# *Discussion*

In the present study, among isolated fungi and bacteria, *Trichoderma asperellum* and *Bacillus pumilus* were the dominant genera respectively. Based on morphological as well as scanning electron microscopic study along with 18S rDNA and 16S rDNA sequencing identity of *T. asperellum* and *B. pumilus* were confirmed. These isolated fungi (*T. asperellum*) and bacteria (*B. pumilus*), when tested against root pathogens- *Macrophomina phaseolina, Fusarium solani, Fusarium graminearum, Fusarium oxysporum* and *Rhizoctonia solani* showed antagonistic activity. Besides antagonistic study, phosphate solubilizing activity of fungal and bacterial isolates were also tested in Pikovaskaya's solid and liquid media. In a studies on soil microbial diversity of North Bengal, Chakraborty *et al.* (2008) obtained seventy fungal isolates of *Aspergillus* spp. from agricultural fields which showed phosphate solubilizing activity. Further, based on quantitative evaluation of phosphate solubilization in liquid medium supplemented with two phosphate source (tricalcium phosphate and rock phosphate) three isolates of *Aspergillus niger* showed high levels of activity. The next best were five isolates of *A. melleus*. One isolate of *A. clavatus* showed a minimum phosphate solubilization activity.

Screening of rhizosphere microflora for antagonism against pathogenic fungi in order to select suitable biocontrol agents has also been previously reported by a large number of workers. Kobayashi *et al.* (2000) isolated three bacteria showing antagonism to *Rhizoctonia solani* from the rhizosphere soil of different crops which they identified as *Pseudomonas fluorescens, Bacillus cereus* and *B. pumilus*. In another study, 11 *Bacillus pumilus* isolates were evaluated by Bargabus *et al.* (2004), of which 2 strains were found to be most effective against *Cercospora beticola*. The potential of various isolates of *Bacillus pumilus* has thus been recorded previously also. Plant growth promoting rhizobacteria (PGPRs) are a common group of bacteria that can actively colonize plant roots and increase plant growth (Kloepper and Schroth 1978). These PGPRs can prevent the deleterious effects of phytopathogenic organisms from the environment. The mechanisms by which PGPRs can influence plant growth may differ from species to species as well as from strain to strain.

The term vesicular–arbuscular mycorrhiza (VAM) was originally applied to symbiotic associations formed by all fungi in the Glomales, but because a major suborder lacks the ability to form vesicles in roots, AM is now the preferred acronym which is characterized by the transfer of nutrients, especially phosphorus, that have been taken up from the soil by the fungi, and in turn they obtain carbohydrates provided by the host plants. In this study rhizosphere soil of mandarin, *Citrus reticulata*, obtained from various sources were initially

screened for the presence of arbuscular mycorrhiza. In general the population of AM fungi in mandarin rhizosphere comprises of *Acaulospora bireticulata, A. capsicula, A. undulate, A. spinosa, Glomus aggregatum,G. constrictum, G. convolutum, G. fasciculatum, G. geosporum, G. microaggregatum, G. mosseae, G. pansihalos, G. albidum, G. ambisporum, Gigaspora albida, Gi. rosea, Gi. gigantea, Gi. margarita, Scutellospora rubra, S. pellucida, S. persica* and *S. calospora*. Range in diameter from 10μm to more than 1,000 μm were found for some *Scutellospora* spp. The spores varied in color from hyaline (clear) to black and in surface texture from smooth to highly ornament. *Glomus* formed spores on the ends of hyphae, while *Acaulospora* formed spores laterally from the neck of a swollen hyphal terminus.*Scutellospora* was identified on the basis of the presence of inner membranous walls and a germination shield (wall structure from which the germ tube can arise) while *Gigaspora* was identified by the absence of these structures.

High population of AM fungi such as species of *Glomus, Gigaspora, Scutellospora* and *Acaulospora* were obtained. Of all of these, *Glomus mosseae* showed highest percentage of occurrence in the foothill regions and it was selected for further tests. The arbuscules formed in mandarin root tisssues were highly coiled with swollen trunks and formed either singly or in clusters. In some root samples vesicles were absent nearer to the arbuscules which indicate that these arbuscules are formed by *Gigaspora* sp. In some root fragments deep blue coloured, thin walled, ellipsoidal structures were found in abundant were known as vesicles. Auxiliary cells were formed by short ramifications occurring at one or simultaneously at both sides of extraradical hyphae.

The diagnostic feature of AMF is the development of a highly branched arbuscule within root cortical cells. The fungus initially grows between cortical cells, but soon penetrates the host cell wall and grows within the cell. As the fungus grows, the host cell membrane invaginates and envelops the fungus, creating a new compartment where material of high molecular complexity is deposited. The arbuscules are relatively short lived, less than 15 days. Other structures produced by some AM fungi include vesicles, auxiliary cells, and asexual spores. Vesicles are thin–walled, lipid–filled structures that usually form in intercellular spaces. Reproductive spores can be formed either in the root or more commonly in the soil. The establishment of mycorrhizal networks in roots and soil constitute a soil-root fungal continuum, which is required for beneficial symbiotic exchanges between fungi and plant. AM mycelium can spread throughout the soil surrounding the root system and increase

the ability to explore soil areas, accessing water and nutrients for plant roots. Arbuscules found in the roots of mandarin were haustoria-like structures which were formed by profuse dichotomous hyphae branching after penetration into inner plant cortical cell walls, forming an interface.

The main benefit to the AMF by the host plant is the provision of an ecological niche because VAM cannot grow independently. Mycorrhizal fungi colonize plant roots and extend the root system into the surrounding soil. The relationship is beneficial because the plant enjoys improved nutrient and water uptake, disease resistance and superior survival and growth. The symbiotic association between AMF and roots provides a significant contribution to plant nutrition and growth. VAM mycelium in soil results in greater efficiency of nutrient absorption particularly for slowly diffusing mineral ions, especially phosphorous as observed by Smith *et al.* (2000). In addition to phosphorous, VAM mycelium also enhances the uptake of nitrogen in the form of $NO_3$ (Morte *et al.*, 2000) and also increase the potassium content in plants ( Maksoud *et al.* 1994). VAM fungi also increase the uptake of Ca, Mg, Cu, Zn and Fe ( Alkaraki and Clark, 1999). AM fungi significantly increase the net photosynthesis by increasing total chlorophyll and carotenoid contents ultimately increasing carbohydrate accumulation. The degree of dependence varies with plant species, particularly the root morphology, and conditions of soil and climate. Plants with thick roots, poorly branched and with few root hairs, are usually more dependent on mycorrhizae for normal growth and development. These species include onions, grapes, citrus, cassava, coffee, and tropical legumes.

When the level of soil fertility and humidity are increased, the dependence on the mycorrhizal condition decreases to a point where the plant becomes immune to colonization. The structural and functional diversity in roots is generally considered to be much lower than that of plant shoots (Fitter, 1987). It is certainly true that roots essentially are elongated cylinders which often appear superficially similar. However. anatomical or chemical variations between the roots of different species can be sufficient to allow their identification in soils collected from natural ecosystems ( Brundrett *et al.*, 1990). Thus with practice it may often be possible to identify roots in mixed samples by examination of their superficial characteristics, or during the course of mycorrhizal assessment. Individual roots can pass through three distinct developmental phases: (i) growth, (ii) maturation and (iii) (in some cases) secondary growth. The importance and duration of these stages varies between plants

and root system components. Plants produce a number of types (orders) of roots, including tap, lateral, basal and adventitious roots, that are physiologically, structurally and genetically distinct. For example, lateral roots typically are narrower in diameter, grow less rapidly and have shorter life spans than roots with higher branching orders .The fine laterals (feeder roots) of trees, especially those forming ECM, are often heterorhizic differentiated into long and short elements, while those with VAM usually have more extensive lateral root systems, without heterorhizy (Brundrett *et al.*, 1990). Tree roots belong to four categories resulting from structural differences between Angiosperms and Gymnosperms and those with VAM or ECM associations. In the later group, distantly related trees have evolved similar, heterorhizic roots with epidermal Hartig nets. Improvements in plant mineral nutrition are mainly related to uptake by extra-radical hyphae from the non-rhizosphere soil region and nutrient transport to the plant root (Schweiger and Jakobsen 2000). After N, P is the most frequently limiting macronutrient for plant growth and is needed in millimolar concentrations in the cellular environment. In order to meet this requirement, and considering the soil P-depleted areas that form around the roots, plants rely upon several mechanisms, such as high affinity transporters, the release of phosphatases and organic acids and association with AMF (Requena 2005), which involves a highly regulated route for P exchange between plants and fungal symbionts (Poulsen et al. 2005).

Spores extracted from the mandarin were examined in water and in polyvinyl alcohol lacto-glycerol (PVLG) through a dissecting microscope and a compound microscope. Spore colour was determined under the dissecting microscope from spores suspended in water. All the spore types formed by members of this order are homologous. Some spores may be asexual, whereas others are very complex in structure, and may be sporangia or sexual structures. Morphological features of isolated AMF spores were critically examined with special reference to variation in size, wall thickness, shape, wall layers viz. germinal wall, coriaceous wall, amorphous wall beaded wall. Scanning Electron microscopy pictures were taken of various spores to study the texture character and ornamentation. The spores were identified up to species level with the help of standard keys.

Root samples collected from eight different locations of mandarin orchards were evaluated for resistance to *M. phaseolina*. Disease assessment was based on percentage loss in dry mass of inoculated roots as well as on the color intensity of infected roots, 15 days after inoculation in relation to control. Two locations, Mirik and Kalimpong where disease

( Chakraborty and Purkayastha, 1983), potato- *Phytophthora infestans* ( Alba and DeVay, 1985), tea – *Bipolaris carbonum* ( Chakraborty and Saha,1994), tea – *Ustulina zonata* (Chakraborty *et.al*, 2002). The cellular location of CRA in tea leaves shared by *Pestalotiopsis theae* (Chakraborty *et.al*, 1995), *Glomerella cingulata* (Chakraborty *et.al*, 1996) and *Exobasidium vexans* (Chakraborty and Sharma, 2000). The present study reports the use of indirect immunofluorescence tests using polyclonal antibodies of *M. phaseolina* as a suitable technique for localization of the CRA shared by the pathogen in mandarin root tissue. Such immunodetection of pathogen is an important requisite for development of management strategies. Its implication has been elaborately described (Chakraborty and Chakraborty, 2003).

Amplification of target DNA through PCR with sequence specific primers is potentially more sensitive and rapid than microbiological techniques, as a number of constraints are removed. Unlike culture, PCR does not require the presence of viable organisms for success and may be performed even when sample volumes are small. Differences in the nucleotide composition of the variable ITS region have been successfully employed to design specific primer sets that amplify DNA selectively among and within species of plant pathogens (Nazar et al, 1991; Moukhamedov et al.,1994; Schilling et al., 1996; Moricca et al., 1998). In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. ITS regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan *et al.*, 1995). These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions within the genome (Hibbett, 1992). They also occur in multiple copies with up to 200 copies per haploid genome (Bruns *et al.*, 1991) arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes.

In the present study, ITS regions of ribosomal genes for the construction of primers were used to identify *Macrophomina*, *Fusarium* and *Trichoderma* spp. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 (for *Macrophomina*); Fcg17F and Fcg17FR (for *Fusarium*) and T/ITS1 and T/ITS4(for *Trichoderma*) primers. Amplified products of size in the range of 550-700bp was produced by the all primers. *Trichoderma* produced a single amplified product ranging from 600-620 bp which is in accordance with Mukherjee *et al.* (2002) who studied the identification and genetic variability of the *Trichoderma* isolates. These results are also in accordance with several workers who observed the amplified rDNA fragment of approximately 600 bp by ITS-PCR in *Trichoderma*

(Muthumeenakshi *et al.* 1994; Lieckfiledt *et al.*, 1999; Ospina *et al.*, 1999.; Venkateswarlu *et al.*, 2008).

The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. PCR products of *T. asperellum* were sequenced at the commercially available automated DNA sequencing facility (Genei, Bangalore). Search for homologies in the GenBank databases (http://www.ncbi.nlm .nih.gov/blast) was carried out using the BLAST program. Sequences were aligned following the ClustalW algorithm. The use of ClustalW determines that, once a gap is inserted, it can only be removed by editing. Therefore, final alignment adjustments were made manually in order to remove artificial gaps. Phylogenetic analyses were completed using the MEGA package (version 4.01; Institute of Molecular Evolutionary Genetics, University Park, PA). In order to identify one potential isolate of *T. asperellum* (RHS/M517) obtained from mandarin rhizosphere, 18S rRNA gene sequence which has been submitted to GenBank databases (Acc. No. HQ 334994) was compared and confirmed with other ten *Trichoderma* 18S rRNA gene sequences from NCBI database.

The ITS PCR has helped to detect polymorphism at ITS region of rDNA among the *T. harzianum* isolates as well as their phylogenetic placement. Based on the results, Chakraborty *et al.* (2010 a,b,c) grouped nineteen *Trichoderma* isolates obtained from soils of North Bengal into two main clusters. One cluster represents *T. viride* and other *T. harzianum*. Again the *T. viride* cluster were sub grouped into three, first subgroup with four isolates, second one with five isolates and third one with two isolates. The second cluster of *T. harzianum* could be further divided into two different groups containing different isolates. The first group consists of four isolates and second group consists of another four isolates of *T. harzianum*. These results are in agreement with those of Latha *et.al* (2002) and Venkateswarlu (2008) who studied genetic variability among the isolates of *Trichoderma* by RAPD using random primers. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The optimal tree with the sum of branch length = 13.33382563 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura and Kumar 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were

eliminated from the dataset (Complete deletion option). There were a total of 194 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

The genetic relatedness among isolates of *M. phaseolina F. solani, F. graminearum, F. oxysporum* were analyzed by random primers OPA-1; OPA-4; OPB2, OPB3, OPD-6; OPD5, A-5; AA-04 and AA-11 to generate reproducible polymorphisms. All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicate the genetic diversity of all isolates. A total of 73 reproducible and scorable polymorphic bands ranging from approximately 200bp to 6000bp were generated in *M. phaseolina, F. solani, F. graminearum* and *F oxysporum*. In the RAPD profiles showed that primer A-5 and OPA4 scored highest bands which ranged between 200bp to 6000bp. Relationships among the isolates was evaluated by cluster analysis of the data based on the similarity matrix. The Dendogram was generated by unweighted pair-group methods with arithmetic mean (UPGMA) using NTSYSpc software. In the case of the RAPD profile of *M. phaseolina, F. solani, F. graminearum* and *F. oxysporum* isolates obtained from mandarin roots can be grouped into two main clusters. One cluster grouped into another two sub groups. First cluster consist of two isolates of *F. graminearum* (RHS/S66 and RHS/S66), two isolates of *F solani* (RHS/M 532 and RHS/M 533 ) and two isolates of *F. oxysporum* (RHS/M 534 and RHS/M 535) and second clades consists with only two isolates of *M. phaseolina* ( RHS/S 565 and RHS/S 566).

Amplification of DNA fragments of *M. phaseolina* and *F. solani* with genus specific primers indicate the usefulness of molecular technique for their detection and identification. Using the specific primers ITS 1 and ITS 4, only a single band of 620 bp was generated in the amplification pattern of all the isolates. *M. phaseolina* as first described by Pearson *et al.* (1986) suggested that isolates from one specific host are more suited to colonize it. Later, Cloud and Rupe (1991) working with isolates of soybean and sorghum, also observed differences in pathogenicity. This has been further confirmed with isolates from soybean, sorghum and cotton (Su *et al.*, 2001). Isolates were clearly grouped according to the host origin. Additionally, no molecular variation could be observed among the isolates tested in PCR of the ITS region.

The present work was also aimed at developing a management strategy to control root rot of mandarin plants by biological means. Antibiosis to *M. phaseolina* by biocontrol agent (*T. asperellum* ) was evaluated *in vitro* and *in vivo* condition. *T. asperellum* tested *in vitro* were effective in causing significant suppression of growth of *M. phaseolina*. After 4 days of incubation *T. asperellum* over grew the pathogen. But in control plate pathogen grew

characteristically. In order to find out the efficacy of this biocontrol agent (*T. asperellum*) to manage the root rot disease of mandarin in glasshouse conditions, experiments were performed using plant materials from two locations which showed high disease incidence. Application of *T. asperellum* in soil was done at least 10 days before inoculation with pathogen. Results revealed that *T. asperellum* was found to be very effective in reducing root rot disease. There are several studies which have focused on mycoparasitic nature of *Trichoderma* species and its contribution to plant health (Chet, 1987; Ousley *et al*, 1994; Harman, 2000; Egberongbe *et al*, 2010).

*Trichoderma* spp. are effective biocontrol agents for a number of soilborne plant pathogens, and some are also known for their ability to enhance plant growth. It has been suggested by the recent workers that *Trichoderma* also affects induced systemic resistance (ISR) mechanism in plants. Analysis of signal molecules involved in defense mechanisms and application of specific inhibitors indicated the involvement of jasmonic acid and ethylene in the protective effect conferred by *Trichoderma* spp. against the leaf pathogen *Pseudomonas syringae* pv. *lachrymans*. Moreover, examination of local and systemic gene expression by real time reverse transcription-polymerase chain reaction analysis revealed that *T. asperellum* (T203) modulates the expression of genes involved in the jasmonate/ethylene signaling pathways of ISR (*Lox1, Pal1, ETR1 and CTR1*) in cucumber plants ( Shoresh *et.al*, 2005). They further showed that a subsequent challenge of *Trichoderma*-preinoculated plants with the leaf pathogen *P. syringae* pv. *lachrymans* resulted in higher systemic expression of the pathogenesis-related genes encoding for chitinase 1, β-1,3-glucanase, and peroxidase relative to non inoculated, challenged plants. This indicates that *T. asperellum* induced a potentiated state in the plant enabling it to be more resistant to subsequent pathogen infection. Efficiency of *Trichoderma* as biocontrol agents against fungal soil pathogens as well as a growth promoter of soybean has been demonstrated by John *et al* (2010), while, endophytic *Trichoderma* isolates obtained from tropical environments delayed disease onset and induced resistance against *Phytophthora capsici* in hot pepper using multiple mechanisms ( Bae *et al* 2011)

Application of *G.mosseae* in the rhizosphere of *Citrus* plants led to an increase in the growth of seedlings in terms of increase in height and number of leaves. Joint inoculation with both the microorganisms (G. *mosseae* and *T. asperellum*) gave most significant results. Defense responses of mandarin plants were demonstrated during early stages of root colonization by *T. asperellum* and *G. mosseae*. Marked reduction in disease development was

evident following dual inoculations of G. *mosseae* and *T. asperellum*. These observed root rot reduction following separate and dual application of *G. mosseae* and *T. asperellum* may be correlated with increase accumulation of defense enzymes such as chitinase, β- 1, 3-glucanase and peroxidase. The induction of systemic resistance was confirmed in the present study since the enhanced activities of defense enzymes were noted not only in the roots which were the sites of inoculation, but also in the leaves as evident in immunological assay using indirect immunofluorescence.

Activation of defense response of mandarin plants against *Fusarium* root rot disease using *Glomus mosseae* and *Trichoderma hamatum* has also been demonstrated. The major defense enzymes and protein showed enhanced activities during disease suppression caused by *Fusarium solani*, which was also confirmed by immunological assays. Staining of peroxidase after native PAGE demonstrated the existence of isoforms. A few of them were constitutively present in healthy roots and leaves. New isoforms were detected in roots inoculated with *G. mosseae*. However, new isoforms were noticed in mandarin leaves following inoculation of roots with *G. mosseae* and *T. hamatum*, singly or jointly. Interestingly dual application of *G. mosseae* and *T. hamatum* induced additional isozyme (Allay and Chakraborty, 2010). Sundaresan *et al.*( 1993) reported that in cowpea plants which had mycorrhizal association, accumulation of phytoalexins was much higher. Increased activity of chitinase, β-1, 3-glucanase and peroxidase were also determined in tea plants following treatments with Josh- a bioformulations of AMF (Chakraborty *et al*, 2007).

*B. pumilus*, a potential PGPR among the isolated bacteria from manadrin rhizosphere showed *in vitro* characteristics of plant growth promoting bacteria such as phosphate solubilization, siderophore production, IAA and volatile production. It was also antagonistic to fungal root pathogens *in vitro*. *B. pumilus* along with *G. mosseae*, either alone or jointly, were applied to the rhizosphere of mandarin seedlings. Both the microorganisms alone, or jointly could promote growth of the seedlings in terms of increase in height and leaf number. However, joint application gave better results. It is well known that microorganisms in soil are critical in maintaining soil functions in both natural and managed agricultural soils and play key roles in suppressing soil borne diseases, in promoting plant growth and in changes in vegetation (Garbeva *et al.*, 2004). It is apparent from the present study as well as studies by a large number of previous workers that PGPRs have the ability to promote growth in plants, which in many cases is associated with pathogen suppression in the soil ( Chakraborty *et. al.*, 2004; 2006; 2007; 2009; 2010b). *B. pumilus*, besides inhibiting growth of test pathogens *in*

*vitro*, when applied to the soil *in vivo*, promoted plant growth and suppressed root rot caused by *M. phaseolina*. Both the microorganisms could also solubilize phosphate *in vivo*. However, the effect was further enhanced when the two were applied jointly, suggesting a synergistic effect. Results of present study indicate that *B. pumilus* and *G. mosseae* could promote growth and suppress root rot of mandarin. While *B. pumilus* acted by both direct and indirect means, *G. mosseae* was responsible mainly for induction of responses within the host. Combined application of both microorganisms gave better results.

Mathivanan *et al.* (2005) also obtained synergistic effect of *Pseudomonas fluorescens* and *Trichoderma viride* in plant growth promotion, yield enhancement and disease suppression in rice. Synergistic effect of *Rhizobium* sp. with either *P. putida*, *P. fluorescens* or *B. cereus* was obtained in pigeon pea, resulting in a significant increase in plant growth, nodulation and enzyme activity (Tilak and Reddy, 2006).Thus, these microorganisms or their products have the ability to elicit responses at molecular level which would include activation of a number of metabolic pathways in the host, the end product of which is finally expressed as increased growth of plant or reduced disease. Dual application of *B. pumilus* and *G. mosseae* for improvement of health status of mandarin plants against *Fusarium oxysporum* was also been demonstrated by Chakraborty *et al* (2011). Mandarin roots were inoculated with *G. mosseae* alone and in combination with *B. pumilus* which was applied as soil drench. Both microorganisms increased growth of the plants but most significant increase was obtained when both were co-inoculated. Similarly, root rot of mandarin caused by *Fusarium oxysporum*, was suppressed to certain extent by *B. pumilus* or *G. mosseae*, but significant suppression occurred when *G. mosseae* was co-inoculated with *B. pumilus*.

Consequently, in order to get a proper insight into the plant growth promotion and induced systemic resistance, analysis of the biochemical changes especially those known to be involved in these mechanisms are essential. Activities of the different enzymes were analyzed in mandarin seedlings following treatments with pathogen and microorganisms as follows: *B. pumilus*, *G. mosseae*, *M. phaseolina*, *B. pumilus* + *M. phaseolina*, *G.mosseae* + *M. phaseolina*, *B. pumilus* + *G.mosseae* + *M. phaseolina* as well as in control. Activities of all the tested enzymes- chitinase and □ β- 1,3 glucanase increased significantly when seedlings were pre-treated either with *B. pumilus* or *G. mosseae* prior to challenge inoculation with the pathogen (*M. phaseolina)*. Peroxidase activity did not increase significantly. However, inoculation with *M. phaseolina* alone did not significantly increase any of the enzyme activities. It is quite evident that, in the present study in addition to other mechanisms of action reported for *B. pumilus* involving siderophore production, IAA production,

antifungal metabolites and phosphate solubilization, induction of defense mechanisms play an important role in disease control and plant growth promotion. The induction of systemic resistance is confirmed in the present study since the enhanced activities of defense enzymes were noted not only in the roots which were the sites of inoculation, but also in the leaves. In a study involving the induction of systemic resistance in rice leaves by *P. fluorescens* (Vidyashekaran *et al.*, 2001), increased activities of PO, PAL, 4-coumarate: 5 CO ligase and increased accumulation of lignin were observed. Sundaresan *et al.*, (1993) reported that in cowpea plants which had mycorrhizal association, accumulation of phytoalexins was much higher. Increased activity of chitinase, β-1,3-glucanase and peroxidase were obtained in sugar beet which was induced by treatment with *B. mycoides* (Bargabus *et al.*, 2002). Induction of defense related enzymes by *P. fluorescens* in black pepper and *Phytophthora capsici* pathosystem was reported by Paul and Sharma (2003). The systemic nature of protection and growth promotion in the present study is also evident as the responses were analyzed in the leaves even when the application was in the rhizosphere. Two isolates of *B. pumilus* were reported to be best plant growth promoters and biocontrol agents against downy mildew disease in pearl millet (Niranjana et al., 2003). They also reported increased activities of PAL, PO and β -1, 3-GLU but not of chitinase activity. In the present study induction of activities of defense enzymes following application of *B. pumilus* or *G. mosseae* was further confirmed by immunological tests using PAbs raised against chitinase and β -1,3-glucanase.

*T. asperellum* further proved to be potential for use in management of root rot disease in the field in combination with AMF (*Glomus mosseae*) and PGPR (*Bacillus pumilus*). A possible long-term benefit of increased implementation of microbial control would be reduced input into agriculture, particularly if seasonal colonization and introduction-establishment come into widespread use. Biological control using agriculturally important microorganisms is simply one of the best potential alternatives for disease control that could be made available in a relatively short time period. Biomass production, their suitable formulation for commercialization of antagonists to check chemical fungicide usage needs to be developed.

Different hypotheses have been proposed to explain bioprotection by AMF. These include (i) improvement of plant nutrition and root biomass in mycorrhizal plants, which could contribute to an increased plant tolerance and compensate for root damage caused by a pathogen, (ii) changes in root system morphology, (iii) modification of antagonistic microbial population in the mycorhizosphere, and (iv) competition between Arbuscular Mycorrhizal

and pathogenic fungi to colonize root tissues, with the possible induction of resistance mechanisms. In the present studies of colonization patterns of mandarin roots by the mycorrhizal fungus ( *Glomus mosseae*) and the pathogen (*Macrophomina phaseolina*), it is evident that proliferation of the pathogen is greatly reduced in mycorrhizal root systems, in comparison with nonmycorrhizal ones. Although improved phosphate nutrition by *G. mosseae* may have contributed to reduced damage by *M. phaseolina* in mandarin roots, other mechanisms must be involved in the bioprotective effects. Benhamou *et al* (1994) have reported that *F. oxysporium* f.sp. *chrysanthemi* development in mycorrhizal Ri T-DNA-transformed carrot roots is accompanied by defense-like host-wall reactions and accumulation of phenolic compounds. The induction of plant wall defense responses reflects the activation of molecular mechanisms during bioprotection against *Phytophthora parasitica* induced by *Glomus mosseae* in tomato (Cordier *et al*, 1998). The cell wall modifications associated with localized resistance and papilla formation characterizing systemic resistance to *P. parasitica* in mycorrhizal tomato root systems are reminiscent of the rapid plant defense responses to pathogens observed in incompatible interactions. Immunocyctochemical investigations have shown that this fungal molecule is released around intercellular hyphae of the pathogen but only in mycorrhizal tissues, so that it could be a putative signal in localized resistance. As far as the induction of defense responses in pathogen-infected nonmycorrhizal parts of mycorrhizal root systems is concerned, this must involve a specific, mycorrhiza-induced, mobile signal. Identification of such signal, which could have a role analogous to salicylic acid or systemin in plant-pathogen interactions, will open new horizons for understanding the molecular basis of bioprotection against fungal pathogens in mycorrhizal roots and for identifying plant genes involved.