistin and five botanicals viz., *Parthenium hysterophorus, Urtica dioea, Cannabis sativa, Polystichum squarrosum* and *Adiantum venustum* at different concentration. Among fungicides only captaf and blue copper had recorded
compatibility to some extent with *T. viride* and suggests that compatible fungicides and botanicals can be used with *Trichoderma* in an integrated disease management package to control soil borne plant pathogens. The compatibility index of blue copper with *T. viride* at different concentrations ranged in between 34.9 and 97.9%, followed by Captaf (16.7 - 25.0%). The per cent compatibility decreased with an increase in the concentration of fungicide. *T. viride* was not compatible with dithane, bavistin and ridomil in any level of selected concentration. Nishant Prakash and Smita Puri (2012) reported that *Trichoderma* spp isolate 1 showed relatively less sensitivity to mancozeb. On the other hand, it showed high sensitivity to fungicides like chlorothalonil, tilt, contaf and bavistin. Tilt, contaf and bavistin at 5μg/ml concentration showed maximum inhibition of *Trichoderma*. However, chlorothalonil at 100 μg/ml showed maximum inhibition (91.66%). Compatibility tests were conducted under *in vitro* condition to find out safer fungicides, pesticides, different cakes and botanicals against *Trichoderma* (Bagwan, 2010). Different fungicide, pesticides, cakes and botanicals were tested against *T. harzianum* (Th 09) and *T. viride* (Tv 11). Results indicate that among the fungicides tested, thiram (0.2%), copper oxychloride (0.2%) and mancozeb (0.2%) were found comparatively safer against *T. harzianum* and *T. viride* as compared to other fungicides. *Trichoderma* isolates were most sensitive to captan, tebuconazole, vitavax, propiconazole and chlorothalonil, but they were tolerant to all the pesticides and weedicides tested (Bagwan, 2010).

Suseela Bhai and Joseph Thomas (2010) evaluated the compatibility of commonly used agrochemicals at recommended dosages with *T. harzianum* that is being used as a biocontrol agent against capsule and rhizome rot diseases of cardamom caused by *Phytophthora meadii* and *P. vexans*, respectively. Three
commonly used fungicides, six insecticides and NPK (75:75:150) were tested under in vitro and in vivo. Cent percent mycelial inhibition was recorded in bordeaux mixture at 1% followed by quinalphos (55.84%). Other fungicides and insecticides under test were found non inhibitory at their respective recommended dosages and were at par with control, thus indicated the compatibility of Trichoderma with tested fungicides and insecticides. In vivo study also showed compatibility of T. harzianum with chemicals and fertilizers. Carbofuran, copper oxychloride and phorate were found highly compatible to T. harzianum. In addition, they were found supportive to increase in population of T. harzianum. Pandya et al. (2012) reported that T. harzianum was found to be highly sensitive to carbendazim, aureofungin, propiconazole and edifenfos by showing minimum growth and inhibition was experienced with copper oxychloride, wettable sulphur and tridemorph and it was followed by propineb, dinocap, captan and metalaxyl-MZ. The fungicide thiram and thiophanate methyl were found to be compatible without inhibition. Mclean et al. (2001) isolated an effective biocontrol agent T. harzianum C52 of the onion white rot pathogen S. cepivorum. The sensitivity of T. harzianum spores to the field rate of eight fungicides commonly applied to onions was determined. Results of in vitro assay indicate that T. harzianum was least sensitive to procymidone and captan and most sensitive to mancozeb, tebuconazole and thiram. A glasshouse pot trial confirmed that T. harzianum was sensitive to mancozeb but tolerant to captan. Mclean et al. (2001) reported that thiram (0.2%), copper oxychloride (0.2%) and mancozeb (0.2%) are compatible with T. harzianum and T. viride. Mishra (1998) observed that bavistin was inhibitory to G. virens, T. virens and T. harzianum as compared to other fungicides. The benzimidazole groups of fungicides (carbendazim and benomyl) were toxic to T. harzianum and T. longibrachiatum even at 1μM
concentration (Viji et al. 1997). Sinha et al. (1983) demonstrated that bavistin was highly inhibitory to *T. viride* at 1.25 ppm. The differential response to biocontrol agents to various fungicides might be due to their inherent resistance to most of fungicides and their ability to degrade chemicals (Papavizas, 1985 and Viji et al. 1997).

**Genetic diversity and molecular markers**

Successful biological control systems commonly employ naturally occurring, antagonistic microorganisms that can effectively reduce activities of plant pathogens (Shishido et al. 2005). Cook (1993) suggested that microorganisms isolated from the root or rhizosphere of specific crop may be better adapted to that crop and may provide better control of diseases than organisms originally isolated from other plant species. Such plant associated microorganisms may prove better biocontrol agents because they are already closely adapted to the plant, plant part or association the particular environmental conditions in which they have to function. *Trichoderma* spp. are well known biocontrol agents against a wide range of soil borne pathogens and some have a plant growth promotion capacity (Ozbay et al. 2004). They are frequently associated with both biocontrol activity and promotion of plant and root growth (Chet et al. 2006; Harman et al. 2004). Screening of diverse population of biocontrol agents is an important requirement for developing efficient biocontrol agents. Therefore, it is imperative to index biocontrol agents prevailing in the area concerned. Virulence and molecular markers are two parameters which are most often used to study fungal diversity. Fungal population structure inferred from physiological data may not reflect the true genetic diversity and evolutionary history of the isolates examined. Molecular marker analysis is a useful technique presently being used to resolve taxonomic problems, identify unknown fungal isolate, analyze
the extent of genetic variability in the population etc. (Bonde et al. 1991). Existence of genetic variability among the strains of same region as well as strains from different regions was well documented (Muthumeenakshi et al. 1998). Chen and Zhang (1994) reported the clustering of Alternaria isolates from Cruciferous hosts into 3 groups and banding pattern was not related to host or geographical distribution.

Identification of species or strains by morphological characters like shape, size and formation of conidia are highly unstable and dependent on media and environment (Hermosa et al. 2000). Phenotypic variation is abundant in Trichoderma, and expertise is required to distinguish between closely related isolates and to recognize variation within the species. With the introduction of molecular technology, it became very easy to identify the closely related organisms. Different molecular markers including randomly amplified polymorphic DNA (RAPD) have been used to characterize genetic diversity in fungal population and for different objectives and desired applications (Larissa et al. 2002, Shanmugam et al. 2008). Thus molecular markers have become enormously important in many areas of fungal biology, including strains typing, epidemiology, population genetics, fungal detection, identification, genetic mapping, gene isolation, genetics and evolutionary biology. These markers are based on minor differences that accumulate in the genomes of members of species as they diversify from one another. In fungi, markers can be developed from chromosomal, extra chromosomal or mitochondrial DNA.

RAPD is a DNA polymorphism assay based on the amplification of random DNA segments with single primers of arbitrary nucleotide sequence (Williams et al. 1990; Welsh and McClelland, 1990). Random markers as products of the PCR-RAPD technique (Williams et al. 1990) have been developed to differentiate numerous fungi,
including *Trichoderma* species (Zimand *et al.* 1994). Molecular markers offer a means of constructing quality control tests that are essential throughout the developmental processes of these biocontrol agents. In the case of *Trichoderma* spp., the quality control test is being evaluated through the production of polymerase chain reaction (PCR) fingerprints by use of semi-random primers designed to primarily target intergenic, more variable areas in the genome (Dubey and Suresh, 2006).

RAPD is an electrophoretic method used for taxonomy at the species level to discriminate species (Hadrys *et al.* 1992). RAPD analysis has been used in many applications and various organisms, especially in the plant sciences (Kapteyn and Simion, 2002). RAPD fingerprinting was used by Arisan-Atac *et al.* (1995) to identify subgroups of *Trichoderma* capable of chestnut blight biocontrol. They studied 11 strains of *T. viride*, 2 strains of *Hypocrea rufa* and 9 other species of *Trichoderma* with relation to the RAPD profile and their ability for controlling *Cryphonectria parasitica* through pairing in vitro. Gomez *et al.* (1997) analyzed the RAPD profiles of *T. harzianum* strains and classified them in different groups according to their capacity for control of plant pathogenic fungi. Muthumeenakshi *et al.* (1998) genetically characterized 15 strains of *T. harzianum*, aggressive for edible mushrooms in the United States and England, using RAPD. The strains were designated “*T. harzianum group 4*”, presenting a high homogeneity degree. Comparison of the molecular data of group 4 with group 2 (the causal agent of the epidemic green mould in industrial mushrooms in England) indicated that the isolates of *T. harzianum* group 4 were different from that of group 2. Fourteen strains of *Trichoderma* were studied by Santos (1992) using molecular markers of RAPD among other techniques, and it was possible to verify the natural genetic variability and to divide the strains in similarity groups, as well as differentiating the original strains of different regions. Zimand *et al.*
(1994) used RAPD markers to distinguish strains of *Trichoderma*, and they were able to distinguish the isolate T-39, used commercially as biocontrol agent of *Botrytis cinerea*. For efficient selection of strains of *Trichoderma* with taxonomic finalities, Fujimori and Okuda (1993) examined 74 strains of *Trichoderma* by RAPD profiles and the results were consistent with the morphological, physiological and ecological data of these strains, and suggested that the technique can aid eliminate strains duplicated in a program for microbial selection. Schlick *et al.* (1994) analyzed strains of *T. harzianum* and mutants induced by gamma radiation originated from one wild isolate with RAPD and was able to differentiate all the mutants strains for at least one primer and concluded that the method was valuable for identification and fast differentiation of strains.

Siddiquee (2002) studied the genetic diversity among among 42 isolates of *Trichoderma* using Random Amplified Microsatellites (RAMS) analysis. The genetic similarity ranged from 12.50 to 85.11% based on a total of 76 bands scored in the *Trichoderma* isolates. Cluster analysis based on UPGMA of the RAMS marker data showed that *T. harzianum*, *T. virens* and *T. longibrachiatum* isolates were grouped into different clades and lineages and found that although *T. aureoviride* isolates were morphologically different when compared to *T. harzianum* isolates, the UPGMA cluster analysis showed that the majority isolates of *T. aureoviride* (seven from nine) were closely related to the isolates of *T. harzianum*. Nine isolates were characterized by RAPD and 24 out of 40 primers produced scorable and reproducible amplifications in all the isolates. Prasad *et al.* (2004) reported that the molecular variability among the five isolates of *Alternaria solani* infecting the tomato using 22 RAPD primers. Nine isolates of *Chactomium globosum Kurize* Fr. having different mechanisms of antagonism against *Bipolaris sorokiniora* and *Ascochyta rabici* was characterized by
random deca primers and generated 336 bands of which 300 bands are polymorphic products (Reshmi Aggarwal et al., 2004). Kuruba Gopala et al. (2008) used RAPD markers to estimate the genetic variation among 17 isolates of *Trichoderma* and reported 91.8% polymorphism. The similarity matrix indicated that TCT6 and TCT13 were genetically distinct as they showed only 22.6% similarity while the isolates TCT4 and TCT10 were found to be genetically similar, as 66.7% similarity was observed between.

The RAPD technique was found to be advantageous over other molecular techniques for the genetic characterization of *Trichoderma* spp. due to the possibility of detecting DNA polymorphisms among very closely related strains (Bardakci 2001; Misra and Gupta, 2009). Siameto et al. (2010) characterized seven isolates using RAPD-and reported genetic similarity ranged from 0.231 between isolates 055E and 011E to 0.857 between isolates 010E and 015E and also suggested that the technique of RAPD was efficient in demonstrating intraspecific genetic variability. Chakraborty et al. (2010) studied genetic variability using RAPD and ITS-PCR among nineteen isolates of *T. viride* and *T. harzianum* obtained from rhizosphere soil of plantation crops, forest soil and agricultural fields of North Bengal region. The genetic relatedness among eleven isolates of *T. viride* and eight isolates of *T. harzianum* were analyzed with six random primers. The similarity coefficient ranged from 0.67 to 0.95. Sagar et al. (2011) used RAPD marker employing 3 decamer primers produced 29 scorable bands of which all (100%) were polymorphic. The co-efficient of gene differentiation (Gst) was 1.00 reflecting the existence of high level of genetic diversity among the isolates. Chakraborty et al. (2011) collected the 8 isolates of *T. viride* from forest soils, 3 from rhizosphere soils of plantation crops, 5 isolates of *T. harzianum* isolated from rhizosphere soil of plantation crops and three isolated from
agricultural soil and were characterized by morphological features, light microscopy and scanning electron microscopy and genetic relatedness by RAPD profile using six random primers. RAPD profiles showed genetic diversity among the isolates with the formation of two distinct clusters of *T. viride* and *T. harzianum* with five sub-groups of each cluster.

The recent emergence of molecular techniques for obtaining evidence from DNA sequences to use in systematic studies raises the question of whether the isozyme approach is now superseded. The clearest advantages of the latter are the fundamental quality of the data (being directly at the DNA level), and their potential use at all levels of the taxonomic hierarchy. The relative advantages of isoenzyme techniques are their lower cost, ease and rapidity. Isozymes are most suited to addressing questions at the level of populations, subspecies and species, and only of limited use at higher levels. Yet it is precisely the species level where the systematist is often seeking a variety of evidence to support taxonomic concepts. The capacity to handle a large number of samples, in a directly comparative fashion, the isozymes are ideal for studying micro-evolutionary processes such as mating system, migration, local differentiation and hybridization. These processes act on all kinds of variation, and knowing about them will assist a taxonomist's approach to other levels of evidence. Finally, isozyme analysis is useful in the design of sampling strategies and the choice of samples for in-depth molecular analysis. Electrophoretic patterns of soluble enzymes represent a direct manifestation of the genome of an organism and can be considered to determine genetic variation among a number of enzyme loci (Micales *et al.* 1992). Isozyme analysis is a useful technique being used to resolve taxonomic problems, identify unknown fungal isolate, analyze the extent of genetic
variability in the population etc. (Bonde et al. 1991). When polymorphism was detected, it reflects directly to the genetic background of the isolates (Shaw, 1965).

Chen and Zhang (1994) reported the clustering of Alternaria isolates from cruciferous hosts into three groups and the banding patterns were not related to hosts or geographical distribution.

Differences in isozyme and protein patterns in various fungal species have been reported (Rani and Kumar, 2007). Lavanya et al. (2008) reported positive activity for 8 of 9 isozymes tested in native PAGE for all isolates of T. harzianum. Esterase, peroxidase, superoxide dismutase, malate dehydrogenase (MDH), catalase and acid phosphotase showed polymorphic banding pattern whereas polyphenol oxidase gave monomorphic pattern. MDH showed maximum activity in terms of maximum number of banding loci and intensity of the bands. Identical protein profiles were observed for all isolates except GP-1 isolate in SDS-PAGE. UPGMA method of cluster analysis grouped the isolates into three main clusters which include isolates from different areas. The genetic similarity varied between 0.22 and 0.61 among the isolates (Lavanya et al. 2008).