STUDIES ON RHIZOSPHERE MICROFLORA OF MANDARIN PLANTS AND THEIR ASSESSMENT AS POTENTIAL BIOCONTROL AGENTS AGAINST ROOT DISEASES

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The significance of the destination reached cannot be of much relevance if the path traveled is not accounted for. The endeavor of undertaking research has been both an intriguing and eventful process. Moreover, the path of exploration, irrespective of the ability of the explorer cannot be embarked upon single handedly. There is the need for assistance, teamwork and above all guidance.

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Contents

Introduction

The genus Citrus of the family Rutaceae, also known as citrus fruit family, comprises of 100 species, many of which are probably hybrid (Ghazanfar *et al.*, 1980). The loose-skinned mandarin orange (*Citrus reticulata* Blanco) is one of the most economically important and popular fruits in the world, constituting about 41% of the total citrus fruits produced in India. The northeastern Himalayan region is one of the three major centres of mandarin orange cultivation in India. About 1600 ha, distributed over nine states -Assam, Arunachal Pradesh, Meghalaya, Manipur, Mizoram, Nagaland, Tripura, Sikkim and the Darjeeling district of West Bengal) is under mandarin orange cultivation, where this high-value crop contributes substantially to the region's small farm economy (Ghosh and Singh 1993). The nutritional value of citrus fruits is well known in our dietary requirements. Presently, it is the third largest fruit industry after mango and banana in India. The North-Eastern (NE) region of India offers favourable climatic conditions for cultivation of various citrus species. Submountain and hilly tracts of states like Meghalaya, Assam, Manipur, Arunachal Pradesh, Mizoram, Nagaland, Tripura, Sikkim and Darjeeling District, West Bengal grow excellent quality citrus fruits. Different citrus species, viz. mandarin, sweet orange, lemon and other limes are cultivated in all the states of the NE region covering 57.2 thousand hectares, with a total production of 306 thousand tones (Mallick *et al.*, 2006).

Citrus reticulata (Blanco) is the principal fruit crop of Darjeeling hills known as 'Darjeeling mandarin' (Plate 1). It is considered as the most important cash crop of the marginal families of the hills (Thapa, 2007). But recently it's decline is posing a threat to the sustainability of citriculture and the stakeholders respectively (Chakraborty *et al.*, 2011a,b). Citriculture in the hilly terrain is undertaken as secondary and tertiary source of income. However, extensive cultivation is seldom undertaken owing to the topography of the terrain. This has resulted in establishment of accountable smaller orange grooves or orchards. Moreover, in the recent past the area under cultivation as well as the production has declined. This can be attributed to various parameters ranging from on-field cultural practices, quality of planting materials, occurrence of diseases that may be due to viruses, fungi, insects or bacteria, and soil quality. The entire citrus orchards in NE India are of seedling origin, with few budded or grafted plants at some experimental research stations. Like many other crops, citrus in this region is plagued with a host of diseases caused by different

Plate 1 (figs. A-B): ***Citrus reticulata*** **in Mirik orchard [A] and harvested mandarin fruits in market place for sale [B].**

The distribution or localization of diseased orchards throughout the hilly slopes of Darjeeling hills is random or mosaic in pattern. The severity of the condition prevalent in the orchards cannot be attributed to one particular disease or factor. The mandarin cultivation in Darjeeling has a massive decline due to various pathological, entomological and nutritional stresses. (Mukhopadhyay *et al*, 1996)

The evident symptoms that can be observed are defoliation of young shoots and twigs and ultimate drying up of the whole plant. The symptomlogy, hence observed is indicative of the orange orchards being affected by "citrus dieback" disease. A multidisciplinary attempt has been made to assess the conditions of the orchards and analyse the relative abundance of the contributing factors for the citrus decline (Mukhopadhyay *et al.*,1985). Lack of proper knowledge on agro-techniques of orchard management of citrus cultivation in terms of nursery management, proper seedling selection, soil health status, plant protection measures, amount of manuring and fertilizer required for the plant, irrigation in winter, mulching practice, pruning practice, intercropping (suitable and unsuitable crop) etc. after the establishment of orchard singly or in combination has lead to citrus decline in Darjeeling hills. It was observed that chronic infection of foot and root rot alone or coupled with the infection of *Fusarium* and *Macrophomina* were associated to large number of dieback plants in combination with nutrient deficiencies. It is a well-known fact that heavy phosphorus application causes zinc deficiency in plant tissue (Marschner, 1995; Cooper and Tinker, 1978). Lambert *et al.* (1979) showed that high phosphorus depressed mycorrhizal inoculation, thus plants were not able to get more zinc.

Biological control is generating a great enthusiasm to play a role in sustainable agriculture. Biological control agents act as biofertilizers or as bio pesticides. Biofertilizers are the preparation containing cells of microorganism which may be nitrogen fixers, phosphorus solubilizers or organic matter decomposers. In short, they are called as bio inoculants which on supply to plants improve their growth and yield. Mycorrhizal fungi provide an effective alternative method of disease control especially those pathogens which affect the below ground plant parts. In mycorrhizal fungi lies enormous potential for use as biocontrol agent for soil borne diseases as the root diseases are one of the most difficult to manage and lead to loss in disturbing proportions. There is evidence that root colonization by Glomalean fungi can reduce disease severity in hosts affected by *Phytopthora parasitica* and other root pathogens (Davies and Mange, 1981). Since more than one Glomalean fungus can occupy a system and according Reed *et al.* (1985) root systems may benefit from the differential resource acquisition of several Glomalean fungi simultaneously. Dual or triple

action with phosphate solubilizing microorganisms (PSM) such as species of *Pseudomonas, Bacillus, Flavibacterium* and *Arthrobacter* have been found to increase the effectiveness of the VAM fungi. PSM produce, mono, -di-, and tri-carboxylic acids which helps in dissolving mineral phosphate by Fe and Al ions. The effect of environmental factors like pH (Reed *et al.*, 1976; Abbot and Robson,1985), light, temperature, moisture, associated microorganisms (Graham *et al.*, 1981) and plant genotype has been well studied. Evidence exist that mycorrhizal plants are capable of resisting the parasitic invasion and minimize the loss caused by soil borne pathogens. Bio-control of *Fusarium* wilt of cotton, jute, tomato and *Macrophomina* root rot of cowpea was achieved by the application of VAM fungi. Ability of an isolate of *Trichoderma harzianum* and arbuscular mycorrhizal fungi (AMF) in enhancing growth of tomato seedlings and control of a wilt pathogen caused by *Fusarium oxysporum* f.sp. *lycopersici* was demonstrated by Mwangi *et.al* (2011).

The dynamism of rhizosphere dwelling bacteria can have a profound effect on plant health and its surrounding environment. Rhizosphere colonization is important not only as the first step in pathogenesis of soil borne microorganisms , but also is crucial in the application of microorganisms for beneficial purposes; most significant among these applications are biofertilization, phytostimulations, biocontrol and phytoremediation (Lugtenberg *et al.*, 2001). The prospect of manipulating crop rhizosphere microbial populations by inoculation of beneficial bacteria to increase plant growth has shown considerable promise in laboratory and greenhouse studies, but responses have been variable in the field. Although non chemical control of pests and diseases seems an attractive option in sustainable agricultural systems, its practical application is severely constrained by lack of reproducible results in comparison to the use of agrochemicals. The biotic and abiotic factors in the environment, which influence the efficiency of pathogen suppression, are not well understood. Nevertheless it is known that some soil become suppressive to fungal, bacterial and nematode pests when susceptible host plants are grown continuously for several years and there is evidence that this is due to the development of soil biological communities which inhibit pests survival or infection of the hosts

Little is known about the microbial endophytic community of citrus (Araujo *et al.*, 2001) and the possible impact of endophytes on yield and especially on the control of citrus plant diseases, making the isolation and study of these microorganism for morphological physiological and genetic studies. A number of fungi are known to colonize plant roots but do

not cause disease. These include Mycorrhizas, binucleate *Rhizoctonia* spp., *Piriformaspora indica*, various plant growth promoting rhizobacteria and *Trichoderma* spp. (Shoresh *et.al*, 2010). Many of these organisms have been known for decades as agents as agents that biocontrol plant diseases, but recent studies have demonstrated that they have many other attributes. These organisms are very clearly endophytic multifunctional plant symbionts (Harman, 2011a). Typically, the fungi penetrate the outer layers of the epidermis and plant cortex, and establish chemical communication with the plant. An initial result is that the fungi are walled off by the plant, but not killed. A relatively large number of the chemical communicants (effectors/elicitors) released by the fungi are known; these include small proteins, peptides and other metabolites, including volatile ones. It is also remarkable that qualitatively similar effects are induced in plants by a variety of plant-associated root colonizing microbes, including plant growth-promoting rhizobacteria (PGPR) and mycorrhizal fungi. Presumably, the ability of these microbes to induce changes in plants, resulting in a large number of healthy roots in which they live, provides a competitive advantage (Harman, 2011b).

The present investigation was undertaken taking into consideration many factors but the focus was mainly on fungal pathogens. The reason being that the on field observation as related to the symptomology on preliminary assessment indicated that the degree severity was more due to fungal pathogens.The goal of this investigation as perceived was to initiate an understanding of the dynamics of micro-flora dwelling in the rhizosphere of the citrus plants and their state of interaction with prevailing pathogens affecting the citrus plants. The present study was designed with an aims to explore the possibility of using beneficial bioinoculant, plant growth promoting rhzobacteria (PGPR) and arbuscular mycorrhizal fungi (AMF) isolated from rhizosphere of mandarin plants (*Citrus reticulata*), specially Darjeeling variety grown in Darjeeling and Sikkim hills and to develop a formulation with bioinoculants for management of root rot disease caused by fungal pathogen (*Macrophomina phaseolina*).

To evaluate and corroborate the goal certain objectives were outlined:

- Investigation on prevalent root diseases of mandarin orange orchards of Darjeeling Hills.
- Screening of Arbuscular mycorrhizal (AM) fungi and other beneficial micro-organisms from rhizosphere of mandarin plants.
- *In vitro* interaction study among frequently associated rhizosphere microorganisms and root pathogens.
- Serological and molecular detection of dominant root pathogen.
- RAPD-PCR and phylogenetic analysis of microorganisms of mandarin rhizosphere.
- Selection and molecular identification of bioinoculants
- Evaluation of bioinoculants for suppression of root rot disease of mandarin
- Activation of defense response of mandarin plants following application of bioinoculants against root rot pathogen and assocaited changes in defense enzymes.
- Cellular location of defense enzyme in root and leaf tissues of mandarin plants following induction of resistance.

Literature Review

Soil is a complex and dynamic biological system which experiences a remarkable set of transformations over time, as energy, chemical elements and water are processed. Over time, primary minerals are weathered and lost. Although new secondary minerals may be formed during soil development, the soil's primary minerals are decomposed and its acid-neutralizing capacity gradually consumed. Moreover, soil is a structured, heterogeneous and discontinuous system, generally poor in nutrients and energy sources (in comparison with the concentrations optimal for nutrient microbial growth *in vitro*), with microorganisms living in discrete microhabitats. The chemical, physical and biological characteristics of these microhabitats differ in both time and space. The microbial population in soil is very diverse. Bacteria, actinomycetes and fungi are three major groups of soil inhabiting microorganisms. Microorganisms are ubiquitous in nature and form vital components of all known ecosystems on earth. One of the fascinating aspects of microorganisms is that some of them have evolved to thrive under conditions that are too harsh for animals as well as plants. The high microbial diversity is due to their ability to survive and multiply in diverse habitats, including anaerobic and other extreme conditions owing to their metabolic versatility and flexibility to utilize whole substrate as nutrient source. It is important to study microbial diversity not only for basic scientific research, but also to understand the link between diversity in relation to community structure and function. There is now a great interest to search for microorganisms which have potential to be used for sustainable crop production by promoting plant growth and suppressing soil borne plant diseases.

The rhizosphere is the critical interface between biota and geologic environment, fundamentally important to soil formation. The plant roots along with the rhizosphere are networks within the bulk soil, biological hotspots where respiration, gas exchange, nutrient and moisture use, and localized supplies of organic matter are most concentrated. In contrast, the bulk soil is a more oligotrophic environment, especially with respect to supply of root-derived organic matter. More than anything, reactive organic reductants and microbial activity are concentrated near roots compared with the soil system as a whole.

Root systems are symbiotic systems in which cells of plants, fungi, and bacteria are intimately associated, both structurally and functionally. This state of existence sometimes renders difficulty in isolation of plant part from that of the microbe. The fact that fungi and bacteria colonize root tissues in "endorhizospheres" suggests that concepts of continuity rather than those of class may be in order for how we think of the rhizosphere and soil. In place of class concepts of rhizoplane, rhizosphere and bulk soil, a continuum seems much more pertinent between (a) root–microbe system, which includes all cells of plant roots,

mycorrhizal fungi and closely associated non-mycorrhizal fungi and bacteria; (b) rhizosphere surrounding these cells, a volume which is immediately affected by the functioning of the root–microbe system and depends on chemical reaction, chemical element, microorganism, and soil type; and (c) bulk soil, the soil not immediately affected by the active functioning of roots, but which may be transformed by rhizospheres over pedogenic time.

Rhizosphere microflora

The study of rhizosphere in the recent times has gained substantial ground and recognition, owing to escalation of recent advances in study of microbial community. Hiltner (1904) defined "the rhizosphere as the zone of soil immediately adjacent to legume roots that supports high levels of bacterial activity". Wallace (2001), defined it as "the rooting zone of the plants including the root, soil attached to the root and adjacent soil. Ryan and Delhaize (2001) further added " the rhizosphere is a densely populated area in which the roots must compete with the invading root systems of neighboring plant species for space, water, and mineral nutrients, and soil- borne microorganisms, including bacteria, fungi, and insects feeding on an abundant source of organic material". However, the term has further been broadened to include both the volume of soil influenced by the root and the root tissues colonized by micro-organisms (Pinton *et al.*, 2001).The original concept has now been extended to include the soil surrounding a root in which physical, chemical and biological properties have been changed by root growth and activity (McCully 2005). Rhizosphere can be divided into ecto and endo rhizosphere. The term endorhizosphere is used to describe the multi-layered microenvironment, which includes a mucoid layer on the root surface, the epidermal layer of the root tissue including the root hairs and the cortical cells.

Rhizosphere has been regarded as 'hot spot' for microbial colonization and activity (Bolton *et al.*, 1993). Actively growing roots release organic compounds, such as sloughed off cells, secretions, lysates and exudates, into the rhizosphere. The activity of microbes in the rhizosphere is expected to be higher and qualitatively different in the rhizosphere as compared to microbes in bulk soil. Such root-released products can be highly specific for a given plant species or even a particular cultivar and plants are thought to selectively enrich their rhizospheres for microorganisms that are well adapted to the utilization of specific released organic compounds (Lynch and Whipps, 1990).

The rhizosphere has been investigated with an increasing awareness that the microbiology of this microhabitat is very closely geared to the health of the plant. There has been accumulation of considerable volumes of literature on soil microorganisms. However, a summarized review has been presented in the following paragraph:

Ansari *et al.* (1986) have studied rhizosphere and rhizoplane mycoflora of barley infected with *Ustilago hordei* and discussed about the biochemical changes that occur due to infection. Higher fungal population and higher number of fungal species were encountered in the infected plants in comparison to their healthy counterparts. Gopinath *et al.* (1987) have reported the colonization of *Fusarium sp.* in sorghum seeds and their significance was observed. Thirty high yielding cultivars of sorghum analyzed, showed severe infection of *Fusarium moniliforme, F. oxysporum, F. semitectum* and *F. solani. F. semitectum* infected the embryonic tissue in 93% seeds, while *F. semitectum* and *F. solani* colonized the embryo in 8 and 5% seeds respectively. But *F. oxysporum* did not colonize the embryo.

Shaik and Nusrath (1987) observed *Trichoderma viridi* and *Aspergillus niger* as a part of microflora of wilt resistant cultivar while susceptible cultivar showed a predominance of *Fusarium udum* and *Fusarium spp.* during all the stages of plant growth. Rangaswami (1988) discussed soil plant microbe interrelationships and concluded that the balance between the rhizosphere micro flora and plant pathogen, vis-à-vis, the soil microflora and the plant pathogens are important in host pathogen relationship. Unless the plant pathogen is capable of competing with other soil organisms and penetrating the barrier of mantle consisting of a wide variety and large number of the rhizosphere microorganisms, it could not reach the root surface, and cause infection.

Watanabe (1988) assayed a total of 22 soil samples from various habitats all over Japan and isolated 10-15 *Pythium* spp. from 21-46 samples in the respective districts by trapping methods, mainly with cucumber seeds as a trapping substrate. Pathogenic species were more in cultivated soil than in the uncultivated one. Among 10 identified species of *Pythium, P. aphanidermatum* and *P. deliense* have been predominantly isolated from the plants and soils of the Thailand by Chamswarng and Gesnara (1988). Neweigy *et al.* (1988) investigated antagonism of microbial isolates from rhizosphere of board bean plants against *F. solani* and *Rhizoctonia solani*. Of the 110 fungal isolates tested 13 isolates antagonized *R. solani* and 9 isolates antagonized *F. solani* . Of the 5 efficient fungal antagonists, 3 were of the genus *Trichoderma* and 2 were of the genus *Aspergillus. Bacillus* species and *Streptomyces* species were also found to be antagonistic against *F. solani* and *R. solani*. A total of 116 fungal species were isolated from rhizosphere, rhizoplane and non-rhizosphere zone of four Indian mangrove plants viz. *Rhizophora mucronata, Avicennia officinalis, Heritiera minor* and *Caropa moluccensis* from the Sunderbans, West Bengal. The highest number of fungi were isolated from *Rhiozophora mucronta* while the least from *Carapa moluccensis*. Salt tolerant fungi were also isolated by supplementing the medium with 3% and 6% NaCl (Garg, 1988).

Out of the ninety six individual strains obtained from the cotton rhizosphere which were tested for bio-control of pre-emergence damping off of cotton by *Phythium ultimum,. Pseudomonas fluorescens* strains 3551 and 3580 controlled the disease (Loper, 1988). Certain other strains (2-79 and 13-79) of *P. flurorescens* applied as a seed treatment, protected wheat against take all disease , caused by *Gaeumannomyces garminis* var. *triticii* and increased the yield at an average of 11% (Weller, 1988). Hyphomycetes fungi including species of *Aspergillus, Penicillium* and *Trichoderma* which showed *in-vitro* antagonism against *Phytopthora*, were effective as agents for biological control of *Phytophthora* root rot of *Azalea* and *Citrus*. Bio-contol of *Phytophthora* which causes bark infection of sweet orange seedlings in the green house and of matured lemon tress in the field was also obtained with *Aspergillus flavipes, A ocharcens, A wentii* and *Penicillum fumicolossum* . Kapoor and Kar (1989) have reported on antagonism of *Azotobactor* and *Bacillus* to *Fusarium oxysporum* f. sp. *lycopersici* and concluded that bacterial antagonists (*Bacillus* sp.) showed greater inhibition of tomato wilt pathogen (*F. oxysporium* f. sp. *lycopersici*) and produced antifungal antibiotic in culture.

Khalis *et al.* (1990) have studied the rhizosphere fungi of umbelliferous hosts and discussed it in the relation to its root exudates. Root exudates affected the growth and activity of fungi in rhizosphere. The rhizosphere mycoflora of coriander and arum have been studied in the relation to qualitative composition of root exudates. Khanna *et al.* (1990) have isolated, characterized and screened antagonists of *Pseudomonas tolasii* for biological control and observed that many isolates caused complete suppression in the appearance of blotch symptoms on whole mushroom cap and blocks, when tried in combination with pathogenic *P. tolasii*. One of these biological antagonist, an isolate of *P. fluorescence* biovar 1 proved quite effective in controlling the blotch incidence on *Agaricus bisporus* beds.

Kapoor and Kumar (1991) have studied the temperature effects on the antagonistic activity of fungal and bacterial antagonists against isolates of *Fusarium oxysporyum* and *Fusarium solani.* They reported that fungal and bacterial antagonists expressed clear antagonistic activity in the temperature regions at 20-27^{0}C and 20-25 ^{0}C respectively, but were most effective at lowest temperature (20^{0}C) tested. In general, antagonistic activity decreases with decrease in temperature. *F solani* isolated KHFs -41 and *F. oxisporyum* isolate DF -13 were most sensitive to fungal and bacterial antagonists, respectively. Amewowor *et al.* (1991)

isolated a number of myxomycetes and associated microorganisms from the root zones of the cabbage and broad bean in the field plots. The soil in the field plots had been studied qualitatively throughout a growing seasons and population compared with those in soil, 30 cm distant from the stem bases. All organisms were more abundant in the root zones than in non-root zone of the soil.

Ten bacteria isolated from the soybean (*Glycine max*) rhizosphere and ten isolates from soil were compared with respect to several characteristics that have been suggested as important to successful root colonization. Rhizosphere and soil isolates differed significantly in their ability to move along soybean root (Hozore *et al.*, 1991). Rhizosphere bacteria were able to colonize root segments further from the point of inoculation in greater number than soil bacteria. Rhizosphere and soil bacteria did not differ significantly in rates and extent of growth, in either exudates collected from germinating seeds or inorganic salt solution.

Hee (1991) conducted studies on the selection and identification of antagonist rhizobacteria in relation to controlling soil borne disease of vegetables. 926 isolates of rhizobacteria from 3 different kinds of selective media and 30 isolates of plant pathogenic bacteria were dual cultured with 10 species of important soil borne plant pathogenic fungi, respectively and their antagonism measured by their inhibition zone. The population density of rhizobacteria in the same field was different according to the crop species planted and the isolation frequency of the antagonistic bacteria from the species of plant was also markedly different according to the fields or regions from where the soils were collected for the effective isolation of rhizobacteria, M523 and King's B media were more suitable than D+ medium. Of 926 rhizobacteria isolated from the soils of 22 plant species. 63 isolates were selected to be antagonistic to *Phytophthora capsici* ,54 to *Rhizoctonia solani* and 17 to *Fusarium oxysporum* f.sp. *lycopersici*, respectively. Of these one isolate RB 173 was finally selected as the most effective antagonist to the 9 species of soil borne plant pathogenic fungi and was identified as *Pseudomonus fluorescens*.

Antagonistic fluorescent *Pseudomonus* have frequently been suggested to be important natural antagonist of plant pathogens. These bacteria have been considered very useful, particularly in relation to the microbial suppression of the "take-all" fungus *Gaeumannomyces graminis* var. *tritici* following wheat monoculture and for the management of rice disease. Arya and Mathew (1993) have studied the rhizosphere microflora of pigeon pea and discussed the qualitative and quantitative incidence of microorganisms after solarization. They isolated microorganisms from non-rhizosphere and rhizosphere seeds of 4

different varieties of pigeon pea after mulching with 300 gauge polyethylene sheet for 15 days. *Fusarium udum* a severe pathogen of *Cajanus cajan* causing wilt disease could not be recovered from the non-rhizosphere soil after mulching for 45 days. They suggested that the suitability of soil for a crop depends not only on its chemical and physical properties but also on its microbial population.

Sen and Gupta (1994) have studied the incidence of the Fusarial wilt of *Robinia psedoacacia* L. in relation to effect of some important edaphic factors. The effect of various soil factors, viz., temperature, moisture , pH and type on the incidence for three species of the *Fusarium* viz., *Fusarium oxysporum, F.equiseti* , and *F. semitectum* was studied. The soil temperature between 20-30^{0}C, moisture of 60% and pH 6.0 were most favourable to all *Fusarium* species. Yephit *et al.* (1995) have worked on *Fusarium* wilt in Carnation and discussed the effect of culture resistance on the propagule persisting in soil. They have taken six carnation cultivars with different degrees of resistance to *Fusarium oxysporum* f. sp *dianthi* race 2 which persisted in naturally infected soil in the field and in containers with artificially infertile soil at three inoculum concentrations.

Soil suppressiveness to *Fusarium* wilt and dry root rot legumes induced by incorporation of the cruciferous crop residues were studied by Sharma *et al.* (1995). They reported that continuous cropping of leguminous crops on the same piece of land has made sandy soils conductive to *Fusarium* wilt and dry root rot (*Macrophomina phaseolina*). Soil suppressiveness may be induced by changes in the microflora environment through addition of antagonistic microorganisms on the nutritional amendments. Bhattacharya and Bora (1995) have studied the rhizosphere microflora of the tea in relation to age of the plants. They have collected the rhizosphere soil of 5, 35 and 75 years old tea plants during different seasons. Fungi were found to be dominant in autumn, while bacteria in rainy-summer, and actinomycetes in spring.

A new solid medium has been developed for the enumeration and isolation of soil and rhizosphere microorganisms. This medium, named rhizosphere isolation medium contains glucose and 15 of the 20 common amino acids. The absence of five other amino acids, namely, aspartic acid, asparagine, cysteine, proline, and threonine, inhibits the growth of *Bacillus mycoides*, a commonly encountered bacterium that rapidly spreads on agar media and complicates the isolation and enumeration of other microorganisms. Compared with a

similar medium containing Casamino Acids, rhizosphere isolation medium had half as many colonies of *B. mycoides*, with each colony approximately half the diameter. The two media had similar total numbers of bacterial colonies. Isolates were divided into taxononomic groups, roughly corresponding to species and genus, by fatty acid methyl ester analysis and numerical methods. There were 24 genera and 41 species were found among the isolates from rhizosphere isolation medium, while 19 genera and 35 species were found in the isolates from the medium prepared with Casamino Acids. No major group of bacteria was found to occur only on one medium or on the other, indicating that the five missing amino acids had no great effect on organisms other than *B. mycoides*. This medium may prove useful in soil and rhizosphere studies in which the growth of *B. mycoides* is undesirable (Buyer, 1995).

Pandey *et al.* (2000, a) isolated four antagonistic bacterial isolates, *Bacillus subtilis*, *Bacillus sp.*, *Pseudomonas corrugata* 1 and *P. corrugata* 2, from the rhizosphere of tea plants growing in different geographical locations in India. These were tested as microbial inoculants for hardening of tissue-cultured tea plants raised in the laboratory prior to the transfer to open land. Bacterial inoculations resulted in enhanced survival (up to 100, 96, and 88%), as against 50, 52, and 36% survival observed in the corresponding control plants, in rainy, winter and summer seasons, respectively. Rhizoplane and rhizosphere soil analyses showed that the major biotic factor responsible for mortality following the transfer of tissue culture raised plants to soil was fungal attack (*Fusarium oxysporum*). Bacterial inoculations also resulted in plant growth promotion of tissue culture as well as seed raised plants of tea.

Pandey *et al.* (2000, b) outlined through a detailed study conducted from various tea growing locations in India, that species of *Penicillium* and *Trichoderma* were dominant in the rhizosphere of established tea bushes. *Penicillium erythromellis, P. janthinellum, P. raistrickii, Trichoderma pseudokoningii* and *T. koningii* were found to be closely associated with tea roots. While seasonal fluctuation in occurrence was observed in the case of *Penicillium spp.*, the population of *Trichoderma* spp. showed less variation during the year. Both species were sensitive to low temperature. In general, fungi associated with the tea rhizosphere were found to prefer a mesophilic temperature range (15 °C to 35 °C). The dominant species of *Penicillium* and *Trichoderma* also exhibited tolerance to lower temperatures, i.e., 5 to 10 °C on agar plates. Most fungi were able to grow in a wide range of pH (4 to 12). Lowering of soil pH in the rhizosphere of tea bushes was positively correlated with the age of the bush and may have affected the development of a specific microbial community in the rhizosphere. Pandey and Upadhyay (2000) reported that rhizosphere of

healthy pigeonpea plant was heavily colonised by *Aspergillus niger* and *Penicillum* sp than those diseased by *Fusarium udum*. *A. niger* also showed moderate antagonism and suppression of the pathogen colony. Resident *Trichoderma* and *Gliocladium* was highly antagonistic but none of the bacterial isolates was antagonistic to the pathogen. *T. viride* formed loops, coiled and ruptured the cell wall of the pathogen. Mechanism of parasitism between *F. udum* and *G. virens* resulted in twisting, air bubbling and disintegration of pathogen hyphae while *T. harzianum* caused severe vacuolation, shrinkage and coagulation of cytoplasm of pathogen hyphae. Kallurmath and Rajasab (2000) isolated that two species of *Aspergilli* along with ten other fungi from rhizophere of onion (*Allium cepa* L.) were isolated. Aspergilli in general were dominant, contributing 38.59% to the total rhizosphere mycoflora. Among the isolates *Aspergilli*, *A.niger* and *A.flavus* were comparatively dominant. *A.niger* was particularly most dominant on onion bulbs with the progress of their maturity.

Pandey *et al.* (2001) reported dominant fungi in the rhizosphere of established tea bushes and their interaction with the dominant bacteria under *in situ* conditions. The population of *Penicillium* and *Trichoderma* species were inversely correlated with the population of two most dominant rhizosphere bacteria, *Bacillus subtilis* and *B. mycoides*. Both *Bacillus* species have been shown to have antagonistic activity against these two fungi under *in vitro* condition. The study demonstrated the existence of a similar antagonism under in situ conditions in the rhizosphere of established tea bushes.

Rhizosphere colonization is one of the first steps in the pathogenesis of soil borne microorganisms. It can also be crucial for the action of microbial inoculants used as biofertilizers, biopesticides, phytostimulators and bioremediers. *Pseudomonas*, one of the best root colonizers is therefore used as a model root colonizer. Lugtenberg et al. (2001) focused on (a) the temporal spatial description of root colonizing bacteria as visualized by confocal laser scanning microscopical analysis of autofluorescent microorganisms, and (b) bacterial genes and the traits used for the colonization of root and of animal tissues, indicating the general importance of a study.

Plant growth promoting rhizobacterial strains belonging to fluorescent *Pseudomonas* were isolated from the rhizosphere of rice and sugarcane by Kumar *et al.* (2002). Among 40 strains that were confirmed as *Pseudomonas fluorescens*, 18 exhibited strong antifungal activity against *Rhizoctonia bataticola* and *Fusarium oxysporum*, mainly through the production of

antifungal metabolites. Genotyping of these *P. fluorescens* strains was made by PCR-RAPD analysis, since differentiation by biochemical methods was limited.

Mathur *et al.* (2004) reported that the plant rhizosphere is an important zone where many micro-organisms both friend and foe exits. The microflora associated with a plant rhizosphere is generally influenced by the soil type, pH, temparature and stages of plant growth. Parveen *et. al.* (2004) studied the mode of antagonism of *Trichodrma viride* against *Alternaria triticina* causing leaf blight of wheat. When studied *in vitro* by employing dual culture technique, *Trichoderma viride* as a biocontrol agent inhibited the growth of the pathogen, its mycelial strands coiled around the hyphae of the test pathogen forming a rope like structure and finally disintegrating the test pathogen, *Alternaria triticina.*

Singh and Sindhan (2004) evaluated four antagonists viz. *Trichoderma viride* , *Trichoderma harzianum, Gliocladium virens* and *Aspergillus nidulans* as seed, soil and combined (seed and soil) treatment for the control of tomato wilt caused by *Fusarium oxysporum* f. sp. *lycoperssici* in green house. *Trichoderma viride, Trichoderma harzianum* and *Gliocladium virens* as seed treatment @10g/kg seed were effective in controlling seedling mortality up to 85% and were at par with carbendazin. After emergence they provided 100% protection; *A. nidulaus* was least effective. All the antagonist treated plants had longer roots and shoots, and more leaves per plant as compared to plants in control.

Aspergillus niger, Trichoderma harzianum, Trichoderma viride, and *Penicillium aurantiogrisem* a bacterium (B1) and *Bacillus subtilis* were isolated from the rhizosphere (Sharma and Champawat, 2004). Amongst the various rhizospheric microorganisms, *Trichoderma viride* and from rhizoplane microorganisms *Gliocladium virens* and bacterium (B2) proved effective against *Fusarium oxysporium* under experimental condition. They also reported four fungi viz. *Aspergillus niger. Trichoderma harzianum, Trichoderma viride* and *Penicillium aurantiogriseum* and two bacteria viz. bacterium (B1) and *Bacillus subtilis* isolated from rhizosphere. The spore of *F. oxysporum* germinated minimum in association with rhizospheric *Trichoderma viride.* The rhizoplane microorganisms *G. virens* and bacterium (B2) exhibited minimum spore germination of *Fusarium oxysporum.*

Oyeyiola (2009) isolated and identified fungi present in the rhizosphere and rhizoplane of Okra (*Hibiscus esculentus*). The fungi were *Penicillium frequetans, Penicillium oxalicum, Penicillium palitans, Rhizopus stolonifer, Rhizopus oligosporus, Rhizopus oryzae, Aspergillus niger, Aspergillus fumigatus, Aspergillus japonicus, Aspergillus clavatus, Mucor hiemalis, Mucor racemosus, Alternaria herbarum and Alternaria triticina.* Among them *R. stolonifer, A. niger* and *A. clavatus* were predominant in both the rhizosphere soil and the

rhizoplane, while *P. oxalicum* and *A. herbarum* were predominant in the rhizosphere soil only. *Mucor hiemalis, Penicillium frequetans, P. oxalicum, A. clavatus, P. palitans* and *A. triticina* were present in the rhizosphere soil and the rhizoplane, but they were absent from the non-rhizosphere soil. The rhizosphere soil contained a greater spectrum of fungal species than either the rhizoplane or the non-rhizosphere soil. The experimental soil was sandy loam in texture. The rhizosphere effect increased progressively with increase in plant age until the 6th week after seed sowing and then declined.

Mulaw *et al.* (2010) reported that the production of *Coffea arabica* in the southwestern is affected by tracheomycosis caused by a soil-born fungus *Gibberella xylarioides*. The use of endemic antagonistic strains of mycoparasitic *Trichoderma* species would be a nature conserving means to combat this disease. They have used molecular methods to reveal that the community of *Trichoderma* in the rhizosphere of *C. arabica* in its native forests, is highly diverse and includes many putatively endemic species. (rRNA) gene fragments.

AM Fungi

Arbuscular Mycorrhizal fungi (AMF) are most ubiquitous in terrestrial ecosystems and form mutualistic relationship with more than 80 percent of major group of vascular plants. An apparently low taxonomic diversity coupled with a broad geographic range had led to the view that AMF are a rather homogenous group, both functionally and morphologically. It is routinely possible, however, to find 10-30 spore types in the soil at a single site. This high local diversity against a background of low global diversity is paradoxical if all fungi are equally capable of colonizing all plants. The apparent high genetic diversity of AMF is similarly paradoxical in relation to the low morphological diversity. Improved methods of isolation from soil, and of microscopy and molecular analysis have shown that there may be substantial diversity present in field soils, but the relationship between morphological and molecular diversity is still unclear.

Root colonization with AMF is a dynamic process, which is influenced by several edaphic factors such as nutrient status of soil, seasons, VAM strains, soil temperature, soil pH, host cultivar susceptibility to VAM colonization and feeder root condition. There has been growing appreciation of the importance of plant and fungal interaction especially AMF on terrestrial ecosystem (Giovannetti *et al.* 2006; Rodrigues, 2008). Mycorrhiza form critical link between the plant and soil structure and make a large direct contribution to soil fertility and quality through contribution of soil organic matter. The assumed primary benefit to plants of the Mycorrhizal symbiosis is an increased uptake of immobile nutrients, especially

phosphorus that are mobilised by the fungus. However, there is increasing evidence that AMF have a range of other effects, for example, protection against plant parasites (Aggarwal *et al.* 2006 a; Bhargava *et al.* 2008) , water stress tolerance (Newsham *et al.*1995) alleviation of salt stress (Evelin *et al.* 2009) and in sustainable maintenance of plant health and soil fertility (Wright and Upadyaya, 1998; Jeffries *et al.* 2003 a). This evidence of multiple functions, host selectively (or non- random distribution) and higher diversity than is apparent from cultures and morphology leads us to conclude that communities of AMF are much more diverse than previously thought. At the community level, recent experiments have shown that AMF are major players and may influence plant species diversity (van der Heijden *et al.* 1998; Klironomos *et al.*2000) and recruitment of plant species into the population (Kiers *et al.* 2000). Colonisation of host plants by AMF exhibits significant spatial and temporal heterogeneity (Merryweather and Fitter, 1998 ; Helgason *et al.*1999). High spore diversity has been found in the field (Bever *et al.* 1996; Eom *et al.* 2000) and the number of spores produced by each AM fungus may be host- dependant (Sanders and Fitter, 1992 ; Bever *et al.*1996).Traditionally , AMF community diversity has been measured using spore counts from field soils, but such counts are a measure of the sporulation activity of the fungi rather than a direct measurement of diversity, and maybe highly variable and almost impossible to correlate among habitat types (Morton *et al.*1995). Molecular genetic analysis provides a way around this obstacle as it has the potential to identify objectively that taxa present in the roots of the plants.

The plant provides the fungal partner with carbon and the fungus improves the plant nutrient uptake from the soil. These fungi have long been considered obligate symbionts with plants, since growing the AM fungus without a host plant has not been possible. Isolated spores can germinate and produce hyphae, but they die if no host root is found. This view has recently been challenged by an experimental study showing that AMF can grow and form spores *in vitro*, if provided with a carbon source and stimulated by particular bacterial strains (Hildebrandt *et al.*, 2006). Whether this can also occur in nature is not yet known. Spores are asexual, multinucleate and are produced directly by the mycelium, either inside or outside the root. In some species, small sporocarps can be produced, where several spores are surrounded by a peridium-like structure. Typical glomeromycotan spores are globose, relatively big (40-800 μm) and with a multilayered wall that can be smooth or ornamented. Evidence of sexual reproduction has not been reported so far in the Glomeromycota. The hyphae lack septa (cross walls between hyphal cells) and can grow both outside (extraradical) and inside the roots (intraradical). This coenocytic structure allows the nuclei to move along the hyphae

(Bago *et al.*, 1999). The intraradical mycelium typically produces highly branched structures called arbuscules, inside the cortical cells of roots (Arum-mycorrhizal type). In some other cases, hyphal coils are formed instead (Paris-mycorrhizal type). The variability of structures along this Arum–Paris continuum has ecological, functional and taxonomic significance that are not fully understood yet (Dickson *et al*, 2007). Many species of Glomeromycota also form large intraradical, globose, storage cells called vesicles. Because of this, glomeromycotan fungi are sometimes also referred to as vesicular-arbuscular mycorrhizal fungi (VAM). Glomeromycotan fungi do not disseminate solely by spores. New plant hosts can be colonised from hyphal fragments present in the soil or growing from colonised root fragments. Alternatively, roots can be colonised by extraradical mycelium extending from a previously established mycorrhiza. The latter may give rise to an extensive mycelial network connecting the root systems of several plants from the same or different species. Nonetheless, the relative importance of the two dispersal methods and the establishment of hyphal networks in nature have not been evaluated. There are some widespread and frequent AM species detected in molecular studies, without any known stage of spore formation. This suggests that there might be species that rarely or never sporulate.

The phylum Glomeromycota was established as a monophyletic group, distinct from the Zygomycota in which they were previously placed. Phylogenetic studies based on molecular data placed the Glomeromycota as a sister group to the Basidio- and Ascomycota (Lutzoni *et al.*, 2004, James *et al.*, 2006). Traditional taxonomy in the Glomeromycota has mainly been based in spore morphology and ontogeny. The structures and characters of the mycelia, e.g. arbuscules, vesicles, coils are of exiguous taxonomical value. Fewer than 200 species, grouped in eleven genera, are described. Most of them have been described after being isolated and grown in pot cultures using a handful of host plant species. Glomeromycotan fungi produce relatively large (40–800 mm) spores with layered walls, containing several hundreds to thousands of nuclei (Becard and Pfeffer 1993). Spores may be formed singly, in clusters or aggregated in so-called sporocarps (Gerdemann and Trappe 1974).

Different functional groups of bacteria such as N_2-fixing bacteria (Secilia and Bagyaraj, 1987), plant growth-promoting rhizobacteria, phosphate-solubilising bacteria (Toro *et al.*, 1996) and antagonists of plant pathogens (Citernesi *et al.*, 1996; Budi *et al.*, 1999) have been reported to be associated with the rhizosphere of different plants colonised by AMF. Some bacteria have also been found to be associated with AM fungal structures such as external hyphae (Toljander *et al.*, 2006) and spore or spore walls (Xavier and Germida, 2003; Roesti

et al., 2005). Bacteria have also been reported to live inside the spores of certain AM fungal isolates (Bianciotto *et al.*, 2003).

Mycorrhizal contribution to agri-ecosystem

Pot experiments were conducted by Louis (1990) to determine the phosphorus response curves of inoculated and uninoculated mungbeans and rice. Two soil types, Luisiana clay and macolod clay loam were used for mungbeans and only Macolod clay loam was used for rice. Phosphorus levels were set at ½, ½, 1, 2x and 4x the recommended rate. In Louisiana clay, mungbean inoculated with mycorrhiza had a better response to P application compared with uninoculated ones. When soil was un-amended with P no significant difference between uninoculated and inoculated plants in all the fermentors used was observed. However, uninoculated plants died two months after sowing, while inoculated plants survived until the maturity stage. Differences on growth improvement attributed to mycorrhiza depended on the amount of P added and the species of VA endophytes used. Uninoculated plants had no response to increasing addition of P . In Macolod clay loam, it was noticeable that inoculated mungbeans were more vigorous, green, and healthier in appearance compared with uninoculated ones. However, the quantitative differences in all the parameters used were statistically insignificant. Both inoculated and uninoculated plants increased similarly in height biomass and pod yield with increasing additions of P. Rice inoculated with mycorrhizae, regardless of VA endophytes, had a better response to P application compared with uninoculated ones when grown in Macolod clay loam. Significant differences among treatments were observed with regard to mean height and biomass of rice. The same trend was observed with regard to group weight. The differences within treatments. At lowest level of P applied, *Gigaspora* sp. and *G. mosseae* were already highly effective as shown by the increase in al parameters used. Optimum growth reasons of uninoculated plants was obtained when P added was at recommended rates and further additions had only slight effect on mean height and no effect on biomass.

Communities of vesicular arbuscular (VAM) mycorrhizal fungi was studied by Johnson *et. al.* (1991) in a along term crop rotation experiment at two location. Spores of mycorohizal fungi were counted and identified in experimental plots with a cropping history of either corn (zea-mays) or Soybean (*Glycine max).* Mycorrhizal fungi communities were affected by both location and cropping history, at Waseca, *Glomus aggregatum.* Schonbeckek & Smith, *G. leptotichum* Schenek & and Smith & *G. occultum* walker spores were more abundant in the soil with a corn history than a soybean history. Densities of *G. aggretatum*

spores were negatively correlated with soil pH at Waseea, but were unrelated to pH at Lamberton where the mean soil pH was lower. Our results indicate that mycorrhiza fungal species are individualistic in their response to cropping history and edaphic factor.

Influence of inoculation with Vesicular-Arbuscualr-mycorhizal fungi on the growth of Asparagus *(Asparagus officienalis)* seedling was examined by Mizonobell *et. al.* (1991) using *Glomus* spp. '301' and '401' which were bred by Kyowa Kakko Kogyo Co. Ltd. Difference in shoot length began to appear clearly between mycorrhizal fungus inoculated and uninoculated seedling 60 days after the inoculation, and higher density (100 spore) of *Glomus* spp. '301' spores gave a higher mycorryzal infection level and made shoots longer. It was revealed that both *Glomus* spp. '30' & '401' had an effect on the growth of asparagus seedlings & *Glomus* spp. '401' promoted the seedling growth more intensively than '301' VAM infection was observed only in the feeder root. In a VAM infected seedling, the number and thickness of storage roots increased, where as feeder roots were shortened.

Screening for the best species of AMF associated with mahogany was done. Growth of host plants at four months significantly increased as a result of AMF inoculation Mahogany seedling inoculated with a mixture *Glomus* inoculated with a mixture of *Glomus etunicatum* and *Glomus mosseae* produced the highest biomas. Availability of soil moisture proved to be the greatest factor in influencing plant growth while AMF inoculation could increase nutrient and water uptake of mahogany seedling resulting to higher growth rates.

AM fungi are an intimate link between the roots of most plants and soils, thereby affecting the development of host plants and host soils. The role of VAM fungi in improving plant nutrition and their interactions with other soil biota have been investigated with reference to host plant growth, but little is known about how these interactions affect soil structure. The impact of cultural practices and the particular role that AMF play in improving soil structure are discussed by Schreiner and Bethlenfalvay (1995) in the context of sustainable farming. Natural mycorrhyzal potential has been carried out in a representative area of a decertified semi arid ecosystem in the southeast of Spain. Many indigenous plants from the field site were mycorrhizal, including the dominat *Anthyllis cytisoides,* which had high levels of colonization by arbuscular mycorrhizal fungi (AMF). Low numbers of AMF spore were present in the soil, although a range of species, including *Scutellospora calospora, Glomus coronatum, Glomus constricim,* and several *Acaulospora* species, was represented.

Crop nutrition and Plant growth

The relationship between the development of arbuscular mycorrhizas and increased growth and nutrition of the host was recognized by Asai as early as 1944. Pioneering work on the potential of mycorrhizas in plant nutrition was carried out by Mosse (1957) on apples, Baylis (1967) on *Griselinia* and other New Zealand plants and Gerdemann (1965) on *Liquidambar* and maize. McGonigle (1988) evaluated seventy-eight field trials with AM fungi and found that inoculation increased yield by about 37%. Though at that time, it was not clear whether the observed increase in yield could be correlated to increased nutrient uptake facilitated by AM fungi, later developments have shown that indeed, AM fungi increase yield through improved nutrition Since roots of most plants are colonized by mycorrhizas, unequivocal proof that AM colonization increases nutrient uptake has been difficult to obtain. Nevertheless, the role of AM fungi in increasing nutrient uptake and plant growth has now been documented. It is now established that even when there is no increase in nutrient uptake efficiency in tissue concentrations or in total plant nutrient content, the fungal partner can make a significant contribution to nutrient uptake.

The development of research on roles of AM fungi in nutrient uptake has been closely linked to the knowledge of soil chemistry, particularly in relation to pools and availability of phosphorus (Van Aarle, 2009). The amount and form of phosphorus in soil and the factors affecting its availability are important in determining the ways in which AM fungi influence uptake by plants (Comerford, 1998). Phosphorus, one of the most essential elements of plants, is not easily available from soil because, in spite of being present in relatively large amounts, much of it is insoluble. Indirect evidence that AM roots can be more efficient in nutrient uptake than non-mycorrhizal roots came from the observation that responsive AM plants are both larger and contain higher concentrations of P in their tissues than uncolonized controls. The explanation first suggested was that AM colonization increased efficiency of absorption by roots. However, this is not the only possible explanation for the increases. Increased total root length or efficiency in AM plants would certainly contribute to increased total uptake, but would not necessarily lead to elevated tissue concentration. If growth keeps pace with P upake, tissue concentrations remain constant, for they are dependent on the relative rates of uptake and growth. It is not known exactly what mechanisms are involved by AM fungi in the P uptake of inorganic and organic P. External phosphatase can make organically bound P available for uptake. It is however not believed that a large amount of phosphatases is excreted by AM fungal extraradical mycelium (Olsson *et.al*, 2002). The

uptake of P by AM fungi must be an active process since the difference in electrochemical potential between soil and extraradical mycelium is large and since the inorganic P concentration inside the mycelium is high compared with the soil solution. It is generally accepted that the uptake of P takes place partly via proton cotransport, which is driven by a membrane-bound proton-ATPase (Becard *et.al*, 2004). Proton-ATPases were found to be expressed in the extraradical mycelium of two *Glomus* species, indicating that P uptake is mediated by a high-affinity transporter of which the functioning depends on the electrochemical gradient of proton generated by a plasma membrane proton-ATPase (Ferrol *et al*, 2002).

Elevated concentrations may also result from increased carbon AM plants, leading to C-limitation and 'luxury' accumulation of P (Smith and Read, 2008). More direct evidence of increased efficiency of P absorption was obtained by expression uptake on the basis of amount of absorbing tissue. The first demonstration of increased inflow of P in AM roots was in highly responsive *Allium cepa* colonized by *Glomus* sp. (Sanders and Tinker, 1971, 1973). They calculated that AM fungi contributed about 70% of the P absorbed by the AM plants. Direct confirmation of the role of AM fungi in improved plant nutrition was obtained by Cavagnaro *et al.* (2006), who, using mutant tomato with reduced AM colonization, showed that AM fungal populations not only contribute to nutrition, but also enhance food quality in terms of nutrient densities. Effects of AM fungi on nutritional value of crops deserve particular attention particularly in the light of concerns that use of highly purified fertilizers and other modern agricultural practices are reducing micronutrient densities below those required for human health (Welch, 2002; Welch and Graham, 2002). Another area of current investigation is to explore the probability of AM fungi increasing beneficial compounds in the plants like antioxidants.

Other than P, evidence is also now accumulating that the AM pathway makes considerable contribution to plant N uptake from soil, regardless of total N uptake and N responses. In most cases, improved nodulation and N_2 fixation in AM plants appears to be the result of relief from P stress and possibly uptake of some essential micronutrients, which result in both a general improvement in growth and indirect effects upon the N_2 fixing system. Experiments using ^{15}N have shown that hyphal N transfer occurs between AM fungi and host roots (Hawkins *et al.* 2000; Mader *et al.* 2000). However, the translocation of N has not been correlated with increased plant N content or growth. There is also evidence now that the efficiency of Zn and Cu is increased in AM plants. Further, the interactions between AM colonization and accumulation of heavy metals and other toxic elements is an area of

considerable interest in relation to both production of safe food and bioremediation programmes.

In order to enhance the sustainability of agro-ecosystems, there is a shift towards low-input instead of conventional high-input agricultural systems. In these low-input systems the Mycorrhizal symbiosis is regaining its importance. This can lead to better fungal development in soil, higher root colonization, and an enhanced nutrient uptake.

Plant protection by AMF

Plants and pathogens interact with a wide variety of organisms throughout their lifecycle. These interactions can significantly affect plant health in various ways. The types of interactions were referred to as mutualism, protocooperation, commensalism, neutralism, competition, amensalism, parasitism, and predation. While the terminology was developed for macroecology, examples of all of these types of interactions can be found in the natural world at both the macroscopic and microscopic level. And, because the development of plant diseases involves both plants and microbes, the interactions that lead to biological control may take place at multiple levels of scale. From the plant's perspective, biological control can be considered a net positive result arising from a variety of specific and non-specific interactions. Mutualism is an association between two or more species where both species derive benefit. Sometimes, it is an obligatory lifelong interaction involving close physical and biochemical contact, such as those between plants and mycorrhizal fungi.

Different hypothesis have been proposed to explain bioprotection by AM fungi. These include (a) 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, (b) changes in root system morphology, (c) modification of antagonistic microbial populations in the mycorrhizosphere, and (d) competition between AM fungi and pathogenic fungi to colonize root tissues, with the possible induction of resistance mechanisms.

Many of the microbes isolated and classified as BCAs can be considered facultative mutualists involved in protocooperation, because survival rarely depends on any specific host and disease suppression will vary depending on the prevailing environmental conditions. Further down the spectrum, commensalism is a symbiotic interaction between two living organisms, where one organism benefits and the other is neither harmed nor benefited. Most plant-associated microbes are assumed to be commensals with regards to the host plant, because their presence, individually or in total, rarely results in overtly positive or negative consequences to the plant. And, while their presence may present a

variety of challenges to an infecting pathogen, an absence of measurable decrease in pathogen infection or disease severity is indicative of commensal interactions. Neutralism describes the biological interactions when the population density of one species has absolutely no effect whatsoever on the other. Related to biological control, an inability to associate the population dynamics of pathogen with that of another organism would indicate neutralism. In contrast, antagonism between organisms results in a negative outcome for one or both. Competition within and between species results in decreased growth, activity and/or fecundity of the interacting organisms. Biocontrol can occur when non-pathogens compete with pathogens for nutrients in and around the host plant. Direct interactions that benefit one population at the expense of another also affect our understanding of biological control. Parasitism is a symbiosis in which two phylogenetically unrelated organisms coexist over a prolonged period of time. In this type of association, one organism, usually the physically smaller of the two (called the parasite) benefits and the other (called the host) is harmed to some measurable extent. The activities of various hyperparasites, i.e., those agents that parasitize plant pathogens, can result in biocontrol. And, interestingly, host infection and parasitism by relatively avirulent pathogens may lead to biocontrol of more virulent pathogens through the stimulation of host defense systems. Lastly, predation refers to the hunting and killing of one organism by another for consumption and sustenance. While the term predator typically refer to animals that feed at higher trophic levels in the macroscopic world, it has also been applied to the actions of microbes, e.g. protists, and mesofauna, e.g. fungal feeding nematodes and microarthropods, that consume pathogen biomass for sustenance. Biological control can result in varying degrees from all of these types of interactions, depending on the environmental context within which they occur. Significant biological control, as defined above, most generally arises from manipulating mutualisms between microbes and their plant hosts or from manipulating antagonisms between microbes and pathogens.

The role of Mycorrhizal fungi in control of various soil borne plant diseases has been reviewed by many workers (Jalali and Jalali, 1991; Jeffries *et al*, 2003, Aggarwal *et al*, 2006; Sharma *et al*, 2009). Besides, the association of VAM fungi with plant nematodes and the beneficial effect of mycorrhizal symbiosis on plant growth had led to investigations into the potential of VAM fungi to limit yield losses due to nematodes (Bhargava *et al*, 2008).

Soil-borne pathogens causing particularly wilts and root-rots, are responsive to differential soil conditions including dynamics of microbial activity in the zone of root influence. V-A mycorrhizal colonization have been shown to promote or inhibit the development of plant diseases, either by marked shifts in the rhizosphere environment to the benefit or detriment of potential pathogen, and /or alternation of the host plant to benefit or hinder, pathogen's progression and development.

It has been demonstrated that changes in the soil environment, particularly in terms of its water and nutrient status, are able to appreciably affect the germination, growth, development and pathogenic behaviour of many soil microorganisms. In pathogenic fungi, quiescence of spores, mycelial lysis, and formation of resistant structures can be induced by microbial competition for nutrient and even for space. And the exogenous addition of nutrients can reverse the impact of such competition.

Numerous studies have clearly shown that mycorrhizal root system are less susceptible to the attack of soil pathogens than non-mycorrhizal systems. Becker and Gerdemann (1977) reported that roots of onion were less susceptible to *Pyrenophore terrestris*, casual agent of pink root disease. In fact, those segments of root system which have mycorrhizal colonization were observed to be directly proportional to disease resistance. Jalali (1988) demonstrated that mycorrhizal inoculations resulted in significant reduction in the host infection by *Fusarium* and *Rhizoctonia.* Drastic growth suppression of *Fusarium oxysporum* f.sp. *ciceri* in chickpea was observed when subjected to soil-inoculation with *Glomus* sp. Similar response was observed on *Rhizotonia solani* when speed-pelleting with sporocarps of the mycorrhizal endophyte was done. Baltruschat and Schonbeck (1975) reported significant reduction in the number of chalmydospores of the root-rotting fungus *Thielaviopsis basicola* on mycorrhizal tobacco roots than non-mycorrhizal ones. On the other hand, multiplication of tomato aucuba mosaic virus increased when tomato plants were subjected to mycorrhizal inoculation. The mycorrhizal colonization alerts the host metabolism, which may result in an increase or decrease in host resistance. Certain chemical, physiological and morphooogical alterations in the host plant are known to be induced by the mycorrhizal infection some of which may be correlated to the alerted host resistance / susceptibility. It is, however, an important feature that mycorrhiza strengthen the cell walls by increasing lignification and the production of other polysaccharides. In such tissue the growth of the pathogen is likely to be suppressed, as was shown with *Fusarium oxysporum*. A stronger vascular system increases the flow of nutrients, confer greater mechanical strength and inhibit effects from potential

pathogen.Vesicular-arbuscular fungus reduces *Rhizoctonia* disease incidence. VAM fungus, *Glomus* sp. was isolated from field with rice-mungbean-corn cropping pattern. It was used to challenge the pathogen *Rhizoctonia solani* on rice, corn and mungbean in pot experiment. *Glomus* sp. was unable to control *R. solani* in mungbean as the pathogen caused damping-off in seedlings a few days after sowing and before the mycorrhizal fungus could establish itself in the soil and roots. On rice, however, sheath blight incidence was reduced by about 30% when the VAM fungus was added to steamed soil at seedling stage or when the sclerotia of the pathogen were added a month after seedling germination. Disease incidence was reduced even (45%) when both *Glomus* and *R.solani* were added to the soil seeding time. With corn grown in steamed soil, the presence of the VAM fungus reduced disease incidence from 66.6% to 8.3% and in natural field soil, from 16.6% to no disease occurrence.

Aspergillus niger (mycoparasite) and VA endophytes jointly as well as individually significantly suppressed occurrence of *Rhizoctonia solani.* The positive influence of endomycorrhizal fungi (possibly *Glomus mosseae*) on the excessive root proliferation in the maize seedlings in *in situ* experiments was established. Evidence was documented for the secretion of growth promoting metabolites like IAA and kinetin on associative growth and development of maize roots and fungal symbionts. Such a study under controlled conditions with micro propagated plants, allowed identification of promoters involved and accurate determination of which step in the rooting process is blocked in recalcitrant crops (Verma,1995). The arbuscular mycorrhiza of *Gigaspora margarita* and transformed Ri T-DNA carrot root was formed by dual culture technology. Some morphological attributes i.e. infection and distribution of hyphae in root, bidirectional movement of hyphal cytoplasm flow, the formation of extrametrical auxiliary cells, hyphal wound healing and spore formation, development and maturity were investigated and the physiological significance in relation to these morphological characteristics was discussed. The structure in symbiosis affected nutrient absorption and translation in plants.

Jalali and Jalali (1991) showed that suppressive effect had direct correlation with the ability of the mycorrhizal symbiont to develop in the absence of available soil phosphate. Soil conditions favourable for V-A mycorrhizal colonization were observed to be not conducive for the growth of test pathogens. High chitinase activity of the mycorrhizal tissue may restrict the growth of the root pathogen in the host. The inhibition of chlamydospore production of *Thielaviopsis basicola* is also found to be due to an increased level of arginine in mycorrhizal roots. In studies of colonization patterns of tomato roots by the Mycorrhizal fungus *Glomus*

mosseae and the pathogen *Phytophthora parasitica*, Cordier *et. al* (1998) showed that proliferation of the pathogen is greatly reduced in Mycorrhizal root system of tomato, compared with non mycorhhizal ones. Moreover, they observed that the host cells containing typical haustoria-like arbuscules structures of the Mycorrhizal fungus were not infected by *P. parasitica* and that pathogen proliferation was reduced not only in mycorrhizal parts but also in nonmycorrhizal parts of mycorrhizal root systems. Cytomolecular phenomena underlying bioprotection against *P. parasitica* in *G. mosseae*-colonized root systems of tomato has been demonstrated.

Various mechanisms also allow VAM fungi to increase a plant's stress tolerance. This includes the intricate network of fungal hyphae around the roots which block pathogen infections. Inoculation of apple-tree seedlings with the VAM fungi *Glomus fasciculatum* and *G. macrocarpum* suppressed apple replant disease caused by phytotoxic myxomycetes . VAM fungi protect the host plant against root-infecting pathogenic bacteria. The damage due to *Pseudomonas syringae* on tomato may be significantly reduced when the plants are well colonized by mycorrhizae (Garcia-Garrido and Ocampo 1989). The mechanisms involved in these interactions include physical protection, chemical interactions and indirect effects. The other mechanisms employed by VAM fungi to indirectly suppress plant pathogens include enhanced nutrition to plants; morphological changes in the root by increased lignification; changes in the chemical composition of the plant tissues like antifungal chitinase, isoflavonoids, etc. alleviation of abiotic stress and changes in the microbial composition in the mycorrhizosphere (Linderman, 1994).

Effectiveness of arbuscular mycorrhizal fungi in the protection of common bean plant (*Phaseolus vulgaris* L.) against Fusarium root rot disease was investigated by Al-Askar and Rashad (2010) under natural conditions in pot experiment. A mixture of arbuscular mycorrhizal fungi consisting of propagated units of *Glomus mosseae*, *Glomus intraradices*, *Glomus clarum*, *Gigaspora gigantea* and *Gigaspora margarita* in suspension form (10^6 unit L^{-1} in concentration) was used at dilution of 5 ml L^{-1} water. The obtained results demonstrated that, arbuscular mycorrhizal colonization significantly reduced the percentage of disease severity and incidence in infected bean plants. On the other hand, mycorrhizal colonization significantly increased the tested growth parameters and mineral nutrient concentrations. While, infection with *Fusarium* root rot disease negatively affected the mycorrhizal colonization level in bean roots. Finally, mycorrhizal colonization led to a significant increase in the phenolic content and the activities of the investigated defense

related enzymes (phenylalanine ammonia-lyase, polyphenol oxidase and peroxidase enzyme). From the obtained results, it can be concluded that the application of arbuscular mycorrhizal fungi as a biocontrol agent played an important role in plant resistance and exhibit greater potential to protect bean plants against the infection with *F. solani*. In studies of colonization patterns of tomato roots by the Mycorrhizal fungus *Glomus mosseae* and the pathogen *Phytophthora parasitica*.

Mycorrhizal colonization significantly reduced the percentage of disease severity and incidence in infected bean plants. These results are in agreement with that of Dar *et al.* (1997), who found that inoculation of common bean plants with *Glomus mosseae* decreased root rot by 34 to 77%. Many authors have reported that the AM colonization can reduce root disease caused by several soil borne pathogens (Abdalla and Abdel-Fattah, 2000;Yao *et al.*, 2002; Chandanie *et al.*, 2009). Among the potential mechanisms involved in the resistance of mycorrhizal systems, the induction of plant defenses is the most controversial (Wehner *et al.*, 2009). Where a number of biochemical and physiological changes has been associated with mycorrhizal colonization.

Alteration in isoenzymatic patterns and biochemical properties of some defense-related enzymes such as chitinases, chitosanases and β-1,3-glucanases have previously been shown during mycorrhizal colonization (Pozo *et al.*, 2002). These hydrolytic enzymes are believed to have a role in defense against invading fungal pathogens because of their potential to hydrolyze fungal cell wall. Stimulating the host roots to produce and accumulate sufficient concentrations of metabolites which impart resistance to the host tissue against pathogen invasion have been reported also (El-Khallal, 2007).

Direct (via interference competition, including chemical interactions) and indirect (via exploitation competition) interactions have been suggested as mechanisms by which AM fungi can reduce the abundance of pathogenic fungi in roots. These have generally been proposed in response to observations of negative correlations in the abundance of AM fungal structures and pathogenic microorganisms in roots (Filion *et al.*, 2003).

Presumably, pathogenic and AM fungi exploit common resources within the root, including infection sites, space and photosynthates within the root (Whipps, 2004). Interference competition may also arise if carbon availability within intercellular spaces and the rhizosphere (Graham, 2001) or the number of infection loci within the root system (Vigo *et al.*, 2000) is reduced as a result of AM fungal colonization. Moreover, increasing the richness

of AM fungal taxa colonizing the root system may result in more intense competition with a pathogenic fungus.

VAM Fungi in biocontrol of fungal pathogens

VAM fungi	Host Plant	Disease	Pathogen	Reference
G. fasciculatum	Tomato	Wilt	*Fusarium oxysporum* f.sp. *lycopersici*	Caron *et al.* 1986
G. fasciculatum	Green gram Black gram Chick pea Sugar cane	Root rot Wilt	*Macrophomina phaseolina* *Fusarium moniliforme and Cephalosporium sacchari*	Kheri and Chandra 1990 ; Jalali *et al.* 1990 : Chandra *et al.* 2000
G. mosseae	Tomato	Blight	*Phytophthora parasitica*	Cordier *et al,* 1998
G.mosseae	Tomato and Egg plant seedlings	Wilt	*Verticillium dahliae*	Karagiannidis *et al.* 2002
G.mosseae *G.intraradices* *G.clarum* *G.aiganlea* *Gigaspora margarita*	Common bean plant	Rot	*Fusarium solani*	Al.Askar and Rashid, 2010
G. fasciculatum	Pea	Root rot	*Aphanomyces enteiches*	Vidyasekharan, 1990
Gigaspora	Pigeon pea	Pegion pea blight	*Phytophthora drechsleri* f.sp.*cajani*	Bisht *et al.* 1985
G. geosporum *G. mosseae*	*Dalbergia sissoo*	Wilt	*Fusarium solani*	Singh *et al.* 2000
G. mosseae *G. fasciculatum*	Soybean	Blight	*Phytophthora megasperma* var.*sojae*	Graham and Menge, 1982
G. mosseae	Poinsettia	Rot Damping off	*Rhizoctonia solani* *Pythium ultimum*	Graham and Menge, 1982

Another group of rhizosphere influencing root-microbe interaction are the plant growth promoting Rhizobacteria (PGPR) which describe soil bacteria that colonise the roots of plants flowing inoculation onto seed and that enhance plant health. PGPR are known to participate ecosystem processes including biocontrol of pathogens. *Pseudomonas* and *Bacillus* are the

genera most commonly known as PGPR, but many other genera also exhibit PGPR activities (Chakraborty *et al.* 2006). Microbial populations in the rhizosphere where a typical response exterted by a kind of bacterial population called " mycorrhiza-helper bacteria (MHB) known to stimulate mycelia growth/ formation of AMF and secretes compound responsible for enhanced root exudation. This in turn stimulates AMF mycelia in the rhizosphere. The bacteria have been found adhering to the AMF hyphae as well as embedded within the spore walls. Bacteria adhering to AMF mycelium may utilize hyphal exudates or use mycelium as vehicle for colonization of rhizosphere.

The behaviour of target and non-target microorganisms at the soil-root interface is largely mediated by factors which manipulate host physiology, and the quantitative and qualitative nature of root exudates. Among the many factors, pesticide applications are able to induce substantial changes in the zone of root influence. Not only are pathogens affected, but changes in the function of mycorrhizal fungi are expected to exert measurable influence on the spectrum of root exudation, as well as the mineral nutrition of host plant favouring a result potential for predisposition to disease.

Synergistic effects of bacteria and mycorrhizal fungi been studied with respect to their combined beneficial impacts on plants. Both ectomycorrhizal (Garbaye, 1994) and endomycorrhizal (Meyer and Linderman, 1986) fungi can interact with different bacterial species. These interactions occur in the zone of soil surrounding the roots and fungal hyphae; commonly referred to as the 'mycorrhizosphere'. The interactions between bacteria and AM fungi have potentially beneficial functions, including the majority of those where PGPR (Meyer and Linderman, 1986; von Alten *et al.*, 1993; Kloepper, 1978) including N_2-fixing bacteria (Secilia and Bagyaraj, 1987) are involved. Plant growth-promoting rhizobacteria are usually in contact with the root surface, or rhizoplane, and increase plant yield by one or more mechanisms such as improved mineral nutrition, disease suppression, or phytohormone production (Weller, 1988; Lugtenberg *et al.*, 2001; Broek and Vanderleyden, 1995). An additional possibility is that the beneficial effects of some PGPR bacteria are due to their interactions with AM fungi. Some reports have shown that PGPR have a strong stimulatory impact on the growth of AM fungi (Linderman, 1997). For example, increased mycelia growth from Glomus mosseae spores caused by an unidentified PGPR has been reported by Azcon (1992). These results suggest that selected PGPR and AM fungi could be co inoculated to optimize the formation and functioning of the AM symbiosis.

Apart from having effects on AM fungal growth, PGPR have been suggested to possess a variety of other direct mechanisms to support the mycorrhizal symbiosis. Garbaye (1994) proposed the term 'mycorrhization helper bacteria' for rhizobacteria that increased the ability of the root to establish symbiotic interactions with ectomycorrhizal fungi. He suggested a number of possible mechanisms for the helper effect, including stimulation of root development, enhanced susceptibility of the root to ectomycorrhizal fungal colonization, or enhancement of the recognition process between root and fungus. Several reports have also demonstrated enhanced AM fungal colonization levels in roots in the presence of PGPR. For example, association of *Pseudomonas putida* with indigenous AM fungi resulted in a clear growth enhancement of clover plants (Meyer and Linderman, 1986), Some PGPR may have properties that support both mycorrhizal establishment and function, supporting the hypothesis that some plant cell programmes may be shared during root colonization by these beneficial microorganisms.

Specific interactions between AM fungi and PGPR most likely occur, and certain groups of bacteria have been shown to be established to a much higher extent in the mycorrhizosphere compared with other groups. This was shown, for example, by Andrade and colleagues (1997) who found that bacteria of the genera Arthrobacter and Bacillus were most frequent in the hyphosphere, the zone of soil surrounding individual AM fungal hyphae, whereas *Pseudomonas* spp. were most abundant in the rhizosphere of *Sorghum bicolor*. This study and others (Artursson *et al* ., 2005) suggest that Gram-positive bacteria may be more commonly associated with AM fungi than Gram-negative bacteria, but this possibility needs to be more rigorously confirmed. It is noteworthy, however, that the bacterial groups most commonly reported to interact synergistically with AM fungi are mainly Gram-positive bacteria and γ-proteobacteria supporting their hypothesis that some members of these phylogenetic groups are more integrally associated with AM fungi than others.

Several PGPR have been shown to be excellent root colonizers (Barea et al., 2002) and a number of surface components have been demonstrated to play a role in the physical interactions between such bacteria and plant roots (Bianciotto and Bonfante, 2002). Several bacteria reported to be good root colonizers, for example, some *Pseudomonas* spp., are also capable of adhering to AM fungal hyphal surfaces, suggesting that the mechanisms involved could be fairly similar. Close cell-to-cell contact between, for example, rhizobia and their host plant roots is an important prerequisite for the formation of the nodules during endosymbiosis, and one may speculate whether similar correlations exist between attachment of bacteria to AM fungal hyphal surfaces and changes in fungal growth or performance.

However, little information is available concerning the extent to which PGPR colonize AM fungal hyphae. Bianciotto and colleagues (1996) reported that some *Rhizobium* and *Pseudomonas* species attached to germinated AM fungal spores and hyphae under sterile conditions, and that the degree of attachment varied with the bacterial strain. However, no specificity for either fungal or inorganic surfaces could be detected among the bacteria tested. Based on their results, these authors suggested that interactions between rhizobacteria and AM fungi were mediated by soluble factors or physical contact.

Endocellular bacteria are reported in only a few fungi including some Glomeromycota species (AM fungi and Geosiphon pyriforme) (Scannerini and Bonfante, 1991; Bianciotto *et al.*, 2000; Perotto and Bonfante, 1997) and in the ectomycorrhizal basidiomycete *Laccaria bicolour* (Bertaux *et al.*, 2003). Regarding the AM fungi, their cytoplasm harbours bacteria-like organisms, which have been observed by microscopy in several AM fungal species (*Glomus versiforme, Acaulospora laevis, Gigaspora margarita*) (Mosse, 1970; MacDonald and Chandler, 1981; Scannerini and Bonfante, 1991; Bonfante *et al.*, 1994). Further investigation of these structures, including the demonstration of their prokaryotic nature,was long regarded as a task too complicated because they could not be cultured. However, by using morphological observations in combination with molecular analyses, Bianciotto and colleagues (1996) succeeded in showing that they actually were of true bacterial origin. They also demonstrated the AM fungal endosymbiotic properties of these bacteria, that they were able to complete their life cycles within fungal cells, and that the bacterial cells were Gram-negative and rod-shaped. Several additional characteristics of the endosymbiotic bacterial genome have since been reported (Ruiz-Lozano and Bonfante, 2000; Minerdi *et al.*, 2001; Minerdi *et al.*, 2002a; Minerdi *et al.*, 2002b).

Endosymbiotic bacteria have been detected in several members of the Gigasporaceae; actually the only fungal species in this family among the evaluated ones, reported not to contain such bacteria was *Gigaspora rosea* (Bianciotto *et al.*, 2000). In the five other species belonging to the Gigasporaceae, intracellular bacteria were detected through all the steps of the fungal life cycle: spores, germtubes, and extra- and intraradical hyphae, except arbuscules (Bianciotto et al., 1996b). The AM fungus most extensively studied for its endosymbiotic bacteria is *G. margarita* isolate BEG 34, which was also the first fungus in which these prokaryotic cells were further investigated

(Bianciotto *et al.*, 1996b). Recent studies have indicated an average of about 20 000 bacteria per *G. margarita* spore (Bianciotto *et al.*, 2004; Jargeat *et al.*, 2004). These bacteria were initially assigned to the genus *Burkholderia* on the basis of their 16S ribosomal RNA gene

sequence, but were recently reassigned to a new taxon termed *Candidatus Glomeribacter gigasporarum* (Bianciotto et al., 2003). In spite of several attempts, these bacteria have never been grown on cell-free media (MacDonald and Chandler, 1981; Scannerini and Bonfante, 1991; Bianciotto *et al.*, 2004; Jargeat *et al.*, 2004), which is the reason why they are assigned to the provisional Candidatus designation for uncultured bacteria (Murray and Schleifer, 1994; Murray and Stackebrandt, 1995).

Soil microorganisms, particularly PGPR, can influence AM formation and function and, conversely, mycorrhizas can affect the microbial populations, particularly PGPR in the rhizosphere (Linderman 1992, 1994; Fitter and Garbaye 1994; Barea 1975,). The analysis of microbe – microbe interactions is crucial to an understanding of the events which occur at the root – soil interface and, particularly, to those related to the microbial colonization of the root surface, or the processes of root infection/colonization by pathogens or mutualistic symbionts (Lynch 1990). These interactions must be taken into consideration when trying to manage AM fungi and PGPR for the biological control of plant pathogens or for the biogeochemical cycling of plant nutrients (Barea *et al.* 2002).

Once the AM status has been established in plant roots, reduced damage caused by soil-borne plant pathogens has been shown. To account for this, several mechanisms have been suggested to explain the enhancement of plant resistance/tolerance in mycorrhizal plants (Linderman 1994, 2000; Azcón-Aguilar and Barea 1992, 1996). One of the proposed mechanisms is based on the microbial changes produced in the mycorrhizosphere. In this context, there is strong evidence that these microbial shifts occur, and that the resulting microbial equilibria could influence the growth and health of the plants. Although this effect has not been assessed specifically as a mechanism for AM-associated biological control, there are indications that such a mechanism could be involved (Azcón-Aguilar and Barea 1992, 1996; Linderman 1994, 2000). In any case, it has been demonstrated that such an effect is dependent on the AM fungus involved, as well as the substrate and host plant (Azcón-Aguilar and Barea 1996; Linderman 2000). Since specific PGPR antagonistic to root pathogens are being used as biological control agents (Alabouvette *et al.* 1997), it has been proposed to try to exploit the prophylactic ability of AM fungi in association with these antagonists (Linderman 1994, 2000;Azcón-Aguilar and Barea 1996; Barea *et al.* 1998; Budi *et al.* 1999).

Several studies have demonstrated that microbial antagonists of fungal pathogens, either fungi or PGPR, do not exert any anti-microbial effect against AM fungi (Calvet *et al.* 1992; Barea et al. 2002; Edwards *et al.* 1998; Vazquez *et al.* 2000). This is a key point to

exploit the possibilities of dual (AM fungi and PGPR) inoculation in plant defense against root pathogens. In particular, Barea *et al.* (1998) carried out a series of experiments to test the effect of *Pseudomonas* spp. producing 2,4-diacetylphloroglucinol (DAPG) on AM formation and functioning. Three *Pseudomonas* strains were tested for their effects on AM fungi: a wild type (F113) producing the antifungal compound DAPG; the genetically modified strain (F113G22), a DAPG-negative mutant of F113; and another genetically modified strain [F113 (pCU203)], a DAPG-overproducer. The results from *in vitro* and in soil experiments demonstrate no negative effects of these Pseudomonas strains on spore germination, and a stimulation of hyphal growth of the AM fungus *Glomus mosseae*. Concentrations of the antifungal compound DAPG which were far in excess of those reached in the rhizosphere of *Pseudomonas*-inoculated plants exhibited negative effects on germination of AM fungal spores, but more realistic concentrations of DAPG did not affect AM fungal development. A soil microcosm system was also used to evaluate the effect of these bacteria on the process of AM formation. No significant difference in AM formation on tomato plants between F113, F113G22 and F113 (pCU203) was observed, with the F113 and F113G22 strains resulting in a significant increase in the percentage of the root system becoming mycorrhizal. Therefore, these strains behaved as MHB. In a field experiment, none of these *Pseudomonas* strains affected: (a) number and diversity of AM fungal native population; (b) the percentage of root length that became mycorrhizal; (c) AM performance. Furthermore, the antifungal Pseudomonas improved plant growth and nutrient (N and P) acquisition by the mycorrhizal plants (Barea *et al.* 1998).

It should be mandatory to detect the cohesiveness of both AMF and PGPR participating in a particular rhizosphere while maintaining the healthy rhizosphere. The key step is to ascertain whether an antifungal biocontrol agent will negatively affect the AMF populations. There is need to exploit the possibilities of dual (AMF and PGPR) inoculation to provide plant defense against root pathogens. The success of these microbes will depend on our ability to manage the rhizosphere to enhance survival and competitiveness of these beneficial microorganisms. Rhizosphere management will require consideration of soil and crop cultural practices as well as inoculants formulation and delivery. It has also been established that combined uses of both AMF and PGPR increases the efficacy towards pathogen control (Sharma *et al.* 2009). But the magnitude of pathogen suppression varies from rhizosphere to rhizosphere, which needs to be deciphered and customized. Hence, there is a need to modulate the mycorrhizosphere to maintain higher hyphosphere activity to mange resident AMF and PGPR for improving the plant and soil health and should be the key aim of

the applied research in the future. With the growing interest in reducing chemical inputs, companies involved in the manufacturing and marketing of bioformulations (AMF-PGPR biocontrol consortium) should experience continued growth. However, stringent quality control measures must be adopted so that farmers get quality products. New, more effective and stable formulations also will need to be developed.

Materials and Methods

3.1. Plant Material

Nursery grown mandarin (*Citrus reticulata*) seedlings, four month old obtained from Citrus Dieback Research Centre, Kalimpong [N 27° 12'457'' E 88° 14' .574''], Singbulli and Mirik Busty Area, Mirik [N 26° 52'.291'' E 88° 11' .174''] West Bengal, were used for experimental purposes. The selected seedlings initially maintained in 6" plastic pots and watered regularly for proper growth. After one year of growth seedlings were transferred in the earthenware pots (12" dia). These were kept in Glass House conditions (Plate 2,figsA-C) and after two years seedlings were planted in the experimental field. Suitable management practices were adopted in the field throughout the years.

3.2. Fungal culture

Fungal pathogen (*Macrophomina phaseolina*) was isolated from samples of diseased roots of mandarin (*Citrus reticulata*) plants grown in Mirik busty by culturing pieces of internal tissues. Infected root tissues were thoroughly washed in sterile water, treated with 0.1% $HgCl_2$ for 2-3 minutes, rewashed with sterile distilled water, transferred to potato dextrose agar (PDA) slants and incubated at 28°C for two weeks. The isolated organism was examined under microscope. Healthy seedlings of mandarin (*Citrus reticulata*) plants (1-year-old) were further inoculated with this isolated organism and incubated for a period of 4 weeks for completion of Koch's postulate. Subsequently, the infected roots were collected, washed, cut into small pieces, treated with 0.1% $HgCl_2$ for 2-3 minutes, rewashed with sterile distilled water, transferred to PDA slants and incubated at 28°C. At the end of two weeks, the reisolated organism was examined, compared with the original stock culture and its identity was confirmed following microscopic observations as *Macrophomina phaseolina.* Mycelia – septate, branched, hyaline when young becoming brown with age. Advancing zone of mycelia mat even and appressed. Sclerotia – black, moderate size (34-78 u in diameter), round or irregular (Plate 7 fig. D) uniformly reticulate with no difference in internal structure. The culture was maintained on PDA slants and stored under three different conditions [5°C , 20°C and 30°C (room temperature)]

Plate 2 (figs. A-C): Seedlings of *Citrus reticulata* maintained in nursery [A & B] and in glass house [C].

in sterile liquid paraffin. The culture was examined at a regular interval to test its viability and pathogenicity of the fungus.

Three other root pathogens- *Fusarium solani, Fusarium oxysporum and Fusarium graminearum* were obtained from Immuno-Phytopathology Laboratory, Department of Botany, N.B.U. which were also maintained with regular sub culturing in PDA for subsequent tests.

3.3. Isolation of microorganisms from Mandarin rhizosphere

Isolation of microorganisms from the rhizosphere of *Citrus reticulata* was carried out following, Warcup's soil plate method (1955) with a few modifications. The method favors isolation and enumeration of soil borne fungi, bacteria and actinomycetes. Five grams of soil particles loosely adhering to the roots were collected from six different locations of Darjeeling hills. The soil suspension was prepared by dissolving the soil sample in 30 ml sterile distilled water using magnetic stirrer for 1 h. The suspension was allowed to settle down till the two distinct layers were clearly visible. Then the upper light brown coloured layer was pipetted out and serial dilutions were made. One ml each of 10^{-3} and 10^{-4} dilutions were used for isolation by dilution plate technique (Kobayashi *et. al*, 2000) using Nutrient Agar (NA), King's B media, Potato dextrose agar (PDA) as well as *Trichoderma* selective media (TSM) as the growth media. The petriplates were then placed in an incubator for observation of the microbial growth after 24, 48 and 96h of incubation.

3.4. Isolation and Identification of AM spores from rhizosphere soil

Spores of arbuscular mycorrhizal fungi were isolated from rhizosphere soil of mandarin by wet sieving and decanting method (Gerdemann and Nicolson, 1963). Approximately 250 g of soil was suspended in 1 L water. Heavier particles were allowed to settle for a few seconds and the liquid was decanted through sieves of decreasing size (BS 60, BS 80, BS 100, BS 150 and BS 200). Pores are fine enough to remove the larger particles of organic matter, but coarse enough to allow the desired spores to pass through. The suspension that passed through these sieves was saved and stirred to resuspend all particles. The heavier particles were allowed to settle for a few seconds and the liquid decanted again through the sieve and spores collected by fine brushes and were kept in different Petri plates according to their size and colours. Moreover for further observations or purification of AMF spores sucrose gradient centrifugation method was used. In sucrose gradient centrifugation (Daniels and Skipper, 1982), spores and minimal amount of organic particles were further purified by

suspending sieving in 40% sucrose solution and centrifuging at 2000 rpm (approximate 370 x g) for 1 minute. The supernatant (with spores) was passed through a sieve of 400 mesh and rinsed with distilled water to remove sucrose residue. With the help of a simple microscope (20X) parasitized spores, plant debris etc were separated and clean spores were stained with Melzar's reagent (50% aqueous solution of chloral hydrate with 2.5-3.75% potassium iodide and 0.75-1.25% iodine) and studied microscopically. For further use, the AMF spores were stored in Ringer's Solution (8.6g NaCl, 0.3g KCl, 0.33g $CaCl_2$ in 1 L of boiled distilled water) at -15°C to -20 °C or in sterile distilled water.

Identification of genera and species was done microscopically using the specific spore characters such as size, colour, shape, wall structure, surface ornamentation and bulbous suspensor by using identification manuals (Trappe, 1982; Schenck and Perez, 1990).

3.5. Screening of root for mycorrhizal infection

Young roots from mandarin plants were dug out manually. The root sample was washed with tap water gently to free them from soil particles and stored in FAA (formaline aceto alcohol) prior to staining. For staining , root segment of 1cm each was put into the test tube and boiled in 10% KOH solution for 15-20 minutes on a water bath (sometimes even 60 minutes for hard roots), washed in tap water and stained with chlorazol black E (Beverage, 1970; Phillips and Hayman, 1970). For confirmation of infection, the presence of intercellular hyphae, vesicles and arbuscules or both characteristics was taken into consideration. Percent root colonization was determined following the method of Giovanetti and Mosse (1980).

3.6. Inoculation techniques and disease assessment

The inoculum of pathogen *M. phoaseolina* was prepared for inoculation of healthy citrus plants in sand maize meal medium supplemented with citrus root pieces, as it increases the survival capacity and viability of the pathogen in the soil. Initially, *M. phaseolina* was grown on PDA in Erlenmeyer flasks (250ml) for 2 days. Subsequently 30 sterilized mandarin root pieces (one inch long) were transferred to each flask and incubated for 15 days. Sand maize meal medium (50g) containing five such pieces covered with the mycelia and sclerotia were inserted in the rhizosphere of each plant. The inoculated plants were examined at an interval of 7 days up to a period of 28 days. Each time, the plants were uprooted, washed and symptoms noted. Finally roots were dried at 60^0C for 96h and weighed. Root rot index was calculated on the basis of percentage root area affected and they were graded into 6 groups and a value was assigned to each group (viz. no. root rot = 0; upto 10% root area affected =

0.10; 11-25% = 0.25; 26-50% = 0.50; 51-75% = 0.75; 76-100% = 1.0). The root rot index in each case was the quotient of the total values of the replicate roots and the number of roots (i.e. number of plants).

3.7. Assessment of mycelial growth.

Mycelial growth of the fungal cultures was assessed in both solid media and liquid media to study and evaluate their cultural characteristics.

3.7.1. Solid media

To assess the growth of fungal culture in solid media, the fungus was first grown on petri dishes, each containing 20ml of PDA followed by incubation for 7 days at 30°C. Agar blocks (6mm diameter) containing the mycelium was cut with sterile cork borer from the actively growing region of mycelial mat and transferred to each Petri dish containing 20ml of different sterilized solid media. The colony diameter was studied at regular interval of time. The media were as follows:

A. Potato dextrose agar (PDA):

Peeled potato - 40.00g, Dextrose – 2.00g, Agar - 2.00g, Distilled water - 100ml

B. Richards agar (RA):

KNO_3 - 1.00g, KH_2PO_4 - 50g, $MgSO_4$. $7H_2O$ - 0.25g, $FeCl_3$ - 0.002g, Sucrose - 3.00g, Agar - 2.00g, Distilled H_2O – 100ml

C. Carrot juice agar (CJA):

Grated carrot - 20.00g, Agar - 2.00g, Distilled water - 100 ml

D. Czapek dox agar (CDA):

$NaNo_3$ - 0.20g, $KHPO_4$ - 0.10g, $MgSo_4.7H_2O$ - 0.05g, KCl - 0.05g, $FeSo_4.7H_2O$ - 0.05g, Sucrose - 3.00g, Agar - 3.00g, Distilled water - 100ml

E. Potato sucrose agar (PSA):

Peeled potato - 40.00g, Sucrose - 2.00g, Agar - 2.00g, Distilled water - 100ml.

F. Malt extract peptone agar (MPA):

Malt extract - 20.00g, Peptone - 1.00g, Dextrose - 20.00g, Agar -

20.00g, Distilled water - 1L.

3.7.2. Liquid media

To assess the mycelial growth in liquid media the fungus was first grown on petriplates, each containing 20ml of PDA and incubated for 5-8 days at 28°C. The mycelial block (5mm) from the actively growing region of the fungus in the petriplate was cut with sterilized cork borer and transferred to Erlenmeyer flask (250ml) containing 50 ml of sterilized Potato dextrose broth (PDB), Richards medium and Nutrient broth (NB) and incubated for 6 - 8 days with constant stirring at room temperature. After incubation the mycelia were harvested through muslin cloth, collected in aluminium foil cup of known weight and dried at 60°C for 96 h, cooled in desiccators and weighed.

3.8. Biochemical tests of microorganisms

3.8.1. Gram reaction

Smears of test organisms prepared from 24h old culture (on nutrient agar slant) with sterile distilled water were made in the centre of clean grease-free slides. The smears were air dried, heat fixed with crystal violet (crystal violet – 2.0g, 95% alcohol- 20ml, ammonium oxalate 1% W/V, aqueous solution – 80ml) stain for 1 min, washed with tap water for 5 sec, flooded with Burke's iodine solution (Iodine 1.0g, KI- 2.0g, distilled water 100ml) and allowed to react for 1 min. Slides were washed for 5 sec in 95% ethanol which was poured drop by drop by holding the slides in slanting position till the smears were decolorised, and then it was rinsed with water and dried. The smears were finally counter stained with safranin (2.5 w/v safranin in 95% ethanol-10ml, distilled water -100ml) for at least thirty seconds, rinsed with water and dried. The gram character and morphological characters were determined under oil-immersion objectives.

3.8.2. Endospore stain

The bacterial smear was prepared. The fixed slide was flooded with the solution of malachite green and the slide was placed over boiling water bath for five minutes. After rinsing, the smear was counter stained with safranin.

3.8.3. Catalase

Bacterial culture (24 h old) was flooded with 0.5 ml 10% H_2O_2 solution and gas bubble production indicated the positive reaction.

3.8.4. Urea digestion

Streaks were made on the slants containing urea medium and incubated at 37° C for 2-7 days. The change in colour of the medium indicates the presence of urease.

3.8.5. Casein hydrolysis

The mild agar was streaked with the bacteria and was incubated at 37° C and observed for the clear zone around the streaks.

3.8.6. Starch hydrolysis

The bacteria were streaked on sterilized starch agar plate (NA + 0.1% soluble starch) and incubated for five days at 37° C. The plates were flooded with Lugol's iodine solution. The clear zone underneath and around the growth indicates the starch hydrolysis.

3.8.7. Indole test

10ml of Davis Mingoli's broth supplemented with 0.1% tryptophan was inoculated with the isolate and incubated anaerobically at 37° C for 7 days. The culture was layered carefully with 2 ml of Ehrlich- Bobme (P-dimethylaminobenzaldehyde 10g, concentrated HCL 100ml) reagent on the surface, allowed to stand for a few minutes and observed for the formation of a ring at the medium reagent interface indicating the production of indole.

3.8.8. Siderophore production

Production of siderophore was detected by standard method of Schwyn and Neiland (1987) using blue indicator chrome azurol S (CAS). The bacteria were spot inoculated at the center of the plate and incubated for 12-15 days. The change in the colour of the medium around the bacterial spot was an indication of siderophore production.

3.8.9.Chitinase production

Production of chitinase was detected by standard method of Hsu and Lockwood (1975). Colonies showing zones of clearance against the creamy background were regarded as chitinase-producing strains.

3.8.10. Cellulase Production

Cellulose overlay agar plates were streaked with the 24h old bacterial cultures and incubated at 37°C for 7 days. The plates were observed for any clearing zone around or underneath the growth.

3.8.11. Protease production

Protease activity was detected on 3% (wt/vol) powdered milk-agar plates according to Walsh *et al.* (1996).

3.8.12. H_2S production

Slants containing SIM agar was inoculated with the test bacteria and incubated for 48h at 37°C. Darkening along the line of the slants indicated the production of H_2S by the organisms.

3.9. *In vitro* testing of rhizosphere microorganisms for antagonism towards root pathogen

3.9.1. Fungi

3.9.1.1. Solid medium

The efficacy of individual fungal isolates, from mandarin rhizosphere was tested *in vitro* for inhibiting growth of the pathogen (*M. phaseolina*) in dual culture using PDA. Each fungal isolate was placed at one side of the agar plate about 1cm away from the edge and a 4mm diameter block of the pathogen, taken from growing edge of the fungal culture was inoculated at the other half of the Petri plate. For each test, three replicate plates were used. The plates were incubated for 7 days (depending upon the growth of the pathogen) at 28°C and inhibition zone towards the fungus colony in individual plate was quantified. Results were expressed as mean % of inhibition in presence of the fungal isolate.

3.9.2. Bacteria

3.9.2.1. Solid medium

The obtained bacterial isolates were evaluated against root pathogen- *Macrophomina phaseolina* in dual culture using NA medium. The bacteria were streaked on one side of the Petri plate and 4mm fungal pathogen block was placed at the other side of the plate, incubation was undertaken for 5-7 days at 28°±2°C and inhibition zone towards the fungal colony in individual plate was quantified. Results were expressed as mean of percentage of inhibition of the growth of the pathogen in presence of the bacterial isolates. For each test three replicate plates were used. Those bacteria, which were antagonistic to *M. phaseolina*, were selected for further evaluation and identification.

3.9.2.2. Liquid medium

To assess the possible antagonism between the root pathogen (*M. phaseolina*) and bacteria, NB was selected for growth of selected bacteria (which showed inhibition in solid medium). Agar block (4mm) containing 7 day old mycelia of the fungus and 0.5ml of bacterial suspension ($1x10^6$ cfu/ml) were used as inoculum for each flask (50 ml NB/250 ml flask). The mycelium grown without bacterial isolates in similar medium was taken as control. The cultures were incubated at 28 ±2°C and after 7 days of incubation mycelia were washed thoroughly with sterile distilled water to remove bacteria as far as possible and

harvested by staining through muslin cloth and mycelial dry weights were determined. Three replicates were taken in each case.

3.10. Scanning Electron Microscopic observation of selected beneficial microorganisms

Selected beneficial microorganisms (BCA and PGPR) were examined under scanning electron microscopy (SEM). Samples were prepared according to a modification of the method described by King and Brown (1983). Test isolates were grown on PDA plates for l0 days in daylight at room temperature. Small pieces of the agar (less than 1 cm), with aerial sporulating culture attached, were excised from each plate and transferred to the interior surface of a dry glass Petri dish lid. Efforts were made not to disturb the attached culture. Steps that involved exposing the samples to the atmosphere were performed quickly to minimize air-drying artifacts. The specimen dishes were then placed in vapour diffusion dehydration (VDD) assembly, and a vacuum was drawn as described by King and Brown (1983). All samples were left in the VDD assembly where a maximum level of dehydration was achieved. The vacuum was released slowly and the specimen dish was removed from the desiccator. Each sample was placed within separate aluminum "disc cup" (20 mm diam x 5 mm deep). Each sample was lifted from the bottom of the specimen dish with fine forceps and was positioned upright in a disc cup. The samples were then dried. All dried samples were mounted on double-sided tape affixed to SEM specimen mounts and were subsequently sputter-coated with gold. Gold coated samples were examined with a Philips 505 scanning electron microscope operating at 9.5-15 Kev.

3.11. Assessment of bacterial growth

For assessment of bacterial growth in liquid medium, 1ml of bacterial suspension was inoculated into the medium and allowed to grow for desired period. Following growth, absorbance was noted in a colorimeter at 600nm. Absorbance was converted into cfu/ml from a standard where known concentration of bacterial suspension was used. The cfu values were counted to log whenever needed. For assessment of bacterial growth, different media were used. These are as follows;

A. Nutrient broth (NB):

Peptone - 5.0g, Beef extract - 3.0g, NaCl - 5.0g, Yeast extract - 3.6g, Water – 1L, pH – 7.4 ± 0.2

B. Nutrient agar medium (NA):

Peptone - 5gm, NaCl - 5gm, Yeast extract - 1.5 gm, Beef extract - 1.5g, Agar - 20g, Water - 1L

C. Luria broth (LB):

Peptone – 10.0 g, NaCl - 5.0g, Yeast extract - 1.5 gm, Distilled H_20 - 1L, pH 7.2 ± 0.2

D. Nutrient sucrose broth (NSB):

Sucrose – 1.5g, Yeast extract - 1.2 gm, Peptone – 1.2.0 g, Beef extract - 0.6g, Distilled H_20 – 300 ml, pH – 7.2 ± 0.2

3.12. *In vitro* Screening and Evaluation of phosphate solubilizing activity of isolated microorganisms

3.12.1. Screening

Preliminary screening for phosphate solubilization was done by a plate assay method using Pikovskaya (PVK) agar medium supplemented with Tricalcium phosphate (TCP) and pH of the medium was adjusted to 7.0 before autoclaving. One gram soil sample was suspended in 9ml sterile distilled water in a tube for serial dilutions, and 1ml aliquots were transferred to PVK medium. The plates were incubated at 28±2°C for 7 days with continuous observation for colony diameter. Transparent (halo) zones of clearing around the colonies of microorganisms indicate phosphate solubilization and each colony was carefully transferred, identified and further used for quantitative determination of phosphate solubilization.

3.12.2. Evaluation

Evaluation of phosphate solublizing activity of fungal isolates was done by growing the isolates in the two sets of Pikovskaya's liquid medium amended with 0.5% tricalcium phosphate and 0.5 % rock phosphate separately over a period of 10 days at 28^0C with constant shaking at 100 rpm in a rotary incubator. Quantitative estimation of phosphate was done following amonium molybdate ascorbic acid method as described by Kundsen and Beegle (1988). Amount of phosphate utilized or solubilized by the isolates were expressed as mg/L phosphate utilized by deducting the amount of residual total phosphate from the initial amount of phosphate source added to the modified Pikovskaya's liquid medium (yeast extract, 0.50 g/L, dextrose, 10.0 g/L, calcium phosphate/rock phosphate, 5.0 g/L, ammonium phosphate, 0.50 g/L, potassium chloride, 0.20 g/L, magnesium sulphate, 0.10 g/L, manganese sulphate, 0.0001g/L, ferrous sulphate, 0.0001 g/L, pH, 6.5)amended with 0.5 % tricalcium

phosphate and 0.5 % rock phosphate. Liquid medium (50 ml) was inoculated with 5 % v/v of the spore suspension prepared from the 7 days old culture grown on PDA slants and incubated at room temperature for 4 days with routine shaking at 100 rpm. The initial pH of the medium was recorded. The mycelia were harvested after 10 days of incubation by filtering and the change in the pH of the culture filtrate was recorded after centrifuging the medium at 5000 x g for 5 min. on a table centrifuge.

Phosphate measurement in the soil was estimated by ammonium molybdate-ascorbic acid method where, 2ml of the aliquot of the soil extract was mixed with 8ml of the colorimeter working solution containing 60 g/L ammonium paramolybdate, 1.455 g antimony potassium tartarate, 700ml/L conc. sulfuric acid and 132 ml/L of ascorbic acid and mixed thoroughly and incubated for 20 min. till the colour developed. Percent transmittance of the solution was taken on a colorimeter with the wavelength set at 882 nm.

3.13. Application of PGPR

3.13.1. Soil drench

The bacteria were grown in NB for 48 h at 28°C and centrifuged at 12,000rpm for 15 minute. The pellet obtained was suspended in sterile distilled water. The optical density of the suspension was adjusted using UV-VIS spectrophotometer following method to obtain a final density of 3 $X10^6$cfu ml^{-1}.

The bacterial suspension was applied to the pots during transplantation of seedling from sleeves. Applications were done @ 0f 100 ml per pot at regular interval of one month for three months subsequently. The rhizosphere of two year's old potted plant was inoculated twice at an interval of 20-25 days.

3.13.2. Foliar spray

The bacterial pellet suspended in sterile distilled water at a concentration of $3x10^6$ cfu ml^{-1} after the addition of a few drops of Tween -20 was sprayed until run off on the foliar part of the ten year old bushes after pruning. The spraying was done forth nightly till the new shoots started appearing. The growth parameters such as number of leaves, branches and height were observed.

3.14. Inocula preparation and application of biocontrol fungi

3.14.1. AMF. Spores of *Glomus mosseae* were separated from the mass of other AM spores by fine tweezers and needles under dissecting microscope and were washed by distilled water

several times to remove the adhered debris followed by inoculation in the roots (7-10 days old) of *Sorghum bicolor*, *Cynodon dactylon*, and *Zea mays* grown in black plastic pots (12inch) having autoclaved soil to discard the presence of other fungal propagules. After 45 days the presence of spores of *G. mosseae* were verified and inocula were prepared by mixing the chopped roots of sorghum plants with the potted soil where extra radical spores of *G. mosseae* were present. Approximately > 175 spores / 100gms could be considered as potent inocula for application.

3.14.2. BCA. Inoculum of *Trichoderma asperellum* was prepared by inoculating wheat bran (sterilized) with 5 mm disc of the fungus and incubating at 28 °C for 10 days. To each pot containing either *F. solani* infested or control soil (2000 g), 10 g of the wheat bran colonized by *T. asperellum* was mixed to give a concentration of 10^5 cfu / g of soil as described by Chakraborty *et al.* (2003).

3.15. Extraction and estimation of soluble proteins

3.15.1. Mycelia

Mycelial protein was prepared following the protocol as outlined by Chakraborty and Saha (1994). The fungal mycelia were grown in 250 ml Erlenmeyer flask each containing 50 ml of potato dextrose broth (PDB) and incubated for 10 days at 30± 1°C. for extraction of antigen, mycelial mats were harvested washed with 0.2% Nacl and rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortor and pestle and homogenized with cold 0.05m sodium phosphate buffer (PH-7.2) supplemented with 0.85% Nacl, 10mM sodium metabisulphite, PVPP (Polyvinyl pyrollidine Phosphate) and 0.5mM magnesium chloride in ice bath. The homogenated mixture was kept for 2h or overnight at 4°C and then centrifuged at 10.000rpm for 30 min, at 4°C to eliminate cell debris. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring in ice bath and kept overnight at 4°C. After this period, the mixture was centrifuged (10,000rpm) for 30 minute at 4° C, the precipitate was dissolved in the same buffer (pH 7.2). The preparation was dialysed for 72h through cellulose tubing (sigma chemical co., USA) against 1L of 0.005 M sodium phosphate buffer (pH 7.2) with six changes. The dialysate was stored at -20°C and used as antigen fro the preparation of antiserum and other experime

3.15.2. Root

Soluble protein was extracted from mandarin roots following the method of Chakraborty *et al.*, (1995). Root tissues were frozen in liquid nitrogen and ground in 0.05 mM sodium

phosphate buffer (pH 7.2) containing 10 mM $Na_2 S_2 O_5$, 0.5 mM $MgCl_2$ and 2mM PMSF was added during crushing and centrifuged at 4°C for 20 min at 12000rpm. The supernatant was used as crude protein extract.

3.15.3. Estimation of protein content

Soluble proteins were estimated following the method as described by Lowry *et al.*, (1951). To 1ml of protein sample 5ml of alkaline reagent (1ml of 1% CuSO4 and 1ml of 2% sodium potassium tartarate, added to 100ml of 2% $Na_2 CO_3$ in 0.1 NaOH) was added. This was incubated for 15 min at room temperature and then 0.5ml of 1N Folin Ciocalteau reagent was added and again incubated for further 15 min following which optical density was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.16. SDS-PAGE analysis of soluble proteins

Sodium dodecyl sulphate polyacrylamide gel electrophoresis was performed for detailed analysis of protein profile following the method of (Laemmli 1970).

For the preparation of gel the following stock solution were prepared

3.16.1. Preparation of stock solution

Following stock solution were prepared

A. Acrylamide and N'N' – methylene bis acrylamide

A stock solution containing 29% acrylamide and 1% bis-acrylamide was prepared in warm water, as both of them are slowly dominated to acrylic and bis acrylic acid by alkali and light. The pH of the solution was kept below 7.0 and the stock solution was then filtered through Whatman No. 1 filter paper and kept in brown bottle, stored at 4°C and used within one month.

B. Sodium Dodecyl Sulphate (SDS)

A 10% stock solution of SDS was prepared in warm water and stored at room temperature.

C. Tris Buffer

i) 1.5M Tris buffer was prepared for resolving gel. The pH of the buffer was adjusted to 8.8 with concentrated HCl and stored at 4°C for further use.

ii) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was adjusted to 6.8 with conc. HCl and stored at 4°C for use.

D. Ammonium Persulphate (APS)

Fresh 10% APS solution was prepared with distilled water each time before use.

E. Tris- Glycine electrophoresis buffer

Tris running buffer consists of 25mM Tris base, 250mM Glycine (pH 8.3) and 0.1% SDS. A 1X solution was made by dissolving 3.02 g Tris base, 18.8 g Glycine and 10 ml of 10% SDS in 1L distilled water.

F. SDS gel loading buffer

This buffer contains 50 mM Tris –HCl (pH 6.8), 10 mM ß- mercaptoethanol, 2% SDS, 0.1% bromophenol blue, 10% glycerol. A 1x solution was prepared by dissolving 0.5ml of 1M Tris buffer (pH 6.8), 0.5ml of 14.4 M ß- mercaptoethanol, 2 ml of 10% SDS, 10 mg bromophenol blue, 1ml glycerol in 6.8 ml of distilled water.

3.16.2. Preparation of gel

Mini slab gel (plate size 8cm x10cm) was prepared for analysis of proteins patterns through SDS-PAGE. For gel preparation, two glass plates (8 cmx10 cm) were washed with dehydrated alcohol and dried to remove any traces of grease. Then 1.5 mm thick spacers were placed between the glass plates at the two edges and the three sides of the glass plates were sealed with gel sealing tape or wax, clipped tightly to prevent any leakage and kept in the gel casting unit. Resolving and stacking gels were prepared by mixing compounds in the following order and poured by pipette leaving sufficient space for comb in the stacking gel (comb +1cm). After pouring the resolving gel solution, it was immediately over layered with isobutanol and kept for polymerization for 1h. After polymerization of the resolving gel was complete, overlay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30-45 minutes. After polymerization of the stacking gel the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris – Glycine buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom of the gel, was removed carefully with a bent syringe.

Name of the compound	10% Resolving gel (ml)	5% Stacking gel (ml)
Distilled water	2.85	2.10
30% acrylamide	2.55	0.5
Tris*	1.95	0.38
10%SDS	0.075	0.030
10%APS	0.075	0.030
TEMED**	0.003	0.003

*For 1.5 M tris pH 8.8 in resolving gel and for 1M Tris pH 6.8 in stacking gel

** N,N,N',N' –Tetramethyl ethylene diamine.

3.16.3. Sample preparation

Sample (50µl) was prepared by mixing the protein extract (35 µl) with 1xSDS gel loading buffer (16 µl) in cyclomixer. All the samples were floated in a boiling water bath for 30 minutes to denature the proteins samples. After boiling, the sample was loaded in a predetermined order into the bottom of the well with T-100 micropipette syringe. Along with the protein samples, a marker protein consisting of a mixture of six proteins ranging from high to low molecular mass (Phosphorylase b-97,4000: Biovine Serum Albumin -68,000; Albumin -43,000; Carbolic Anhydrase -29.000; Soybean Trypsin inhibitor- 20,000; Lysozyme - 14,300) was treated as the other samples and loaded in separate well.

3.16.4. Electrophoresis

Electrophoresis was performed at 18mA current for a period of two to three hours or until the dye reached the bottom of the gel.

3.16.5. Fixing and staining

After completion of electrophoresis, the gel was removed carefully from the glass plates and the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid: methanol: water (10:20:70) for overnight.

The staining solution was prepared by dissolving 250mg of Coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. When the stain was completely dissolved, 45ml of water and 10ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No.1 filter paper.

The gel was removed from the fixer and stained in this stain solution for 4 h at 37 °C with constant shaking at low speed. After staining the gel was finally destained with destaining solution containing methanol, water and acetic acid (4.5: 4.5:1) at 40 °C with constant shaking until the background become clear

3.17. Immunological studies

3.17.1. Preparation of antigen

3.17.1.1. Fungal antigen

Mycelial protein was prepared following the method as outlined by (Chakarborty and Saha 1994). Mycelia mats were harvested from 7-10 days old culture and washed with 0.2% NaCl then again rewashed with sterile distilled water. Washed mycelia were crushed with sea sand

using a chilled mortar and pestle and homogenized with cold 0.05 M sodium phosphate buffer (pH 7.2) supplemented with 0.85% NaCl, 10 mM sodium metabisulphite and 0.5 mM $MgCl_2$ in ice bath. The homogenate mixture was kept for 2h or overnight at 4 °C and then centrifuged at 10.000rpm for 30 min at 4 °C to eliminate cell debris. The supernatant was collected and stored in -20 °C and used as antigen for the preparation of antiserum.

3.17.1.2. Root antigen

Root antigen was extracted from mandarin roots following the method of Chakraborty *et al.*, (1995). Root tissues were frozen in liquid nitrogen and ground in 0.05 mM sodium phosphate buffer (pH 7.2) containing 10 mM $Na_2 S_2 O_5$, 0.5 mM $MgCl_2$ and 2mM PMSF was added during crushing and centrifuged at 4°C for 20 min at 12000 rpm. The clear supernatant was used as antigen.

3.17.2. Raising of polyclonal antibodies

3.17.2.1. Rabbits and their maintenance

Polyclonal antibodies were prepared against fungal and bacterial antigens in New Zealand white male rabbits of approximately 2kg of body weight. Before immunization, the body weights of rabbits were recorded and observed for at least one week inside the cages. Rabbits were maintained in Antisera reserves for plant pathogens, Immuno-Phytopathology Laboratory, Department of Botany, NBU. They were regularly fed with green grass, soaked gram, green vegetables and carrots etc. twice a day. After each bleeding they were given saline water for three consecutive days and kept in proper hygienic conditions.

3.17.2.2. Immunization

Before immunization, normal sera were collected from each rabbits. For developing antisera , intramuscular injections of 1ml antigen(protein extracted) mixed with 1ml of Freund's complete adjuvant (Genei) were given into each rabbit 7 days after pre- immunization bleeding and repeating the doses at 7 days intervals for consecutive week followed by Freund's incomplete adjuvant (Genei) at 7 days intervals upto 12-14 consecutive weeks as required. Method of (Alba and Devay, 1985) and (Chakraborty and Saha, 1994) were followed for immunization.

3.17.2.3. Bleeding

Bleeding was performed by marginal ear vein puncture, three days after the first six injections, and then every fourth injection. In order to handle the rabbits during bleeding, they

were placed on their back on a wooden bored fixed at an angle of 60°, and held the rabbits tight so that it could not move during the bleeding. The hairs from the upper side of the ear was removed with the help of a razor and disinfected with alcohol. The ear vein was irritated by the application of xylene and an incision was made with the help of a sharp sterile blade and 5 -10 ml of blood samples were collected in sterile graduated glass tube. The blood samples were incubated at 37°C for 1hr for clotting. After clotting; the colt was loosened with a sterile needle. Finally, the serum was classified by centrifugation. (2000g for 10 minute at room temperature) and distributed in 1 ml vials and stored at -20°C as crude antisera. The serum was used for double diffusion analysis, dot blots analysis and Enzyme Linked Immunosorbent Assay (ELISA)

3.17.3. Purification of IgG

3.17.3.1 Precipitation

IgG was purified as described by (Clausen, 1988). Crude antiserum (2ml) was diluted with two volume of distilled water and an equal volume of 4M (NH_4 SO_4) ammonium sulphate was taken and pH adjusted to 6.8, stirring the mixture for 16h at 20° C in magnetic stirrer. The precipitate thus formed was collected by centrifugation at 12,000 rpm for 1h at 22 ° C for 1 h. Supernatant was discarded and pellet was used for further steps.

3.17.3.2 Column preparation

Eight gram of DEAE cellulose (Sigma Co. USA) was suspended in distilled water for overnight. The water was poured off and the DEAE cellulose was suspended in 0.005M phosphate buffer (pH 8.0) and the washing was repeated for 5 times. The gel was then suspended in 0.02 M phosphate buffer, (pH 8.0) and was transferred to a column (2.6 cm in diameter and 30cm height) and allowed to settle for 2h. After the column material had settled 25ml of buffer (0.02M sodium phosphate, pH 8.0) washing was given to the column material.

3.17.3.3 Fraction collection

At the top of the column, 2ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02 m to 0,03 M. the initial elution buffer (1) was 0.02 M sodium phosphate (pH 8.0). The buffer was applied in the flask on which rubber connection from its bottom was supplying column. Another connection above the surface of buffer (1) was connected to another flask with buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to column buffer (2) was soaked into buffer (1) thereby producing a continuous raise in

morality. Ultimately, 40 fractions each of 5ml were collected and the optical density values were recorded at 280nm using UV-Vis spectrophotometer (DIGISPEC-200GL).

3.17.4. Immunological assays

3.17.4.1. Agar gel double diffusion

3.17.4.1.1. Preparation of agarose slides

The glass slides (6cm x 6cm) were degreased using ethanol 90%v/v: diethyl ether (1;1v/v) and ether, then dried in hot air oven. After drying the plates were sterilized inside the petriplate each containing one plate. Conical flask with Tris-Barbiturate buffer (pH 8.6) is placed in boiling water bath. Agar/ agarose (0.9%) was boiled over water bath to dissolve the agar at 90 ° C for next 15 min. Then pinch of 0.1% (w/v) sodium azide was added and mixed well. For the preparation of agarose gel, the molten agarose is poured (6 to 10 ml) on the grease free sterilized slide with the help of a sterile pipette in laminar air flow chamber and allow it to solidify, after solidification cut 3-7 wells (6mm diameter) with sterilized cork borer distance of 1.5 to 2cm away from central well and 2.0 to 2.5 cm from well to well.

3.17.4.1.2. Diffusion

Agar gel double diffusion tests were carried out using antigen and antiserum following the method of Ouchterlony (1967). Antigen plus undiluted antisera appropriately diluted were poured into wells with micropipette (50µl/well) antisera in middle. Slides were kept in moist chamber at 25°C for 72h. Precipitation reaction was observed in the agar gel only in cases where common antigen was present.

3.17.4.1.3. Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9% NaCl and 1% NaN_3) for 72 h with 6 hourly changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. Then the slides were stained with Coomassie brilliant blue (R250, Sigma: 0.25g Coomassie blue, 45ml methanol, 45ml distilled water and 10ml glacial acetic acid) for 10 min at room temperature. After staining, the slides were washed in destaining solution (methanol: distilled water: acetic acid in 45:45:10 ratios) with changes until background become clear. Finally slides were washed with distilled water and dried in hot air oven for 3 h at 50° C

3.17.4.2. Plate trapped antigen coated (PTA)- ELISA

Plate trapped antigen coated (PTA)-ELISA was performed following the method as described by (Chakraborty *et al.*, 1995) with modifications. Antigen were diluted with coating buffer and the antigens were loaded (200µl per well) in ELISA plate (Coaster EIA/RIA, strip plate, USA) arranged in12 rows in a (Cassette) ELISA plate. After loading, the plate was incubated at 25 °C for 4 h. then the plate was washed four times under running tap water and twice with PBS-Tween and each time shaken well to dry. Subsequently, 200 µl blocking reagent was added to each well for blocking the unbound sites and plate was incubated at 25 °C for 1h. After incubation, the plate was washed as mentioned earlier. Purified polyspecific IgG was diluted in antisera dilution buffer and loaded (200 µl per well) and incubated at 4°C overnight. After a further washing, antirabbit IgG goat antiserum labeled with alkaline phosphatase diluted 10000 times in PBS, was added to each well (100 µl per well) and incubated at 37 °C for 2 h. The plate was washed, dried and loaded with 200 µl of p-Nitrophenyl Phosphate substrate in each well and kept in dark for 1 h. color development was stopped by adding 50 µg per well of 3 N NaOH solution and the absorbance was determined in an Multiscan Ex (Thermo Electron) ELISA Reader(Multiskan, ThermoLabsystems) at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

3.17.4.3. Dot immunobinding assay

Dot blot was performed following the method suggested by Lange *et al.* (1989) with modifications. Following buffers were used for dot immunobinding assay.

a. Carbonate –bicarbonate (0.05 M, pH 9.6) coating buffer.
b. Tris buffer saline (10mM pH 7.4) with 0.9% NaCl and 0.5% Tween 20 for washing.
c. Blocking solutions 10% (w/v) skim milk powder (casein hydrolysate, SLR) in TBST (0.05 M Tris-HCl, 0.5 M NaCl) 5% v/v Tween 20 , pH 10.3.
d. Alkaline phosphatase buffer (100 mM tris HCl, 100 mM NaCl, 5mM $MgCl_2$

Nitrocellulose membrane (Millipore, 7cm x10cm, Lot No. H5SMO 5255, pore size 0.45µm, Millipore corporation, Bedford) was first cut carefully into the required size and fix between the template with filter paper at the bottom. 0.5M carbonate- bicarbonate buffer (pH 9.6), 4µl, was loaded in each well and allowed to dry for 30 min at room temperature. Antigen (5µl) was loaded on to NCM and allowed to dry for 30 min at room temperature. Template was removed and blocking of NCM was done with 19% non fat dry milk (casein hydrolysate, SRL) prepared in TBST for 30-60 minutes on a shaker. Respective polyclonal antibody (IgG 1:500) prepared against that antigen was added directly in the blocking solution and further incubated at 4 °C for overnight. The membrane was then washed gently in running tap water for three min, thrice

followed by washing in TBST (pH 7.4), (Wakeham and White, 1996). The membrane was then incubated in alkaline phosphatase conjugated goat antirabbit IgG (diluted 1:10,000 in alkaline phosphatase) for 2h at 37°C. The membrane was washed as before. 10 ml of NBT/BCIP substrate (Genei) was added next and color development was stopped by washing the NCM with distilled water and color development was categorized with the intensity of dots.

3.17.4.4. Western blot analysis

Protein samples were electrophoresed on 10% SDS-PAGE gels as suggested by Laemmli (1970) and electrotransferred to NCM using semi-dry Trans-blot unit (BioRad) and probed with PAbs of the pathogen (*M. phaseolina*) following the method of Wakeham and White (1996). Hybridization was done using alkaline phosphatase conjugate and 5-bromo-4-choloro-3-indolylphosphate (NBT-BCIP) as substrate. Immunoreactivity of the proteins was visualized as violet coloured bands on the NCM.

3.17.4.5. Fluorescence antibody staining and microscopy

Indirect fluorescence staining of fungal mycelia, cross- section of mandarin roots and leaves were done using FITC labeled goat antirabbit IgG following the method of (Chakraborty and Saha, 1994)

3.17.4.5.1. Fungal mycelia

Fungal mycelia were grown in liquid Richards's medium as described earlier. After five days of inoculation young mycelia were taken out from flask and taken in Eppendorf tube and washed with PBS (pH 7.2) by centrifugation at slow speed. Then mycelia was treated with normal sera or antisera diluted (1:50) in PBS and incubated for 1 h at room temperature. The mycelia was washed thrice with PBS- Tween (pH 7.2) as mentioned above and treated with Goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma chemicals) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 45 min at room temperature. After incubation mycelia was washed thrice in PBS and mounted in 10% glycerol. A cover slip was placed and sealed. The slides were observed and photograph under both phase contrast and UV fluorescence condition using Leica Leitz Biomed microscopy with fluorescence optics equipped in (UV) filter set 1-3.

3.17.4.5.2. Cross section of mandarin roots and leaves

Initially, cross section of healthy mandarin roots and leaves were cut and immersed in PBS (pH 7.2). These section were treated with normal serum or antiserum diluted (1:50) in PBS

and incubated for 1 hour at room temperature. After incubation, cross sections were washed thrice with PBS- Tween (pH 7.2) for 15 minute and transferred to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC).The sections were incubated for 45 minutes in dark. After that sections were washed thrice with PBS- Tween as mentioned above and then mounted on a grease free slide with 10%glycerol. Fluroescence of the root section were observed using Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and photograph was taken.

3.18. Isolation of genomic DNA

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. For bacteria, the growth was taken for 24 hr. Liquid nitrogen was used for crushing the cell mass for both cases.

3.18.1. Preparation of genomic DNA extraction buffer

The following buffers for DNA extraction were prepared by mixing appropriate amount of desired chemicals with distilled water and adjusted the desired pH.

Lysis Buffer
50 mM Tris, pH 8.0
100 mM EDTA
100mM NaCl
1% SDS

Genomic DNA Buffer
10 mM Tris, pH 8.0
0.1 mM EDTA

CTAB Buffer
2% CTAB
1.5% PVP K 30
1.4 mM Nacl
20 mM EDTA
100mM Tris HCL pH 8.0
0.1% B-mercaptoethanol

3.18.2. Genomic DNA extraction

Isolation of fungal genomic DNA was done by growing the fungi for 3-4 days. The mycelia were incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS, for 1 hr at 60^0C followed by centrifugation at 12,000 rpm for 15 min., whereas genomic DNA was extracted from isolates of bacteria and actinomycetes using CTAB buffer. The supernatant was then extracted with equal volume of water saturated phenol and further centrifuged at 12,000 rpm for 10 min; the aqueous phase was further extracted with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and centrifuged at 12,000 rpm for 15

min; the aqueous phase was then transferred in a fresh tube and the DNA was precipitated with chilled ethanol (100%). DNA was pelleted by centrifuging at 12000 rpm for 15 min, washed in 70% ethanol and air dried.

3.18.3. Purification of genomic DNA

The extraction of total genomic DNA from the isolated microorganisms as per the above procedure was followed by RNAase treatment. Genomic DNA was resuspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse (60µg). After incubation the sample was re-extracted with PCI (Phenol: Chloroform: Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

3.18.4. Measurement of DNA concentration using Spectrophotometry

The pure sample was (without significant amounts of contaminants such as a proteins, phenol, agarose, or other nucleic acids), used to quantify DNA. For quantitating DNA absorbance at wavelengths of 260 nm and 280 nm were taken. Quantification was done as follows:

1 O.D. at 260 nm for double-stranded DNA = 50 ng/ul of dsDNA

1 O.D. at 260 nm for single-stranded DNA = 20-33 ng/ul of ssDNA

Pure preparations of DNA have OD_{260}/OD_{280} value 1.8. If there is contamination with protein or phenol, this ratio will be significantly less than the value given above, and accurate quantitation of the amount of nucleic acid will not be possible.

3.18.5. Agarose gel eletrophoresis to check DNA quality

Gel electrophoresis is an important molecular biology tool. Gel electrophoresis enables us to study DNA. It can be used to determine the sequence of nitrogen bases, the size of an insertion or deletion, or the presence of a point mutation; it can also be used to distinguish between variable sized alleles at a single locus and to assess the quality and quantity of DNA present in a sample.

3.18.5.1. Preparation of DNA samples for electrophoresis

Agarose (0.8%) in 1X TBE buffer was melted, cooled and poured into the gel casting tray with ethidium bromide. Gels solidify in 15-20 min.

3.18.5.2. Run gel electrophoresis for DNA fraction

15µl of sample and 5µl of DNA loading dye mixed properly was loaded in each well of agarose gel (1%).The electrical head of the gel tank was attached firmly and electric supply was applied at constant current 90 mA and voltage 75 volt (BioRAD Power Pac 3000) at least for 90 min. The DNA migrated from cathode to anode. Run was continued until the bromophenol blue had migrated an appropriate distance through the gel. Then electric current was turned off and gel was removed from the tank and examined on UV transilluminator and photographed for analysis.

3.19. RAPD PCR analysis

For RAPD, random primers were selected (Table-1). PCR was programmed with an initial denaturing at 94°C for 4 min. followed by 35cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 70°C for 90 s and the final extension at 72°C for 7 min. in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25 % bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis.

3.19.1. RAPD primers

The following primers were used for RAPD analysis in the study:

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC
RAPD primers				
AA-04	CAGGCCCTTC	10	38.2	70%
OPA-4	AATCGGGCTG	10	39.3	60%
A-11	AGGGGTCTTG	10	31.8	76%
A-5	AGGGGTCTTG	10	36,8	73%
OPD6	GGGGTCTTGA	10	32.8	83%
OPA1	CAGGCCCTTC	10	38.2	70%

3.19.2. Amplification conditions

Temperature profile, 94°C for 4 min followed by 35cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 70°C for 90 s and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler.

3.19.3. Analysis of RAPD bands

RAPD band patterns were initially assessed by eye and isolates were grouped according to their shared band patterns.

3.19.4. Scoring of individual bands

Two methods of scoring bands were assessed. The first method involved scoring bands using the computer programme NTSYSPc and the second method was to score the number of shared bands (i.e. bands of equal size) on a gel by eye. For both methods, photographs of the gels were scanned into a computer and saved as graphics files.

3.19.5. Reconstruction of the phylogenetic tree

As with sequence data, RAPD data can be analysed in a number of different ways. The simplest form of analysis is to group isolates with identical band patterns for a given primer. More complex analyses involve cladistic analysis of data and reconstruction of the phylogenetic tree.

3.19.6. UPGMA method

The image of the gel electrophoresis was documented through Bio-Profil Bio-1D gel documentation system and analysis software. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed *In Silico* into similarity matrix using NTSYSpc (Numerical Taxonomy System Biostatistics, version 2.11W). The SIMQUAL program was used to calculate the Jaccard's coefficients. The RAPD patterns of each isolate was evaluated, assigning character state "1" to indicate the presence of band in the gel and "0" for its absence in the gel. Thus a data matrix was created which was used to calculate the Jaccard similarity coefficient for each pair wise comparison. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc.

3.20. ITS PCR analysis

All isolates of *Trichoderma* were taken up for ITS-PCR amplification. Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 30 cycles of denaturation at 94° C for 30 sec, annealing at 59°C for 30 sec and extension at 70°C for 2 min and the final extension at 72 °C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25%

bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

3.20.1. ITS- PCR primers

The following primers were used to amplify ITS regions:

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC	Amplicaon size (bp)	References
Macrophomina sp.						
ITS 1	TCCGTAGGTGAACCTGCG	18	61	56%	~620	White *et al.* (1990)
ITS4	TCCTCCGCTTATTTGATATGC	21	63	59%		
Fusarium sp.						
Fcg17F	TCGATATACCGTGCGATTTCC	21	65	47%	~570	Nicholson *et al.* (1998)
Fcg17R	TACAGACACCGTCAGGGGG	19	66	63%		
Trichoderma sp.						
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57%	~600	White *et al.*, (1990)
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45%		

3.20.2. Amplification conditions

Temperature profile, 94°C for 5 min. followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 59°C for 30 sec and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler.

3.20.3. Sequencing of rDNA gene

The rDNA was used for sequencing purpose. DNA sequencing was done bi-directionally using the ITS primer pairs by Genei Bangalore.

3.20.4. Sequence analysis

DNA sequence information was analyzed using bioinformatic algorithms tools e.g. Bioedit, MEGA 4, NTSYSpc as well as the few online softwares.

3.20.5. Chomatogram of sequence

The chromatogram of the DNA sequence was analysed by the software Chromus.

3.20.6. Editing and alignment of sequence data

All the DNA sequences edited by using the software BioEdit and aligned with Clustral W algorithms.

3.20.7. BLAST analysis of the sequences

The DNA sequences were analyzed using the alignment software of BLAST algorithm (http://ingene2.upm.edu.my/Blast, Altschul *et al.*, 1997) for the different characteristic of DNA sequence for the identification of microorganism Identification of microorganism was done on the basis of homology of sequence.

3.20.8. Submission of rDNA gene to NCBI genbank

The DNA sequences were deposited to NCBI GenBank through BankIt procedure and approved as the ITS sequence after complete annotation and given accession numbers.

3.21. Denaturing Gradient Gel Electrophoresis (DGGE)

3.21.1. Materials

(A) 40% Acrylamide:bisacryl-amide (37.5:1)

(B) 50 x DGGE/TAE buffer solution

Trizma-Base:	484.4 grams
Sodium-Acetate:	272.0 grams
trisodium EDTA	37.2 grams
H_2O	2 liters

pH 7.40 adjusted with about 230 ml of glacial acetic acid.

(C) Preparation of Denaturants

100% Denaturant

Urea	42.0 grams
38.5% Acrylamide (makes a 6.5% gel)	16.9 ml
50x DGGE/TAE	2.0 ml
Formamide	40.0 ml

Filled up to 100 ml with distilled H_2O.

0% Denaturant

38.5% Acrylamide	16.9 ml
50x DGGE/TAE	2.0 ml
Ammonium Persulphate	10% (w/v)
TEMED	20µl

3.21.2. Methods

3.21.2.1. Creating the gel sandwich (DCode System BioRad)

Large glass-plates were cleaned with soap and a soft sponge and rinsed with tap water. After drying, they were cleaned again with 96% ethanol. Both 1mm spacers were also cleaned with

96% ethanol and placed on the large glass plates. The clamps were screwed to the sides of the sandwich, in order to be sure that the spacers, 2 glass-plates and especially the glass plates were aligned at the bottom side of the sandwich and placed in the holder. The clamps were unscrewed and the alignment card slid between the glass plates to align the spacers. The clamps were screwed and the alignment of the glass-plates was checked. Then the sandwich was placed on top of the rubber gasket and the handles pressed down.

3.21.2.2. Preparing the gel

One tube of APS 10% per gel was prepared. The tubing needle was replaced with a new one, the screw between the compartments was opened and the compartments rinsed with water using pump at a uniform speed. The system was completely drained and flushed with compressed air. The gel solutions were prepared as required. Stacking gel was also prepared according to following table.

UF solution	[UF](%)	[Acrylamid/Bis] (%)	Volume UF solution (ml)	Volume APS 10% (µl)	Volume TEMED (µl)
Low	30-45	6	13	78	6
High	60	6	13	78	6

APS and TEMED was added to the low and high solutions according to table, stirred gently by hand and proceeded immediately for pouring the high concentration solution in the compartment closest to the outlet of the gradient mixer and the low concentration solution in the other compartment by the delivery system. The whole system was kept for polymerization.

3.21.2.3. Running a gel

Fresh 0.5x TAE buffer was added to the buffer tank to the mark "Fill". The DCode™ Universal Mutation Detection System (Bio-Rad) was switched on at least 60 min before electrophoresis, so that the buffer can heat up to 60°C. After 2-3 hours of polymerization, the comb was removed carefully and the bottom of the sandwich was rinsed with tap water to remove non-polymerized gel .The sandwich was set in the sandwich-holder. A dummy sandwich was also set at the other side to get a closed upper buffer compartment. (A dummy consists of a large and small glass plate stuck together with no spacers in between). The DCode™ was then switched off and the lid taken off after 1 minute. The sandwich holder was slided into the buffer tank, with the red dot of the cathode at the right side. The DCode™ pump and the stirrer underneath the tank were switched on (300 rpm) until samples were loaded.

3.21.2.4. Staining of gels and photography

Before taking DGGE units out from the tank, the run - evaporated H_2O was replaced up to the marked level. Carefully the DGGE unit was dismantled. The ethidium bromide stain was added into a tray with 50XTAE buffer and the gel was stained for 5 minutes and distained with running buffer. The gel was photographed under UV transilluminator.

3.22. Extraction and Assay of defense enzyme activity

3.22.1. ß-1, 3- glucanase (E.C. 3.2.3.39)

Extraction of ß-1,3- glucanase (E.C. 3.2.3.39) was done following the method described by Pan *et al.* (1991). Mandarin root and leaf samples (1g) were crushed in liquid nitrogen and extracted using 5ml of chilled 0.05 M sodium acetate buffer (pH 5,0) by grinding at 4 °C using mortar and pestle. The extract was then centrifuged at 10000 rpm for 15 min at 4 °C and the supernatant was used as crude enzyme extract.

Estimation of the ß-1,3-glucanase was done by following the Laminarin dinitrosalicylate method (Pan *et. al.*, 1991): The crude enzyme extract of 62.5µl was added to 62.5 µl of laminarin (4%) and then incubated at 40°C for 10minutes. The reaction was stopped by adding 375µl dinitrosalicylic reagent and heating for 5 min on boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexed and absorbance was recorded at 500nm. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as µg glucose released min^{-1} g^{-1} fresh tissues.

3.22.2. Chitinase (E.C. 3.2.1.14)

Extraction of chitinase (E.C. 3.2.1.14) was done by following the method described by Boller and Mauch (1988) with modifications. 1g root and leaf sample from mandarin plants were crushed in liquid nitrogen and extracted using 5ml of chilled 0.1M Sodium Citrate buffer (pH5). The homogenate was centrifuged for 10minutes at 12,000rpm and the supernatant was used as enzyme source.

Chitinase activity was measured according to the method described by (Boller and Mauch, 1988). The assay mixture consisted of 10µl Na-acetate buffer (1M) pH 4, 0.4ml of enzyme solution, 0.1ml of colloidal chitin (1mg). Colloidal chitin was prepared as per the method of (Roberts and Selitrennikoff, 1988). After 2h of incubation at 37 °C the reaction was stopped by centrifugation at 10,000g for 3minutes. An aliquot of supernatant (0.3ml) was pipetted into a glass reagent tube containing 30µl of potassium phosphate buffer (1M) pH7.1 and incubated with 20µl of (3%w/v) desalted snail gut enzyme Helicase (Sigma) for 1hour.

After 1h, the pH of the reaction mixture was brought to 8.9 by addition of 70µl of sodium borate buffer (1M) pH9.8. The mixture was incubated in a boiling water bath for 3minutes and then rapidly cooled in an ice water bath. After addition of 2ml of DMAB (ρ-dimethylaminobenzaldehyde) reagent. The mixture was incubated for 20 min at 37 °C.

Immediately therefore absorbance value at 585nm was measured using a UV-VIS spectrophotometer. N-acetyl glucosamine (GlcNAc) was used as standard. The enzyme activity was expressed as µg GLcNAc min^{-1} mg^{-1} fresh tissues.

3.22.3. Phenylalanine ammonia lyase (PAL) (E.C. 4.3.1.5)

Extraction of PAL (E.C. 4.3.1.5) was done by following the method described by Chakraborty *et al.* (1993) with modifications. 1gm root and leaf sample was crushed in 0.1M sodium borate buffer pH 8.8 (5ml/gm) with 2mM of ß mercaptoethanol in ice cold temperature. The slurry was Centrifuge in 15000 rpm for 20 minutes at 4°C. Supernatant was collected and after recording its volume, was immediately used for assay or stored at-20°C.

Phenylalanine ammonia lyase activity in the supernatant was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The reaction mixture contained 0.3 ml of 300µM sodium borate (pH 8.8), 0.3 ml of 30 µM L- phenylalanine and 0.5ml of supernatant in a total volume of 3ml. Following incubation for 1 h at 40 °C the absorbance at 290nm was read against a blank without the enzyme in the assay mixture. The enzyme activity was expressed as µg cinnamic acid produced in 1 min g^{-1} fresh weight of tissues.

3.22.4. Peroxidase (E.C. 1.11.1.7)

For the extraction of peroxidase (E.C.1.11.1.7) the plant tissues were macerated to powder in liquid nitrogen and extracted in 0.1 M Sodium borate buffer (pH 8.8) containing 2 mM ß mercaptoethanol under ice cold conditions, the homogenate was centrifuged immediately at 15000 rpm for 20 minutes at 4 °C. after centrifugation the supernatant was collected and after recording its volume was immediately used for assay or stored at -20 °C (Chakraborty *et al.*, 1993).

For determination of peroxidase activity, 100µl of freshly prepared crude enzyme extract was added to the reaction mixture containing 1 ml of 0.2 M sodium phosphate buffer (pH 5.4), 100µl of 4mM H_2O_2, 100 µl O-dianisidine (5mg ml-1 methanol) and 1.7ml of distilled water. Peroxidase activity was assayed spectrophotometrically at 460 nm by monitoring the oxidation of O- dianisidine in presence of H_2O_2 (Chakraborty *et al.*, 1993). Specific activity was expressed as the increase in absorbance at 460 nm g^{-1} tissue/ min^{-1}.

4.1. Survey of prevalent diseases of *Citrus reticulata* caused by pest and pathogens

The present study was undertaken to study the relation between prevalent diseases of mandarin orange (*Citrus reticulata*) and strategies for bio control of the same from area specific screened microorganisms. Though other diseases were also prevalent but due consideration was given to symptoms of fungal origin as related works on disease caused by fungal pathogens is comparatively negligible to the study on diseases of viral and bacterial origin. Darjeeling district is located in the northern-most part of West Bengal and is bordered by Sikkim and Bhutan in the North, districts of West Dinajpur and Purnia in the south, Nepal in the West and Bhutan, Jalpaiguri and Bangladesh in the east. Geologically, the southern portion is covered with sedimentary rocks and the northern part by metamorphic rocks. The soil is residual and derived by the withering of the underlying rocks. The entire hill region has a more or less forest-based ecosystem which include different types of citrus species. Mandarin orchards in Darjeeling hills started to show severe dieback causing huge economic loss to the farmers. In order to know the root cause of the disease, extensive survey of citrus orchards in Darjeeling District of North Bengal was conducted during fruiting (October-December) as well as non fruiting season (March- July). The nurseries and orchards screened were mostly private enterprises; hence random screening and sample collection was undertaken in various villages which include 7 in Mirik, 2 in Kurseong, 3 in Gorubathan and 5 in Kalimpong. Cultivation in these villages was almost completely abandoned due to dieback. In general, the soil was found to be poor in nutrition. There were several fungal pathogens contributing to the dieback (Plate 3, figs A-G). Repeated surveys were conducted and an inventory was outlined, keeping in view many factors ranging from disease symptomology to host pathogen interaction. Symptoms and development of some commonly occurring fungal diseases of *Citrus reticulata* have been described below in brief.

Root

Symptoms of Charcoal root rot caused by *Macrophomina phaseolina* were characterized by a gradual decay of the root tips, lateral roots and root crown. This gradual destruction of the root system causes the seedlings to become stunted and chlorotic, and finally to die. Phytopthora foot rot/Gummosis caused by *Phytophthora nicotiane* was noticed on the bark at or just above the bud union on susceptible scions. Lesions first occur as a drop of gum on the surface of the bark which appears to be brown, discolored, necrotic and slippery. In some cases, the margin of the infected area breaks away from the healthy area and may curl back. Lesions can eventually girdle the entire tree trunk leading to the death of the tree.

Plate 3 (figs. A-G): Mandarin tree in Mirik orchard [A] Survey of diseases prevalent in Mirik orchards [B]. Different diseases of mandarin plants [C - G].

Phytophthora spp. also infects the cortex of feeder roots. The root system gets a stringy appearance. This leads to yield loss and general prolonged tree decline. Cotton root rot caused by *Phymatotrichopsis omnivore,* appears on trees that are more than three years old. The fungus kills the tree so quickly that most of the dried leaves remain attached to the branches. Besides, root rot complex caused by *Fusarium solani* and *Fusarium oxysporum* were also observed in various nursery grown seedlings.

Leaf

White powdery growth of *Oidium tingitaninum* on different surfaces of the plant specially on the young actively growing twigs causing Powdery mildew were seen. Infected leaves became yellow, dried up and fell off resulting in dying back of the branches. *Mycosphaerella citri* primarily attacks leaves but can also infect fruits. Greasy spots appear first on the upper surface of the leaf as yellow colour. The corresponding surface on the undersides turn dark and appear slightly raised and greasy. The swollen tissue starts to collapse and turn brown and eventually leaves drop prematurely. The first symptom caused by *Diaporthe citri* on leaves were small, circular, dark depressions with a yellow margin. Later, the spots became raised and turned dark brown. Leaves turn yellow and may drop prematurely. Raised spots are also found on twigs and fruits. Spots on the fruits were at first small, light brown and dunken. Later they became dark and raised. Spots sometimes develop in a tear-streaked pattern resulting from infection caused by spores which wash down over the fruit surface during heavy dews of light rains.

Fruits

Alternaria alternata attacks fruit, leaves and young shoots causing *Alternaria* brown spot disease. The first symptoms appear as small, slightly depressed black spots which can cause the young fruit to fall from the tree. Fruit is usually immune to infection after reaching 3 to 4 months of age. On susceptible varieties only the young leaves and shoots can be infected. They produce brown, necrotic, blighted areas of various sizes usually surrounded by yellow halos on the plant tissue. Anthracnose or wither-tip is caused by *Glomerella cingulata.* Brown soft decay of fruit, or discolored streaks on the rind (called tear staining) are symptomatic of anthracnose. *Alternaria citri* causes Black rot. The infection starts while the fruit is still on the tree. The fruit is infected through cracks or openings on the stylar end of fruit. The fruit colours prematurely and drops (Plate 4,figs A-G). Infected tissue is often relatively firm on navels. This decay develops mostly during storage but can be identified in the field. The same

Plate 4 (figs. A-G): *Citrus reticulata* tree bearing fruits in the experimental plot of Immuno phytopathology laboratory [A-D], Fruit drop symptoms after 4 months of fruiting [E-G].

species of *Phytophthora* that causes foot rot, also infect fruits during periods of excessive rain resulting in decayed areas that are brown, firm and leathery. Later a white velvety growth is seen on the surface of the fruit accompanied by a strong fermenting odour. Fruits are also contaminated with another fungus (*Diplodia natalensis*) in the field but diseases commonly appear at the packing house or transit. Decay occurs around the stem end and advances in streaks down the side of the fruit. There is no fungal growth on the surface of the root. *Penicillium digitatum* causes a rapid breakdown of fruit punctured of bruised during harvesting and packing operations. The fungus enters the fruit only through wounds. The fruit becomes soft and shrinks in size. White mold that later turns green can be seen on the surface of the fruit. *Leptothyrium pomi* keeps citrus fruits from turning yellow in the infected spots. Small black specks are formed on the rind in areas immediately surrounding the oil glands. There is no effect on fruit or juice quality. Postbloom fruit drop caused by *Colletotrichum acutatum* appears as peach to brown colored necrotic spots on petals of flowers and produces fruit drop and the formation of persistent buttons which remain attached to stems. The pathogen survives on the surface of leaves, twigs and buttons between flowering periods.

Viral diseases include Cachexia, Citrus tatter leaf, Exocortis and Tristeza. The pathogen *Xyloporosis* causing Cachexia makes the inner bark surface bumpy. The bark projections are smooth in contrast to the sharp projections produced by the citrus tristeza virus (CTV). CTV is a virus that is limited to the phloem tissues of its host. It is transmitted by vectors that penetrate the phloem to extract sap, mostly the aphid species that colonize the crop. The brown citrus aphid is considerably more efficient at transmitting the virus than are other aphids that infest citrus. The adult tree turns yellow (Plate 5,fig.D) and wilts rapidly, and dies within a few years. Fruit typically remain smaller than normal. Leaf symptoms including yellow foliage (Plate 5,fig A&B) and sparse shoot growth are also often apparent. Tatter leaf-citrange stunt virus causes a bud union crease, while *Citrus exocortis* causes bark-shelling and stunting of trees. In the early stages of the disease, gum exudes from pustules at the base of the trunk at the base of the trunk and may extend from below the soil line to the bud union. New bark forms beneath the pustules and the outer bark sloughs off forming the characteristic bark-shelling. The tree eventually declines.

Different insects such as mealy bug, leaf miner, fruit fly, trunk borer and citrus dog were prevalent. In the nursery grown plants *Papilio demoleus* was most abundant during the rains when its larvae (caterpillars) cause damage to citrus leaves. Caterpillars body is cylindrical,

Plate 5 (figs. A-D): Yellowing of leaves indicating appearance of virus in nursery grown seedlings [A] and in the experimental field [D] in comparison with healthy plant [C].

distinctly segmented and in young larvae pigmented black and white. Older larvae are predominantly green above and whitish below. Young larvae resemble bird droppings (Plate 6, fig.C) while older larvae appear to have no shadow and blend with the citrus leaves. The newly emerged female butterfly is attracted to the nectar of flowers, to urine and faeces, rotten fruit and other food sources, while the mature mated female is attracted to citrus oils on the leaves on which she lays her eggs. The early larvae are black and white in colour and do not attempt to hide, while the mature larva with its different colouring withdraws to parts of the plant where its light under parts cancel its natural shadow and allow it to blend with the leaves. Bright light and high temperature cause the larva to leave its resting position and actively seek shade. As a defence against predators, the caterpillar expands the third thoracic segment which bears "eye-spots". At the same time, a forked, tongue-like *osmaterium*, normally withdrawn into the first thoracic segment, is extruded and vibrated. These changes give the larva a snake-like appearance and together with a pungent, aromatic secretion from the mouth, frighten off animals that might eat it. Adult butterflies can distinguish between certain colours only and are most sensitive to the red end of the spectrum (Plate 6,fig.D).

Planococcus citri, commonly known as mealy bug occurring on ventral surface of foliage (Plate 6, fig.A) cause nearly the same damage as aphids. Body oval; slightly rounded in lateral view; light orange to pink; body contents crushed are reddish brown; mealy wax covering body, not thick enough to hide pink body color; without longitudinal lines on dorsum; ovisac ventral only; with 17 lateral wax filaments, becoming progressively longer posteriorly, anterior pair about 1/4 width of body, straight, except posterior pair often curved apically, thin, posterior pair longest, varying from 3/4 to 1 time length of body. Surface of lateral filaments smooth. Like aphids, they suck plant sap and secrete honey dew, on which fungi can easily grow. This dirtiness gives ornamental plants a loss of quality. Viruses, however, are less transmitted by mealy bugs than by aphids. Apart from yellowing, defoliation, and cosmetic damage, mealy bugs also reduce the vigour of the plant.

Psocoptera indet, free-living insects were found under tree bark. They have long antennae, broad heads and 'bulging' eyes may grow up to 10mm in length, though they're usually less than 6mm, and the adults are often winged. The wings are held roof-like over their bodies look a little like aphids but their long antennae, broad heads and biting jaws show that they are not. Sometimes the colonies seem to move in coordinated fashion (Plate 6, fig. B).

Plate 6 (figs.A-D): Insects that feed on citrus plants. *Planococcus citri* on ventral surface of mandarin leaf [A] *Psocoptera indet* accumulated on the stem of mandarin plant [B] Young larvae [C] and adult stage of *Papilio demoleus*[D].

4.2 Root rot disease of *Citrus reticulata*

Mandarin seedlings were collected from eight different locations, viz. Rangali Rangliot, Bijanbari, Sukhia Pokhari, Kurseong, Mirik, Kalimpong Block I, Kalimpong Block II and Gorubahan and maintained in the Glass house conditions. Charcoal root rot pathogen (*Macrophomina phaseolina*) isolated from mandarin orchards of Darjeeling hill was used for present study after completion of Koch's postulate. Mycelia – septate, branched, hyaline when young becoming brown with age. Advancing zone of mycelia mat even and appressed. Sclerotia – black, moderate size (34-78 u in diameter), round or irregular (Plate 7 fig.D) uniformly reticulate with no difference in internal structure.

Healthy seedlings of mandarin (*Citrus reticulata*) plants (1-year-old) grown in earthenware pots were inoculated with this isolated organism and incubated for a period of 4 weeks. Pathogenicity of *M. phaseolina* was tested on twenty mandarin plants each of eight different locations. The inoculated plants were examined after 4 weeks. Colour of root, root rot index and percentage loss in dry weight of roots were noted. Young seedlings showed light brown discolouration of the root at the soil line initially which gradually turned dark brown to blackish brown and finally to black. In advanced stage of disease symptoms also appeared at ground level. Lower leaves turned yellow and remained attached, sometimes showed wilting symptoms. In advanced stage defoliation of lower leaves were evident (Plate 7 figs. E-G; Plate 8 figs. F-H). When epidermis was removed, small, black bodies (sclerotia) were discerned. These propagating bodies were abundant enough to impart a grayish black colour like charcoal to the tissues.

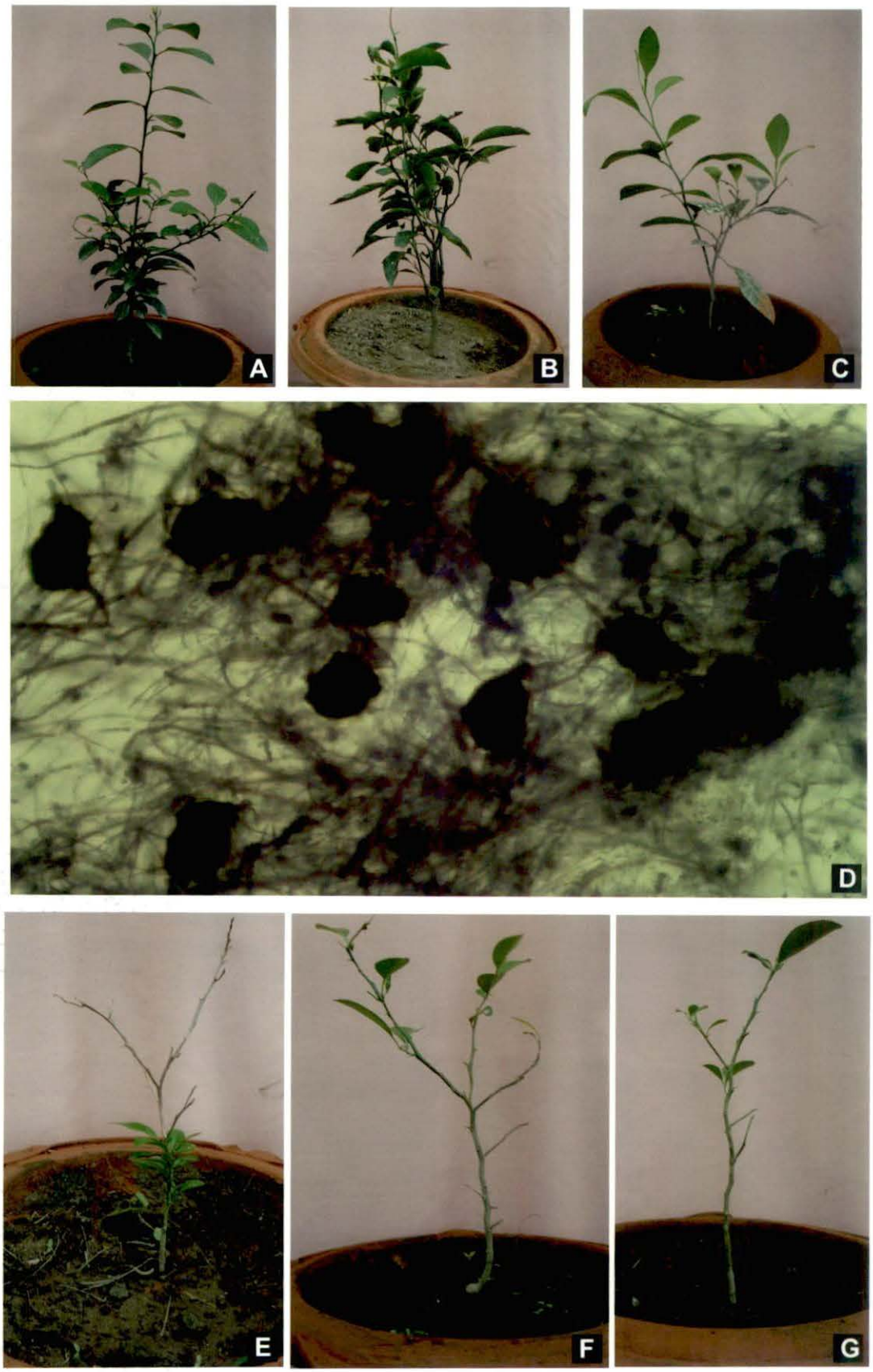


Plate 7 (figs. A-G): Healthy mandarin plants in pots [A-C], *Macrophomina phaseolina* (root rot pathogen) [D], Infected mandarin plants showing symptoms 30 days following artificial inoculation with *M. phaseolina* [E-G].

Table 1 : Pathogenicity test of *Macrophomina phaseolina* on different root samples of *Citrus reticulata*

Locality of *C. reticulata* saplings	* Root rot index	**Colour intensity
Rangli Rangliot	0.10	+
Bijanbari	0.25	++
Sukhia Pokhari	0.75	++++
Kurseong	0.25	++
Mirik	0.75	++++
Kalimpong Block I	0.75	++++
Kalimpong Block II	0.50	+++
Gorubathan	0.50	+++

* On the basis of root area affected; 0-10% (0.10); 11-25% (0.25); 26-50% (0.50); 51-75% (0.75); 76-100% (1.0).
** + Light brown, ++ Deep brown , +++ Blackish brown, ++++ Black

The root rot index as well as percentage loss in dry weight of roots were very low at the initial stage of infection which increased significantly with time in compatible interaction. Mandarin seedlings of three locations (Mirik, Kalmpong Block-I and Sukia Pokhri) were found to be highly susceptible (Table 1).

Culture filtrate of the pathogen (*M. phaseolina*) following two weeks growth in Richards' media at 28^0C, was collected and the young seedlings of mandarin (*Citrus reticulata*) of three different locations ((Mirik, Kalmpong Block-I and Sukia Pokhri) which showed susceptible reaction, were further tested in *in vitro* conditions in comparison with sterile distilled water control (Plate 8, figs A-D). It is interesting to note that seedlings showed same symptoms in this case also. Wilting followed by chlorosis and browning reaction of green leaves were evident in seedlings grown in culture filtrate of the pathogen. Wilting symptoms first appeared 7 days after treatment. However within two weeks leaves of all the seedlings turned into brown colour.

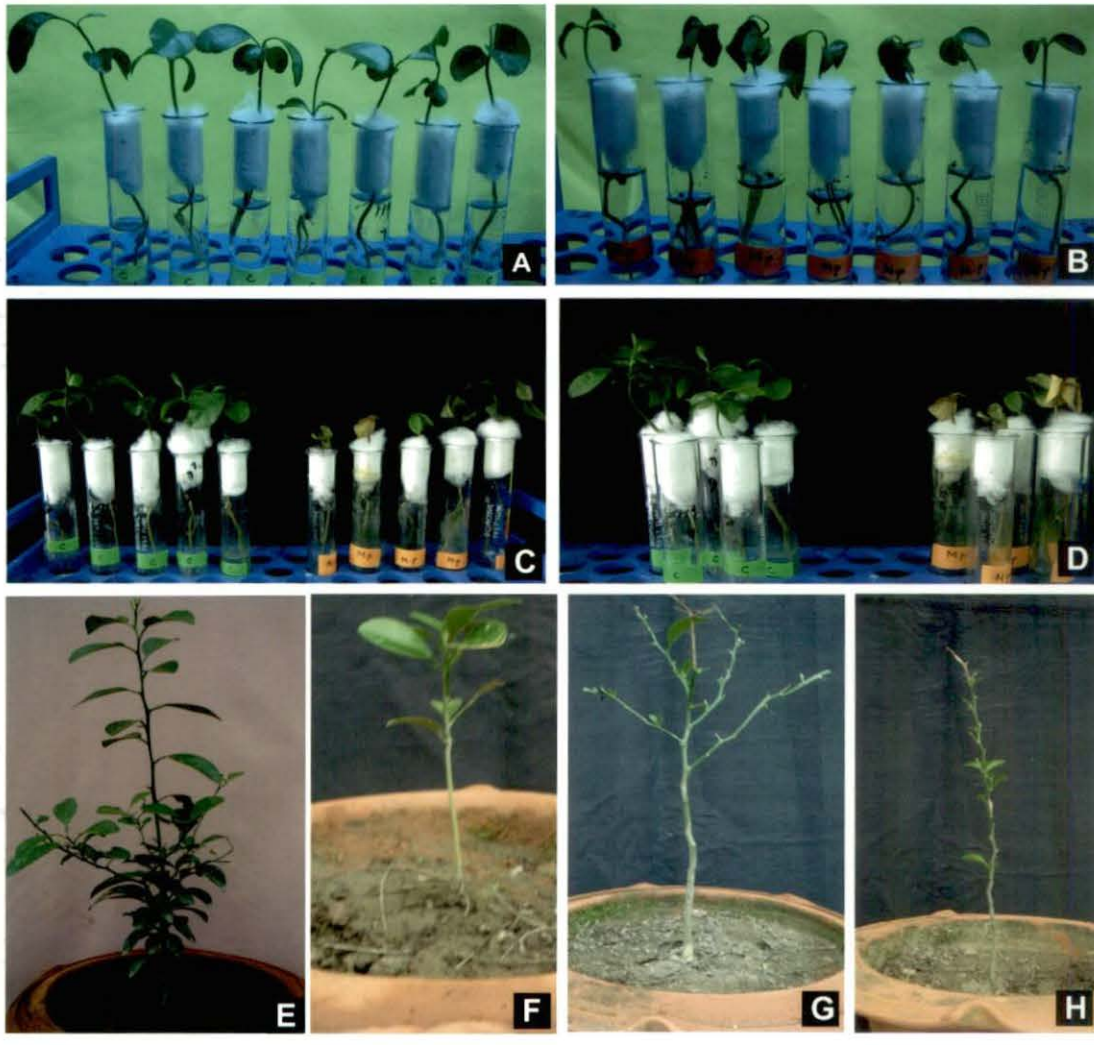


Plate 8 (figs. A-H): Mandarin seedlings grown in distilled water [A, C & D - left] and culture filtrate of *Macrophomina phaseolina* [B, C & D - right]. Healthy [E] and *M. phaseolina* infected plants [F-H].

4.3 Cultural conditions affecting growth of the pathogen (*M. phaseolina*)

Macrophomina phaseolina infect mandarin plant roots and their interactions affect the development of root rot disease. Initially it was considered worthwhile to study the effects of some major factors such as incubation time, temperature and pH of substrate on growth of the pathogen *in vitro*.

4.3.1. Effect of incubation time

The effect of incubation time on the growth of *M. phaseolina* was studied *in vitro*. *M. phaseolina* was grown in Richard's media for a period of 24 days at 28^{0}C. Mycelial growth of the fungus was recorded after 2,4,8,12,16,20, and 24 days. The results are embodied in Table 2. Maximum growth of *M. phaseolina* (755 mg) was observed after 12 days of incubation and then the rate of growth declined. Mycelial growth increased by 24% from 8 to 12 days of incubation and decreased by 5% from 12 to 16 days.

Table 2 . Effect of incubation time on growth of *M. phaseolina*

Incubation Time (days)	Average dry weight of mycelia (mg)
2	97.20 ± 2.24
4	214.35 ± 4.44
8	618.00 ± 3.72
12	755.00 ± 1.81
16	717.52 ± 1.58
20	689.32± 2.86
24	653.62± 2.43

Average of 3 replicates/treatment; Temperature 28^{0}C; pH of medium – 5.4

4.3.2. Effect of pH on growth

It is well known that the pH of the medium usually plays an important role in the growth of microorganisms. The utilization of nutrients depends partially upon the pH of the culture medium. Therefore, it was considered imperative to use a buffer system to stabilize the pH of the culture medium during incubation. In the present study, buffer solutions with pH values ranging from 4-8 (4.0, 5.0, 6.0, 7.0 and 8.0) were prepared by mixing KH_2PO_4 and K_2HPO_4 each at a concentration of M/30. The pH of the medium was adjusted using N/10 NaOH or N/10 HCl to obtain the corresponding range of pH values (4.0-8.0). Both the medium and the phosphate buffer were sterilized. Equal parts of the buffer solution and medium were mixed before use. Each flask containing 50ml of the medium was inoculated with fungus and

incubated for 12 days at 28^{0}C. The results are given in Table 3. It appears that *M. phaseolina* grew well over a range of pH (4.0-8.0) and optimum growth was recorded at pH 5.5. It is necessary to mention that mycelia growth increased up to pH 5.5 and then gradually declined.

Table 3. Effect of different pH on the growth of *M. phaseolina*

pH	Average dry weight of mycelia (mg)
4.0	310.50 ± 2.33
4.5	581.25 ± 3.40
5.0	616.00 ± 3.12
5.5	695.00 ± 2.61
6.0	437.55 ± 2.53
6.5	409.32± 3.86
7.0	325.12± 2.63
8.0	310.33± 1.95

Average of 3 replicates/treatment; Temperature 28^{0}C; Incubation time – 12 days

4.3.3. Effect of temperature on growth

Temperature is also a major factor affecting growth of a pathogen. Therefore; the effects of different temperatures (15, 20, 25, 28, 30, 35, 40^{0}C) on growth of *M. phaseolina* was studied *in vitro*. Maximum mycelial growth was noted at 30^{0}C with a decline at 40^{0}C (Table 4).

Table 4. Effect of different temperature on the growth of *M. phaseolina*

Temperature (^{0}C)	Average dry weight of mycelia (mg)
15	95.00 ± 2.43
20	181. 05 ± 2.40
25	246.00 ± 3.22
28	595.00 ± 2.82
30	737.55 ± 3.53
35	665.42± 2.85
40	125.25± 3.63

Average of 3 replicates/treatment; pH adjusted to 5.5 ; Incubation time – 12 days

4.4 Isolation of microorganisms from mandarin rhizosphere and their identification

4.4.1. Analyses of soil samples of mandarin orchards

The soil samples were collected from eight different locations- Rangli Rangliot, Bijanbari, Sukhia Pokhari, Kurseong, Mirik, Kalimpong Block I , Kalimpong Block II and Gorubathan. These samples were given for analysis in Soil Testing laboratory, Institute of Plantation Science and Management, North Bengal University before the isolation of microorganisms. Moisture content, pH, soil type, soil texture, carbon and nitrogen ratio, available K and P etc were determined for all eight soil samples. Results have been presented in Table 5.

Table 5: Analyses of soil samples collected from mandarin orchards

Sample area	Moisture %	pH	Organic carbon	nitrogen	Soil type	Soil texture			Available	
						Silt %	Clay %	Sand %	K_2O (ppm)	P_2O_5 (ppm)
Rangli Rangliot	16.67	4.38	1.05	0.11	Clay	10	51	39	120.96	38.09
Bijanbari	18.35	4.11	1.15	0.12	Sandy Clay	5	45	50	154.57	40.26
Sukhia Pokhari	15.85	3.90	1.10	0.11	Sandy Clay	2	43	55	88.70	31.56
Kurseong	26.35	4.43	1.27	0,13	Sandy Clay	4	40	50	123.65	26.12
Mirik	18.62	4.69	1.35	0.14	Sandy Clay	10	34	56	177.41	50.06
Kalimpong Block I	20.28	4.76	1.09	0.11	Sandy Clay	13	42	52	168.00	54.41
Kalimpong Block II	22.75	4.58	1.08	0.12	Sandy Clay	15	45	54	165.55	53.35
Gorubathan	21.22	3.86	1.32	0.11	Sandy Clay	12	40	55	146.22	50.75

4.4.2. Fungal isolates

The mycelial growth and sporulation behavior of fungi isolated from rhizosphere of *Citrus reticulata* were examined separately on PDA medium. Nature and rate of mycelial growth as well as sporulation time were recorded. Radial growth patterns of different fungal isolates have been presented in Plate 9 Morphological characters and microscopic observations under bright field of the isolated fungi have been presented in Table 6 . On the basis of hyphal character, nature of conidiophore and conidia these were identified. It was found that most of the fungal isolates belonged to the genera *Aspergillus, Fusarium, Penicillium, Sporotrichum, Rhizopus, Macrophomina, Emenicella* and *Trichoderma.*

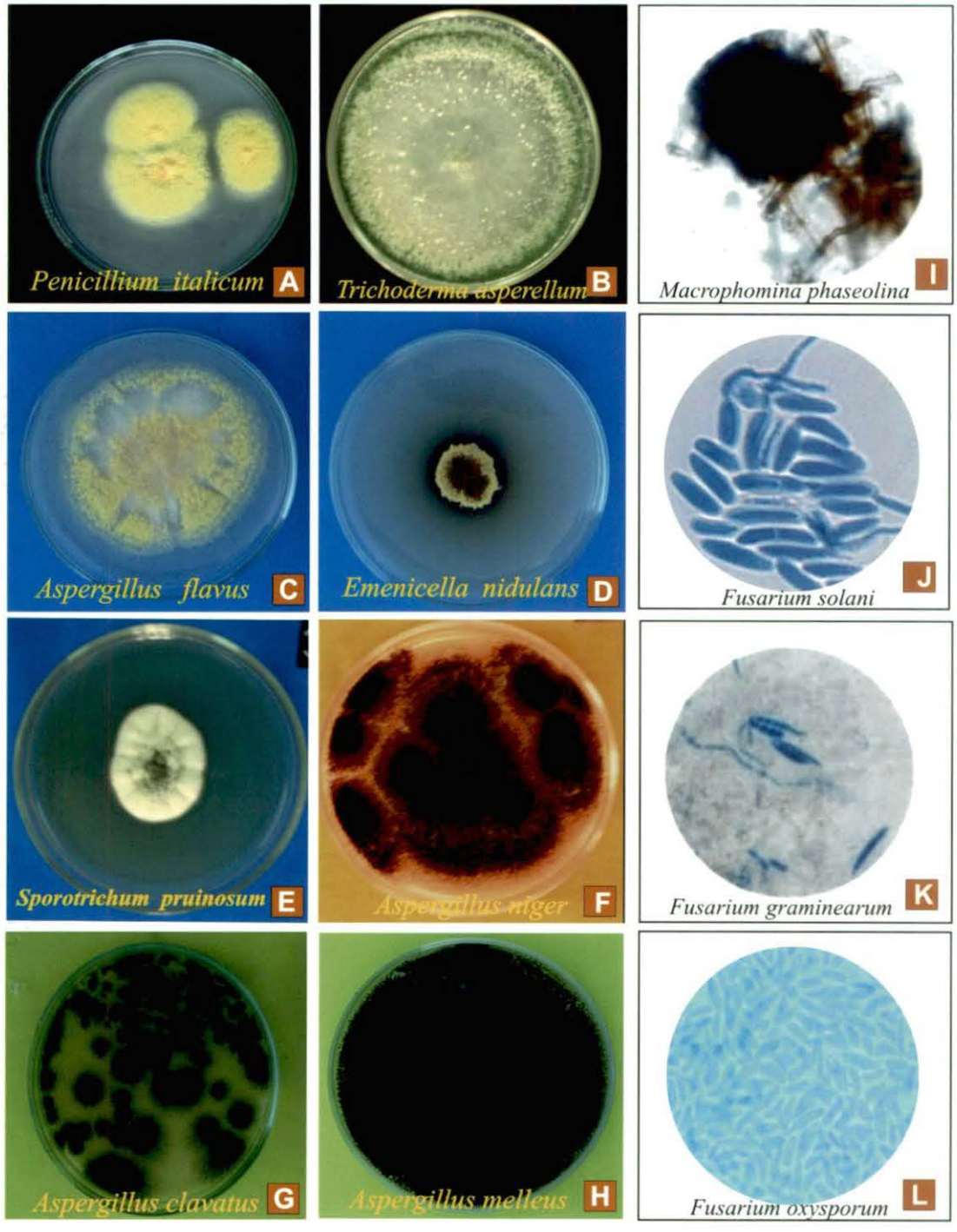


Plate 9 (figs. A-L): Radial growth [A-H] and microscopic observation [I - L] of fungi isolated from rhizosphere of mandarin plants.

Table 6 : Morphology and Microscopical Characters of fungi isolated from mandarin rhizosphere

Organisms identified	Morphology and Microscopical Character
A. niger RHS/M492	**Colonies:** Black on PDA mediam **Mycelia:** Hyaline, aseptate **Conidia:** Conidial heads radiate. Conidia brown, ornamented with warts and ridges, subspherical, 3.5-5.0 µm diam. **Conidiophore:** Consisting of a dense felt of conidiophores. Conidiophore stipes smooth-walled, hyaline. Vesicles subspherical, 50-100 µm diam
Aspergillus flavus (RHS/M495)	**Colonies:** Colonies on Czapek and PDA usually spreading, yellow green, reverse colourless to dark red brown, occasionally dominated by hard sclerotia, white at first, becoming red brown to almost black with age, 400-700 µm diam **Mycelia:** hyaline, aseptate **Conidia:** Conidial heads typically radiate, splitting into several poorly defined columns, rarely exceeding 500-600 µm diam., mostly 300-400 µm, smaller heads occasionally columnar up to 300-400 µm. Conidia typically globose to subglobose, conspicuously echinulate, variable, (3-) 3,5-4,5 (-6) µm diam., sometimes elliptical or pyriform at first and occasionally remaining so, and then 4.5-5.5 x 3.5- 4.5 µm. **Conidiophore:** Conidiophores thick-walled, hyaline, coarsely roughened, usually less than 1 µm long, 10-20 µm diam., just below the vesicle; vesicles elongated when young, becoming subglobose to globose, 25-45 µm diam.; both metulae and phialides present; metulae usually 6-10 x 5,5 µm but sometimes up to 15-16 x 8-9 µm; phialides 6,5-10 x 3-5 µm.
A. mellus RHS/M493	**Colonies:** Black on PDA mediam **Mycelia:** Hyaline, aseptate **Conidia:** Conidia globose to subglobose , smooth walled or irregularly roughened, 2.8-3.5 µm diam. **Conidiophore:** Conidiophore usually 0.5-2 µm tall, thickwalled, roughened.
A. clavatus RHS/M494	**Colonies:** Colonies on Czapek and malt agar usually spreading, occasionally floccose, blue-green, mycelium white, inconspicuous. **Mycelia:** Hyaline, aseptate **Conidia:** Conidial heads clavate, usually splitting into several divergent columns. Conidia smooth-walled, ellipsoidal, 3-4,5 x 2,5-3,5 µm diam. **Conidiophore:** Conidiophores very long, 500-900 µm long, smooth-walled, hyaline to slightly brown near vesicle. Vesicle clavate, 15-75 µm diam. Phialide 7-10 x 2-3,5 µm; metulae absent.
A. fumigatus RHS/M498	**Colonies:** Colonies (CzA) dark blue-green, consisting of a dense felt of conidiophores, intermingled with aerial hyphae. **Mycelia:** hyaline, aseptate **Conidia:** Conidia verrucose, (sub)spherical, 2.5-3.0 µm diam Conidial heads columnar; conidiogenous cells uniseriate. **Conidiophore:** Conidiophore stipes smooth-walled, often green in the upper part. Vesicles subclavate, 20-30 µm wide.

Organisms identified	Morphology and Microscopical Character
Aspergillus oryzae RHS/M499	**Colonies:**. Colonies on potato dextrose agar at 25°C are white to yellow to drab gray to brown, but never green **Mycelia:** Hyphae septate and hyaline **Conidia:**. Conidial heads are radiate to loosely columnar and biseriate Conidia are globose, 3-4.5 µm, with very rough walls **Conidiophore:** Conidiophores 30-350 µm, smooth-walled, and brown. Vesicles are globose to subglobose, 7-16 µm in diameter. Metulae and phialides cover the upper portion of the vesicle.
Fusarium graminearum RHS/S566	**Colonies:**. Raddish white **Mycelia:** Hyaline, aseptate **Conidia:** Conidia often formed sparsely, falcate, sickle-shaped or markedly dorsi-ventral, 3-7-septate, 25-50 x 3-4 µm, with a well developed, often pedicellate foot cell. Microconidia absent. Macroconidia produced from doliiform phialides 10-14 x 3,5-4,5 µm, formed laterally or on short multibranched conidiophores; sporodochia may form in older cultures **Conidiophore:** Chlamydospores, when present, are intercalary, single, in chains or clumps, globose, thick-walled, hyaline to pale brown with a smooth or slightly roughened outer wall, 10-12 diam. Many strains fail to develop chlamydospores on standard media
Fusarium solani RHS/M532	**Colonies:**. Colonies growing rapidly, with white to cream-coloured aerial mycelium, usually green to bluish-brown **Mycelia:** hyaline, aseptate **Conidia:** Microconidia usually abundant, produced on elongate, sometimes verticillate conidiophores, 8-16 × 2.0-4.5 µm. Chlamydospores frequent, singly or in pairs, terminal or intercalary, smooth- or rough-walled, 6-10 µm diam **Conidiophore:** Conidiophores arising laterally from aerial hyphae. Monophialides mostly with a rather distinct collarette. Macroconidia produced on shorter, branched conidiophores which soon form sporodochia, usually moderately curved, with short, blunt apical and indistinctly pedicellate basal cells, mostly 3-septate, 28-42 × 4-6 µm, occasionally 5-septate
Fusarium oxysporum RHS/M535	**Colonies:**. Colonies growing rapidly, with white mycelium **Mycelia:** hyaline, aseptate **Conidia:** Microconidia usually abundant, produced on elongate. **Conidiophore:** conidiophores, 8-16 × 2.0-4.5 µm. **Chlamydospores:** frequent, singly or in pairs, terminal or intercalary, smooth- or rough-walled, 6-10 µm diam
Sporotrichum pruinosum RHS/M496	**Colonies:**. Distinct greyish or pinkish hue; **Mycelia:** hyaline, aseptate **Conidia:** Blastoconidia from unbranched conidiophores ellipsoidal to ovoid pyriform or nearly cylindrical, 5.8 x 3.5 µm. Chlamydospores terminal or intercalary, hyaline, (sub)globose to broadly ellipsoidal or more rarely pyriform, 11- 60 µm diam or 11 x 7.5 µm, with granular contents and thick walls (up to 4.5 µm). **Conidiophore:** Conidiophores simple or typically branched. Branching racemose, each branch forming a terminal blastoconidium.

Organisms identified	Morphology and Microscopical Character
Macrophomina phaseolina RHS/S565	**Colonies:**. Pycnidia dark brown, solitary or gregarious on leaves and stems, immersed, becoming erumpent, 100-200 µm diam **Mycelia:** hyaline, aseptate **Conidia:**. Conidia hyaline, ellipsoid to obovoid, 14-30 x 5-10 µm **Conidiophore:** Conidiophores (phialides) hyaline, short obpyriform to cylindrical, 5-13 x 4-6 µm.
Trichoderma asperellum RHS/M517	**Colonies:**. Dark green **Conidia:**. subglobose to ovoidal, 3.5 to 4.0 µm long, smooth, green **Conidiophores**: Typically with paired branches forming over 150 µm of the length of terminal branches. Cells supporting the phialides equivalent in width to, or at most only slightly wider than, the base of phialides arising from them. **Phialides:** 6.5-6.7 µm long, 2.5-3.5µm wide at the widest point 1.6-2.5 µm at the base; supporting cell 2.4-3.6 µm; Terminal phialides in a whorl or solitary, typically cylindrical or at least not conspicuously swollen in the middle and longer than the subterminal phialides.
Penicillium italicum RHS/M510	**Colonies:**. Velutinous to fasciculate, crustose **Mycelia:** hyaline, aseptate **Conidia:**. Smooth-walled, ellipsoidal to cylindrical, 3.5-5 x 2.2-3.5 µm **Conidiophore:** Terverticillate, appressed elements, born from subsurface hyphae
Emenicella nidulans RHS/M509	**Colonies:**. Colonies (PDA) growing rapidly, green, cream-buff or honey-yellow; reverse dark purplish **Mycelia:** Hyaline, aseptate **Conidia:**. Conidial heads short, columnar, up to 80 µm long **Conidiophore:** Conidiophore stipes brownish, 60-130 x 2.5-3.0 µm. Vesicles hemispherical, 8-10 µm diam. Conidiogenous cells biseriate, 5.9 x 2-3 µm. Metulae 5.6 x 2.3 µm. Conidia spherical, rugulose, subhyaline, green in mass, 3-4 µm diam
Rhizopus oryzae (RHS/M 497)	**Colonies:**. Colonies (MEA, 30°C) expanding, up to 1 cm high, whitish to greyish-brown. **Mycelia:** Hyaline, aseptate **Sporangiophore:** Singly or in tufts, brown, 1-2 mm high, 18 µm wide, mostly unbranched, sometimes with brownish swellings up to 50 µm diam. Rhizoids sparingly branched, up to 250 µm long, brownish. Sporangia spherical, 50-250 µm diam, brownish-grey to black; columella comprising 50-70% of sporangium, spherical; apophysis short, 3-12 µm high. Sporangiospores greyish-green, angular, subspherical to ellipsoidal, longitudinally striate, 6-8 x 4.5-5.0 µm **Chlamydospore:** Single or in chains, spherical to ovoidal, 10-35 µm diam, hyaline, smooth-walled. Zygospores. Zygospores red to brown, spherical or laterally flattened, 60-140 µm, with flat projections. Suspensors unequal, spherical and conical. Heterothallic

4.4.3. Bacterial isolates

A list of bacteria isolated from the rhizosphere soil of mandarin plants along with their codes have been presented in Table 7.

Isolated bacteria were studied under microscope after suitable staining and characterized based on morphological and biochemical studies following Bergey's manual of Systematic Bacteriology. Bacterial identification was performed on the basis of morphological, physiological and biochemical tests. Isolates were characterized for H_2S production, phosphate solubilization, starch hydrolysis, casein hydrolysis, chitin degrading, siderophore production, catalase production, protease production, urase production, cellulase production and indole production. Results (Table 7) revealed that out of 13 bacterial isolates, 10 bacteria showed gram positive reaction and rest were gram negative, where as 5 bacterial isolates showed phosphate solubilizing activity and 8 isolates showed cellulase activity. All isolates showed positive result in catalase activities. Overall, *Bacillus sp.*, *Bacillus cereus*, *Bacillus pumilus* and *Pseudomonas sp.*, were found to be more abundant.

Table 7 : Morphology and biochemical tests of isolated bacteria

Code	Shape	Pigment	Spore	Gram reaction	H_2S production	Phosphate solubilization	Starch hydrolysis	Casein hydrolysis	Chitin degrading	Siderophore production	Catalase production	Protease production	Urase production	Cellulase Production	Indolae Production	Identification
B/RHS/C1	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus pumilus*
B/RHS/M2	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus cereus*
B/RHS/M3	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus cereus*
B/RHS/M4	Rod	W	+	+	-	-	+	+	+	+	+	+	_	-	-	*Bacillus sp.*
B/RHS/M5	Rod	W	+	+	-	-	+	+	+	+	+	+	_	-	-	*Bacillus sp.*
B/RHS/M6	Rod	W	+	-	-	-	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/RHS/M7	Rod	W	+	-	-	-	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/RHS/M8	Rod	W	+	-	-	-	+	+	+	+	+	+	_	+	-	*Pseudomonas sp.*
B/RHS/M9	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus sp.*
B/RHS/M10	Rod	W	+	+	-	+	+	+	+	+	+	+	_	+	-	*Bacillus cereus*
B/RHS/M11	Rod	W	+	+	-	-	+	+	+	+	+	+	_	-	-	*Bacillus sp.*
B/RHS/M12	Rod	W	+	+	-	-	+	+	+	+	+	+	_	-	-	*Bacillus sp.*
B/RHS/M13	Rod	W	+	+	-	-	+	+	+	+	+	+	_	-	-	*Bacillus sp.*

4.5. Occurrence of Arbuscular Mycorrhizal fungi in rhizosphere of mandarin plants

Population of different species of AM fungi isolated from the rhizosphere of mandarin from different regions were determined. Sites selected were hilly regions of Kalimpong, Mirik, Bijanbari and Kurseong of Darjeeling hill. Among the AM fungi, *Glomus mosseae* could be determined as the most predominant, followed by other genera such as *Gigaspora, Acaulospora* and *Scutellospora.* Microscopic observations of selected AMF sores and root colonization in *Citrus reticulata* have been presented in Plate 10 (figs.A-O). Some of the characteristic features were considered for identification of all those isolates which were found as consistent association and maximum colonization with mandarin roots.

Acaulospora bireticulata . Spores single in the soil; develop laterally; sessile; light orange to yellowish brown; globose to subglobose; approx. 190µm diam; sometimes irregular; 130-180 x 170-250 µm. Subcellular structure of spores consists of a spore wall and two inner germination walls.Spore wall contains three layers. Layer 1, forming the spore surface, 1.1µm thick, closely attached to wall 2, continuous with the wall of a sporiferous saccule. Layer 2 laminate, ornamented, light orange yellowish brown, ornamentation consists of hyaline to yellowish white round-tipped polygonal structures.

Acaulospora spinosa. Color: cream to pale orange-brown. Shape: Globose or subglobose, size distribution: 140-220 µm, spore wall consists of two layers. Layer 1: Hyaline and1.2-1.6 µm thick. Layer 2: thickens by formation of pale yellow sub layers followed by synthesis of closely packed rounded spines. Layer 3: A single hyaline layer, 0.6-1.2 µm thick either is adherent to L2 or more often slightly separable (where it resembles a flexible inner wall).

Glomus fasciculatum. Colour: pale yellow to bright brown with globose to subglobose in shape. Spores produced directly with one or more subtending hyphae attached to it. Spore wall is continuous. Spore wall consisting of three layers (L1, L2, and L3). Spore size ranges from 70-120µm in diameter.

Glomus aggregatum . Spores globose to oval in shape. Size ranges from 40-120µm in diameter, color- pale yellow. Formed singly or in sporocarps. Spore wall consist of 1-2 layers. Sporocarps formed in loose clusters, from a single stalk, diameter ranges from 200-1800 x 200-1400 µm in size.

Glomus mosseae. Brown to orange-brown in colour, shape, globose to sub-globose with an average diameter of 200µm. Presence of three hyaline layers with subtending hyphae attached. Hyphae are double layered.

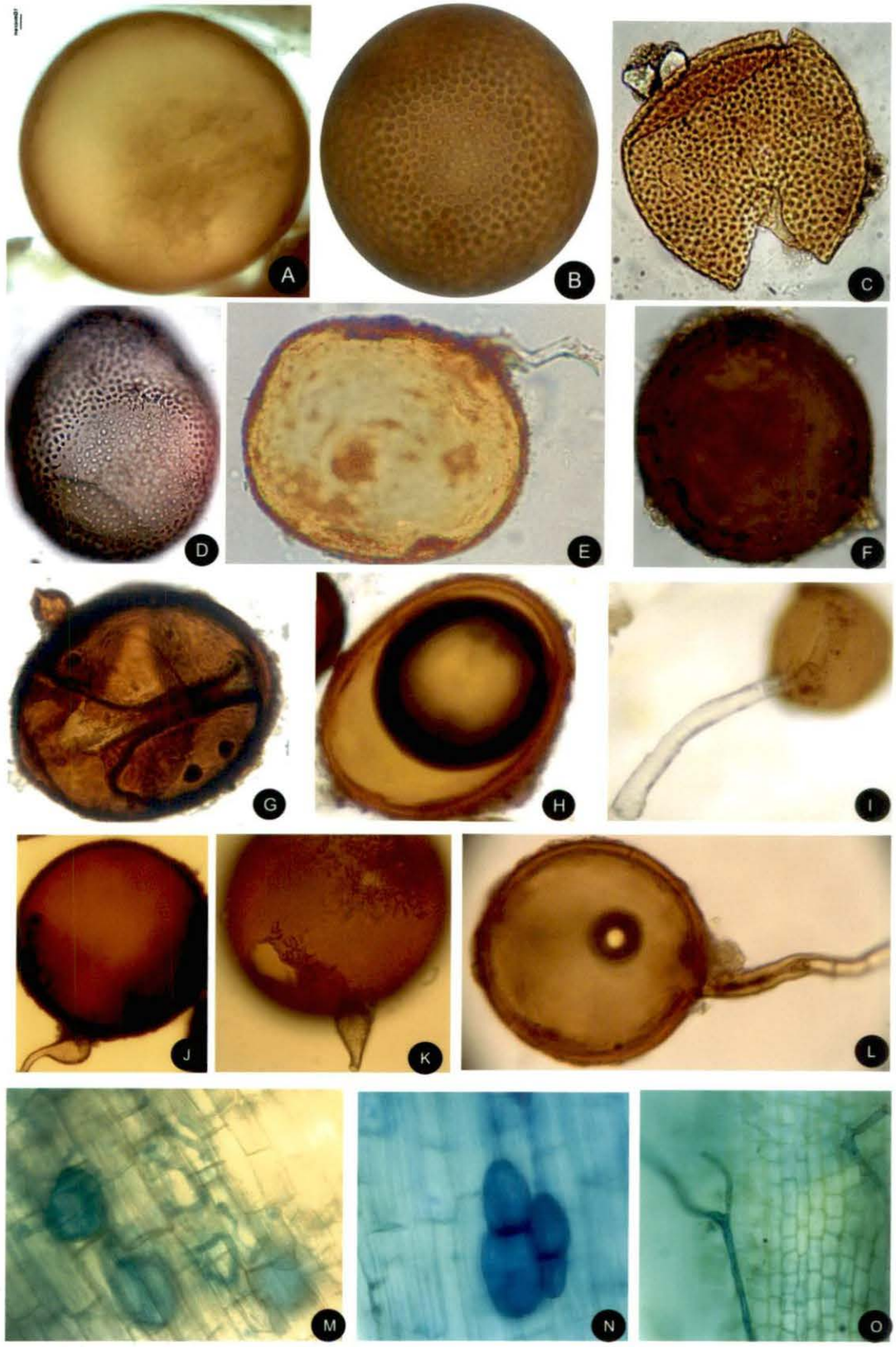


Plate 10 (figs. A-N): AMF spores and root colonization in *Citrus reticulata*. *Glomus sp.* [A], *Acaulospora bireticulata* [B & C], *Acaulospora spinosa*[D], *Glomus mosseae* [E], *Glomus constrictu*m [F], *Gigaspora gigantea*[G], *Scutellospora rubra* [H], *Glomus badium* [I], *Gigaspora margarita* [J & K] ,*Glomus fasciculatum* [L] and colonization of *Citrus reticulata* roots with AM fungi [M, N and O].

Glomus drummondii. Spores occur singly in the soil; develops from the tip of extraradical hyphae of mycorrhizal roots. Spores are golden yellow, globose to subglobose, average diameter 70µm, single subtending hypha attached with the spore. Spore wall consists of three distinct layers.

Glomus constrictum. Spores single in the soil with one subtending hypha, colour brownish orange to dark brown globose to subglobose; 160µm diam in average. Spores consists of one wall containing two layers, most juvenile spores with spore wall layer 1 only. Subtending hypha brownish orange to dark brown; straight or curved; usually markedly constricted at the spore base, sometimes cylindrical, flared to funnel-shaped; composed of two layers continuous with spore wall layers 1 and 2.

Glomus clarum . Spores single in the soil; hyaline to pale yellow, globose to subglobose; 150 µm diam; sometimes ovoid; 90-100 x 140-180 µm; with one subtending hypha. hyaline to pale yellow straight to curved; wall of subtending hypha hyaline to pale yellow ,thick at the spore base; composed of three layers

Glomus aggregatum . Spores formed singly in the soil, in aggregates, in roots, aggregates ranges from 160-1600 x 250-1900 µm, without a peridium, with two to over one hundred spores loosely distributed. Colour of spores are pastel yellow to yellowish brown; mostly globose to subglobose; rarely pyriform to irregular; usually with a single subtending hypha, rarely with two.

Glomus badium. Spores occur in dense sporocarps in the soil and on the surface of vesicular-arbuscular mycorrhizal roots. Sporocarps brownish orange to reddish brown; mainly ovoid to irregular; sometimes globose to subglobose; 250-320 µm diam; with 4-43 spores, radially originating from a hyphal plexus and separated by an interspore mycelium and occasionally by cystidium-like structures.

Gigaspora gigantean. Spores single in the soil; formed terminally or laterally on a bulbous sporogenous cell; greenish yellow (globose to subglobose; 300µm diam; sometimes ovoid; 250x 270 µm. Subcellular structure of spores consists of a spore wall with two layers and one germinal wall.

Gigaspora margarita. Spores produced singly in the soil, blastically at the tip of a bulbous sporogenous cell. Spores yellowish white to sunflower yellow; globose to subglobose; 357 µm diam; sometimes ovoid; 320 X 370 µm. Sporogenous cell orange to brownish yellow. Structure of sporogenous cell composed of two layers. Layer 1 hyaline, 1.7 µm thick approximately. Continuous with spore wall layer 1. Layer 2 orange to brownish yellow, 5.6 µm thick, continuous with spore wall layer 2.

Plate 11 (figs. A-C): Scanning Electron Micrograph of AMF spores obtained from mandarin rhizosphere. *Glomus fasciculatum* [A] Gigaspora gigantea [B] *Acaulospora bireticulata* [C].

Scutellospora pellucid. Spores single in the soil; formed terminally on a bulbous subtending hypha; hyaline to yolk yellow; globose to subglobose; 195µm diam; sometimes ovoid; 130-155 x 160-235 µm.

Scutellospora rubra. Spores color: dark orange-brown to red-brown at maturity, immature spores are white to cream with a rose tint under a dissecting microscope. Shape: globose to subglobose. Size 180 µm in average.

Scanning electron microscopic observation was made of three genera (*Glomus, Gigaspora* and *Acaulospora*) and presented in Plate 11(figs. A-C). Surface of *Glomus fasciculatum* showed adhered hyphae and few pores in the outer surface. The outer hyaline surface was sloughed and eroded (Plate 11,fig.A). SEM image of *Gigaspora gigantea* showing the outer hyaline layer and the conspicuous curved hyphal attachment is evident in Plate 11 fig.B. *Acaulospora bireticulata* with ornamentation consists of hyaline to round-tipped polygonal structures and the attached sporiferous sacule (Plate 11, fig.C).

Percentage of AM population in mandarin rhizosphere obtained from four different locations have been presented in Figure 1. Survey of Mycorrhizal fungi from the rhizosphere soil of mandarin plants grown at different places in Darjeeling hills (Mirik, Kalimpong, Biajanbari and Gorubathan) indicated the genus *Glomus* comprising of six different species, *Gigaspora* and *Acaulospor*a comprising four different species each and *Scutellospora* comprising two different species were found to be predominant AMF.

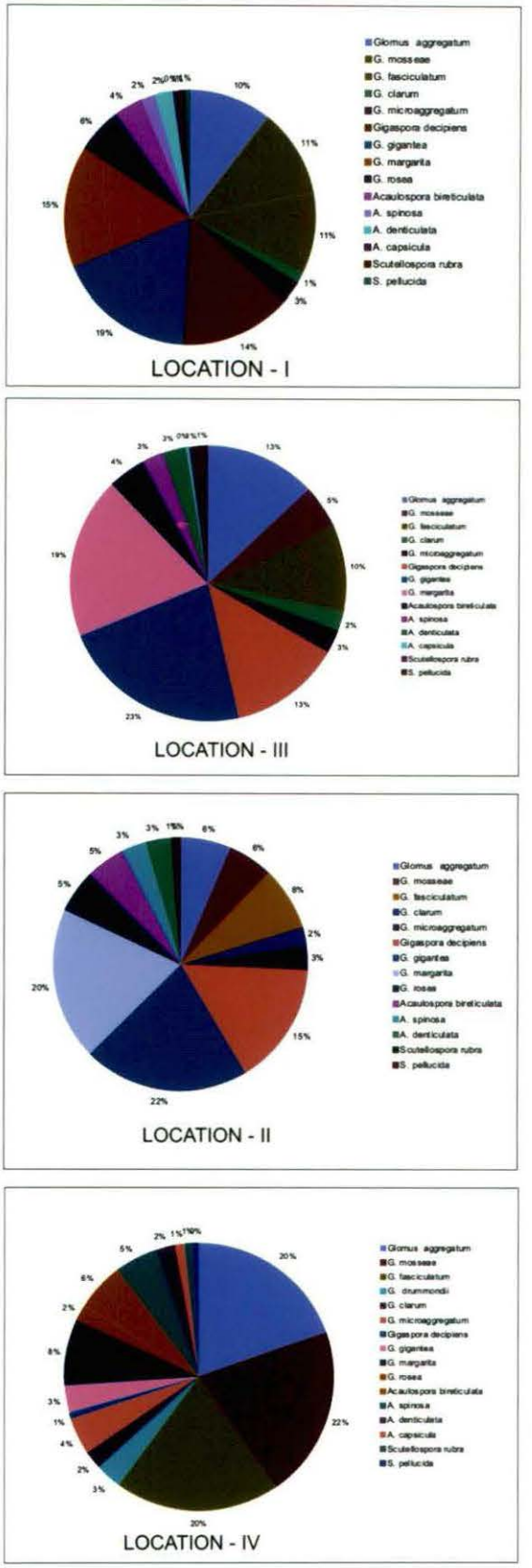


Figure 1: Percentage of AMF population in mandarin rhizosphere of four different locations of mandarin orchard

4.6. *In vitro* screening of fungal and bacterial isolates of mandarin rhizosphere for phosphate solubilizing activities

Phosphorus solubilizers produce clearing zones around the microbial colonies in solid medium. Insoluble mineral phosphates such as tricalcium phosphate or hydroxyapatite are contained in the media. The principal mechanism for mineral phosphate solubilization is the production of organic acids and acid phosphatases play a major role in the mineralization of organic phosphorus in soil. It is generally accepted that the major mechanism of mineral phosphate solubilization is the action of organic acids synthesized by soil microorganisms. Production of organic acids results in acidification of the microbial cell and its surroundings.

4.6.1. Screening in PVK medium

The fungal and bacterial isolates were screened for phosphate solubilizing activity in Pikovskaya (PVK) medium supplemented with tricalcium phosphate (TCP). The pH of the media was adjusted to 7.0 before autoclaving. Sterilized PVK medium was poured into sterilized Petri plates; after solidification of the medium, a pinpoint inoculation of fungal and bacterial isolates was made onto the plates under aseptic conditions. They were incubated at 28±2°C for 7 days with continuous observation for colony diameter. Formation of halo zones around the colony indicated positive results . Solubilization index was evaluated according to the ratio of the total diameter (colony + halo zone) and the colony diameter.

Phosphate solubilizing activities of both fungal isolates and bacterial isolates as evident in PVK medium have been presented in Plate 12, (figs.A-F). Five isolates each among fungal and bacterial isolates showed phosphate solubilizing activities. In case of *Aspergillus niger* (RHS/M492) activity reached to a maximum on the day fourth then remained constant till the end of the week whereas in *Aspergillus mellus* (RHS/M493) solubilization started within 24 h, reached maximum value on day three and remained constant throughout the week (Table 8).

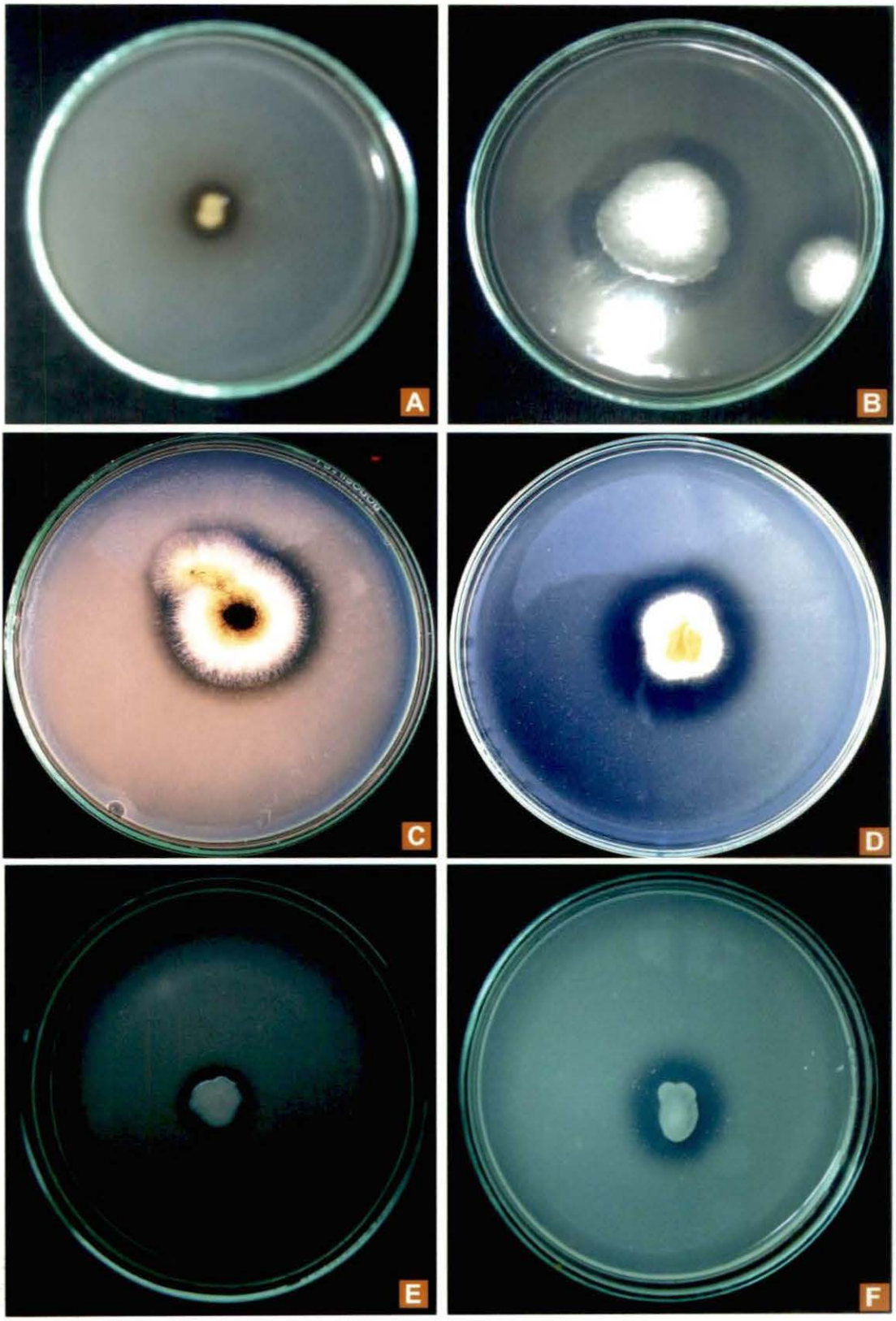


Plate 12 (figs. A-F): *In vitro* characterization of microorganisms for phosphate solubilization in PVK medium. *Aspergillus fumigatus* [A] *A. clavatus* [B] *A. niger* [C] *A. melleus* [D] *Bacillus cereus* [E] and *Bacillus pumilus* [F].

Table 8 : *In vitro* screening for phosphate solubilizing activities by fungal and bacterial isolates of mandarin rhizosphere.

Fungal and Bacterial isolates	Diameter of Clear zone (cm)*					
	24 h	48 h	72 h	96h	120h	144h
Aspergillus niger (RHS/M492)	-	0.1	0.6	0.7	0.7	0.7
A. mellus (RHS/M493)	0.1	0.3	0.5	0.6	0.6	0.6
A. clavtus (RHS/M494)	-	0.2	0.3	0.3	0.5	0.5
A. fumigatus (RHS/M498)	-	0.3	0.3	0.4	0.5	0.6
A. oryzae (RHS/M499)	0.2	0.2	0.3	0.3	0.3	0.4
B. pumilus (B/RHS/C1)	-	0.2	0.4	0.4	0.5	0.6
B.cereus (B/RHS/M2)	0.2	0.4	0.5	0.5	0.5	0.6
B. cereus (B/RHS/M3)	0.1	0.2	0.3	0.4	0.5	0.5
Bacillus sp. (B/RHS/M9)	0.2	0.3	0.5	0.5	0.5	0.5
B. cereus (B/RHS/M10)	0.1	0.2	0.3	0.4	0.5	0.5

*Average of three replicates.

4.6.2. Evaluation in liquid media

Five PSF isolates *Aspergillus niger* (RHS/M492), *A. mellus* (RHS/M493), *A. clavtus* (RHS/M494), *A. fumigatus* (RHS/M498) and *A. oryzae* (RHS/M499) were further evaluated in PVK liquid media amended with tricalcium phosphate (TCP) and rock phosphate (RP) to assess their phosphorus solubilization capacity. Results have been presented in Table 9 ; .The pH of the cultural broth samples dropped significantly as compared to the control where it remained constant around pH 7.0. *Aspergillus niger* (RHS/M492) caused decrease in pH from 7, at the beginning to 3.7 which was attributed to the varying diffusion rates of different organic acids secreted by the tested organisms. *Aspergillus oryzae* (RHS/M499) showed better efficiency of TCP solubilization after seven days of incubation (Table 9)

Table 9 : Evaluation of phosphorus solubilization by fungal isolates in liquid media amended with tricalcium phosphate (TCP) and rock phosphate (RP)

PSF isolates	TCP (mg/l)	pH	RP (mg/l)	pH
A. niger (RHS/M492)	864	3.1	351	3.6
A. mellus (RHS/M493)	875	3.2	346	3.4
A. clavtus (RHS/M494)	883	3.1	355	3.2
A. fumigatus (RHS/M498)	871	4.1	345	3.5
A. oryzae (RHS/M499)	903	3.2	363	2.8

PSF = Phosphate solubilizing fungi; TCP = tricalcium phosphate (P=997mg/l); RP= rock phosphate (P=500mg/l)

The increase of P concentration in the later stages might be due to the action of the fungi on the substrate for demands of nutrients, thus releasing more P from insoluble sources. Increase in the released P during the later stages was also attributed to cell lysis and P precipitation brought about by organic metabolites. The tested isolates reached their maximum biomass level after seven days of incubation. Such result indicated the ability of the fungal strains to solubilize P and change it to available form. Culture media with no TCP produced poor growth.

4.7. *In vitro* interactions of rhizosphere microorganisms of mandarin plants with *M. phaseolina*

Fungal isolates obtained from rhizosphere of mandarin plants excluding the root pathogens as well as bacterial isolates were grown in solid and liquid media and these were tested for their antagonistic activity against root rot pathogen (*M. phaseolina*) by dual paring tests. Their interactions were categorized into three types (A): Homogenous; free intermingling between pairing microorganisms, (B): Overgrowth; pathogen overgrown by the test organisms, (C): Inhibition; a clear zone of inhibition and of growth at time of contact. Different types of reactions developed in the pairing experiments are enlisted in Table10

Table: 10 *In vitro* interactions of fungal and bacterial isolates with *M. phaseolina*

Name of isolates	Type of reactions in test against *Macrophomina phaseolina*
Aspergillus niger (RHS/M492)	Inhibition
A. mellus (RHS/M493)	Overgrowth
A. clavtus (RHS/M494)	Overgrowth
A. fumigatus (RHS/M498)	Overgrowth
A. oryzae (RHS/M499)	Overgrowth
Trichoderma asperellum (RHS/M517)	Inhibition
Emenicella nidulans (RHS/M 509)	Homogenous
Penicillum italicum (RHS/M 510)	Homogenous
Bacillus pumilus B/RHS/C1	Inhibition
Bacillus cereus B/RHS/M2	Overgrowth
Bacillus cereus B/RHS/M3	Overgrowth
Bacillus sp. B/RHS/M4	Overgrowth
Bacillus sp. B/RHS/M5	Overgrowth
Pseudomonas sp. B/RHS/M6	Inhibition
Pseudomonas sp. B/RHS/M7	Homogenous
Pseudomonas sp. B/RHS/M8	Homogenous
Bacillus sp. B/RHS/M9	Overgrowth
Bacillus cereus B/RHS/M10	Overgrowth
Bacillus sp. B/RHS/M11	Homogenous
Bacillus sp. B/RHS/M12	Homogenous
Bacillus sp B/RHS/M13	Homogenous

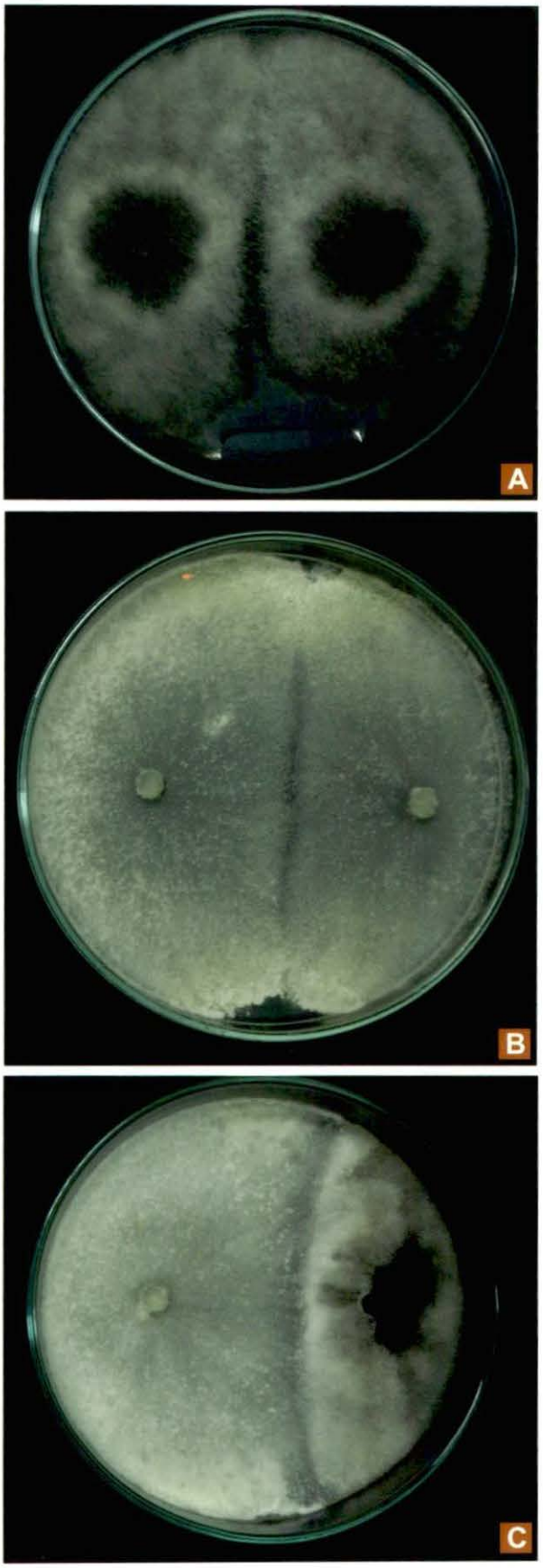


Plate 13 (figs. A–C): *In vitro* antagonism of *Trichoderma asperellum* against *Macrophomina phaseolina*. Homologous pairing of *M. phaseolina* [A] and *T. asperellum* [B]. Pairing of *T. asperellum* and *M. phaseolina*[C].

In pairing experiment against the root rot pathogen (*M. phaseolina*), *Trichoderma asperellum* inhibited the growth of the pathogen (Plate 13, figs A-C). Another fungal isolate, *Aspergillus niger* also inhibited growth of *M. phaseolina* in pairing experiment.

In vitro pairing experiment was further performed against isolated fungi and bacteria against three root pathogens (*Fusarium solani*, *F. graminearum* and *F. oxysporum*). Inhibition percentage has been calculated against these pathogens and presented in Table 11. *Trichoderma asperellum* was found to be most effective inhibiting growth of the pathogens.

Table 11: Effects of interactions between the fungal isolates and root pathogens

Antagonists	***Fusarium graminearum*** **dia(mm)**			***Fusarium oxysporium*** **dia(mm)**			***Fusarium solani*** **dia(mm)**		
	Antagonist	Pathogen	Inhibition (%)	Antagonist	Pathogen	Inhibition (%)	Antagonist	Pathogen	Inhibition (%)
A.niger RHS/M492	41	30	66.7	27	34	62.2	58	29	67.8
A. mellus RHS/M493	58	29	67.8	58	29	67.8	28	36	60.0
A. clavtus RHS/M494	28	36	60.0	27	36	60.0	29	36	60.0
A. fumigatus RHS/M498	29	36	60.0	58	29	67.8	42	30	66.7
A. oryzae RHS/M499	76	14	84.4	78	15	83.4	67	33	57.8
Trichoderma asperellum RHS/M517	29	36	60.0	42	38	57.8	58	29	67.8
Emenicella nidulans RHS/M 509	27	34	62.2	44	39	56.7	59	29	67.8
Penicillum italicum RHS/M 510									

In vitro pairing experiments were also performed with bacterial isolates against root pathogens. *Bacillus pumilus* inhibited the growth significantly (Plate 14, figs A-D). Maximum percentage inhibition was noted using *Bacillus pumilus*, followed by *Bacillus cereus* and *Pseudomonas* sp. (Table 12).

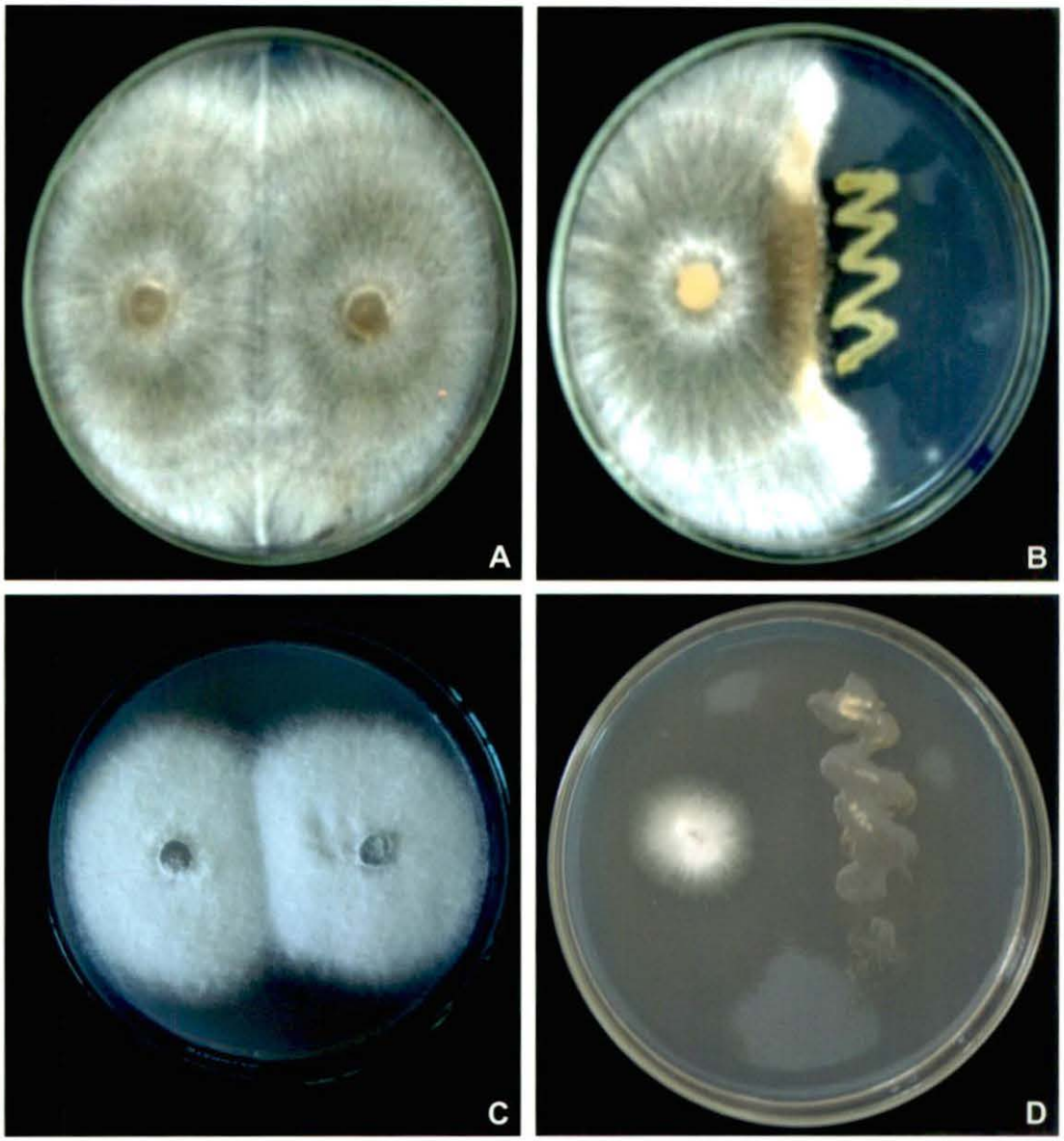


Plate 14 (figs. A–D): *In vitro* homologous pairing of *Rhizoctonia solani* [A] and *F. solani* [C]. *In vitro* antagonism of *Bacillus pumilus* with *R. solani* [B] and *F. solani* [D].

Table 12: Effects of interactions between the bacterial isolates and pathogens

Antagonists	*Rhizoctonia solani* Colony dia(mm)			*Fusarium oxysporum* Colony dia(mm)			*Fusarium solani* Colony dia(mm)		
	Antagonist	Pathogen	Inhibition (%)	Antagonist	Pathogen	Inhibition (%)	Antagonist	Pathogen	Inhibition (%)
Bacillus pumilus B/RHS/C1	77	12	86.7	76	14	84.4	79	12	86.7
Bacillus cereus B/RHS/M2	76	14	84.4	71	19	78.9	73	17	81.1
Bacillus cereus B/RHS/M3	75	13	85.6	61	23	74.4	76	14	84.4
Bacillus sp. B/RHS/M4	75	13	85.6	76	14	84.4	71	19	78.9
Bacillus sp. B/RHS/M5	71	19	78.9	79	12	86.7	73	17	81.1
Pseudomonas sp. B/RHS/M6	79	19	78.9	78	14	84.4	79	13	85.6
Pseudomonas sp. B/RHS/M7	76	14	84.4	76	14	84.4	76	14	84.4
Pseudomonas sp. B/RHS/M8	79	12	86.7	79	12	86.7	79	12	86.7
Bacillus sp. B/RHS/M9	59	29	67.8	26	31	65.6	28	36	60.0
Bacillus cereus B/RHS/M10	42	39	56.7	27	36	60.0	29	36	60.0
Bacillus sp. B/RHS/M11	42	30	66.7	58	29	67.8	27	34	62.2
Bacillus sp. B/RHS/M12	42	38	57.8	28	36	60.0	58	29	67.8
Bacillus sp B/RHS/M13	44	39	56.7	29	36	60.0	27	36	60.0

4.8. Serological and molecular detection of *M. phaseolina*

4.8.1 Soluble protein

Mycelial antigen prepared from the pathogen (*M. phaseolina*) was analysed initially by SDS PAGE. The molecular weight of protein bands visualized after staining with coomassie blue wer determined from the known molecular weight marker. Mycelial protein exhibited 23 bands in SDS PAGE ranging in molecular weight (Ca. 95.4 kDa to 10.5 kDa). Bands were of varying intensitie and more proteins of lower molecular were present (Plate 15,Fig C) .

4.8.2. Immunological assays

Immunological assays were performed using Polyclonal antibodies (PAb) raised agains mycelial protein of *M. phaseolina* in rabbit. Effectiveness of antigen in raising antibodies wer checked initially using agar gel double diffusion technique followed by dot immunobinding assa and western blot analysis. Finally Optimization of ELISA was done by considering two variables dilution of the antigen extract and dilution of the antiserum to obtain maximum sensitivity.

4.8.2.1. Immuno-diffusion

Agar gel double diffusion tests was performed using the mycelial antigens of *M. phaseolin* and homologus PAb. Strong precipitation reactions occurred in homologous reactions i immunodiffusion test which was evident by intense precipitation (Plate 15,fig. A). Cross reactiv antigen shared between root antigens of manadarin (*C. reticulata*) and polyclonal antibody of tw fungal pathogens (*Macrophomina phaseolina* and *Fusarium solani*) were detected usin immunodiffusion tests. Antigens were prepared from root samples collected from eight differer locations of Darjeeling hills as well as from root pathogens (*M. phaseolina* and *F. solani*). Cros reaction of root antigens prepared from eight different locations were performed with PAbs of *M. phaseolina* and *F. solani* . Strong precipitin reactions were noticed in root samples of four locations Mirik, Sukhia pokhri, Kalimpong Block I and Block II) when reacted with PAb of *M. phaseolin* while root samples of other four locations showed weak precipitin reaction. In the pathogenicity te also root rot index were higher in three locations. Positive reactions were also noticed i heterologous reaction of root antigens and PAb of *F. solani.* However, root antigens prepared fro two locations gave no such precipitin reactions using PAb of *F. solani* (Table 13). Commo antigenic relationship shared between host and pathogen was evident in such cases where susceptibl reactions were noticed in pathogenicity test.

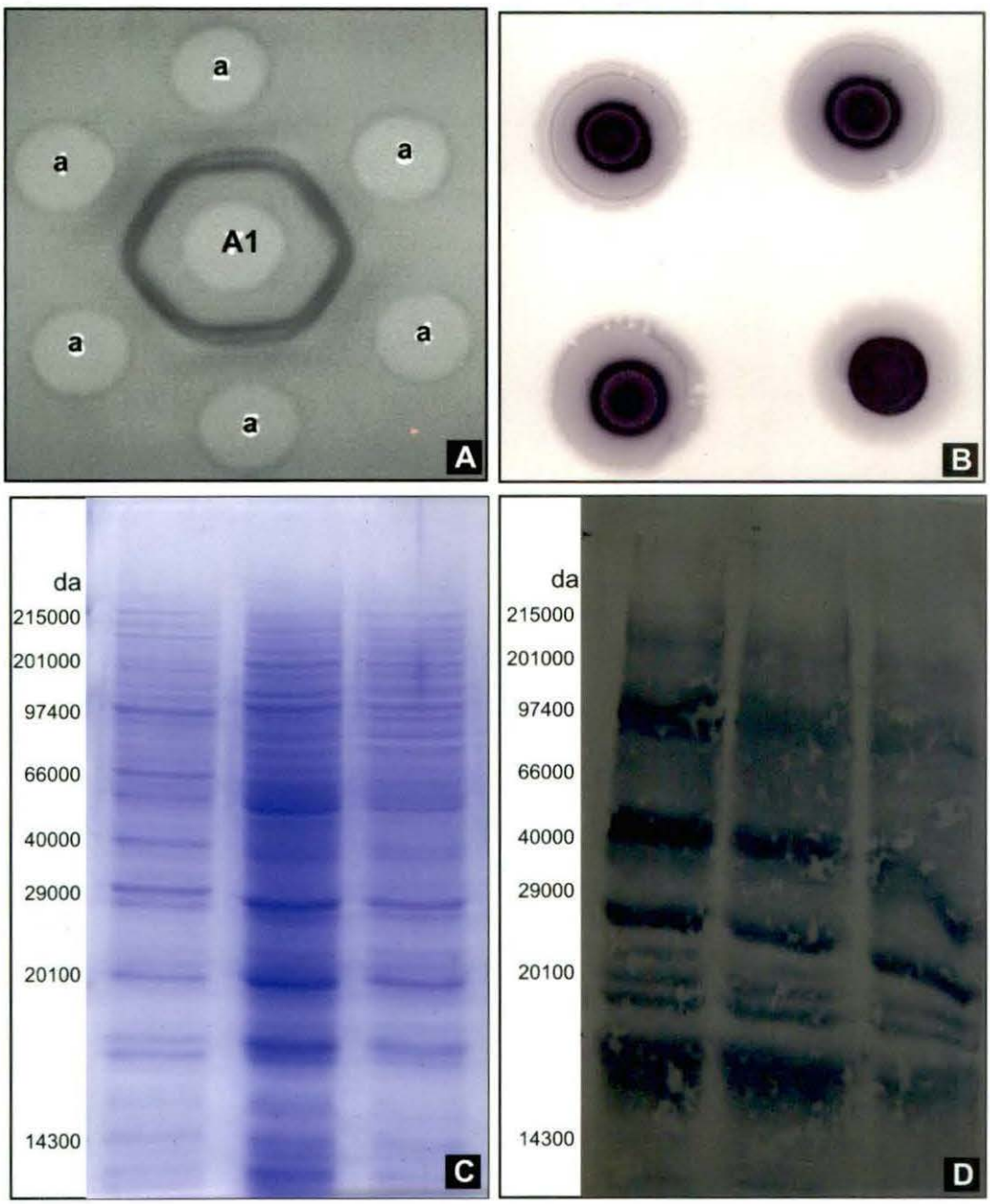


Plate 15 (figs. A-D): Immunodiffusion [A], Dot immunobinding assay [B], SDS PAGE analysis [C] and Western blot analysis[D] of *Macrophomina phaseolina* using mycelial antigen (a) and PAb of *M. phaseolina* (A1)

Table 13 : Detection of cross reactive antigens among *C. reticulata* and *F. solani* and *M. phaseolina* using agar gel double diffusion

Root antigens of *C. reticulata* and root pathogens	PAb	
	F. solani	*M. phaseolina*
Rangli Rangliot	-	±
Bijanbari	-	±
Sukhia Pokhari	+	+
Kurseong	±	±
Mirik	+	+
Kalimpong Block I	+	+
Kalimpong Block II	+	+
Gorubathan	±	±
M. phaseolina	-	+
F. solani	+	-

Common precipitin band (+) present, (-) absent, (±) weak

4.8.2.2. PTA-ELISA

Optimization of PTA- ELISA was done using purified IgGs of known concentration which was predetermined using the referred formula. Goat antirabbit IgG (whole molecule) alkaline phosphatase (Sigma) conjugate (1:10000) and p-nitrophenyl phosphate (100 mg ml^{-1}) were used for PTA-ELISA as enzyme substrate (pNPP), reaction was terminated after 60 min and the absorbance values were recorded as mean of five adjacent wells measured at 405 nm. Root antigens were prepared from healthy as well as artificially inoculated plants of *C. reticulata*. Three days and seven days following inoculation with *F. solani* and *M. phaseolina*, root antigens were prepared along with healthy root antigens and reacted with PAbs of *M. phaseolina* and *F. solani* for comparison. Absorbance values were higher in those root samples which showed susceptible reaction when tested against root pathogens. Following inoculation with the pathogens absorbance values were always higher in artificially inoculated plant roots in comparison with healthy root antigens when tested against PAbs of the respective pathogens (Table 14).

Table 14 : PTA-ELISA values showing reaction of PAbs of *M. phaseolina* and *F. solani* with antigens of healthy and inoculated roots of *C. reticulata*

Citrus saplings Locality	Antigen concentration (40 mg/L)		
	Healthy	Inoculated	
		M.phaseolina[a]	*F. solani*[b]
Rangli Rangliot	0.812	1.264	1.182
Bijanbari	0.890	1.149	1.139
Sukhia Pokhari	1.115	1.774	1.345
Kurseong	0.972	1.880	1.265
Mirik	1.064	1.993	1.876
Kalimpong Block I	1.007	1.887	1.766
Kalimpong Block II	1.187	1.932	1.980
Gorubathan	0.938	1.872	1.765

PAb of *M. phaseolina* and *F. solani* were used at 1:125 dilution
[a] 7 days after inoculation
[b] 3days after inoculation
absorbance at 405 nm

4.8.2.3. Dot immunobinding assay

Dot immunobinding assay using mycelia antigen and PAb of *M. phaseolina* was also standardized. For this, soluble protein obtained from seven-day-old mycelia of *M. phaseolina* were reacted on nitrocellulose paper with PAb of the pathogen (*M. phaseolina*). Results shows development of deep violet colour indicating a positive reactions suggestive of effectiveness of mycelial antigen in raising PAb against the pathogen (Plate 15, fig B).

4.8.2.4. Western blot analysis

Western blot analysis using PAb of *M. phaseolina* was also performed to develop strategies for rapid detection of the pathogen. For this total soluble protein of young mycelia was used as antigen source and SDS-PAGE was performed as described previously followed by probing of the localized antigen with alkaline phosphatase conjugate. The bands on nitrocellulose membrane was compared with corresponding protein bands on the SDS-PAGE. Bands of varying intensities was observed ranging from 14 KDa to 95 KDa (Plate 15, fig. D). Bands of lower molecular weight were more in number. Hence the result suggests that Western blot formats could be used as one of a refined tool for detection of pathogen.

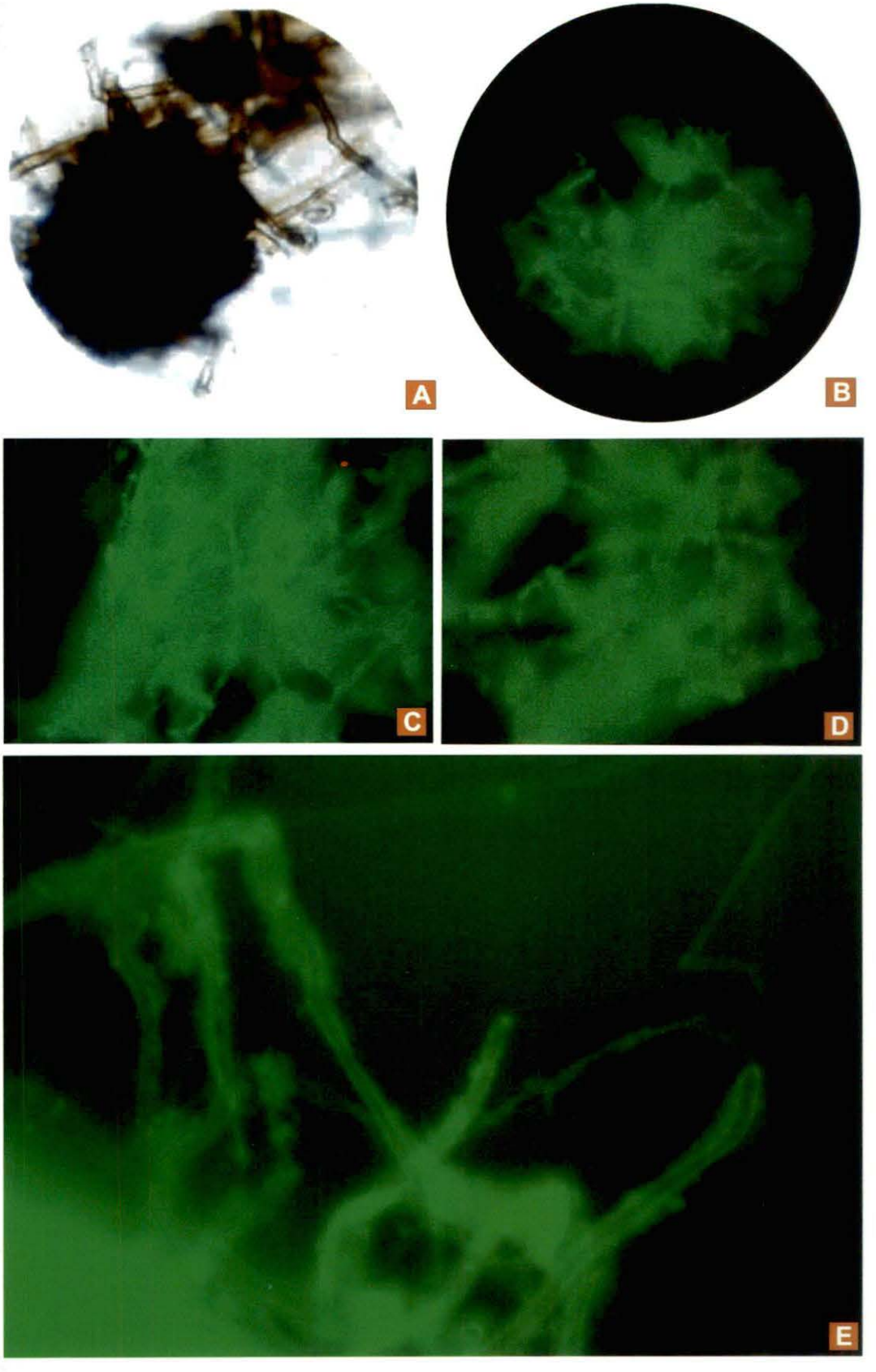


Plate 16 (figs. A-E): Microscopic observation of *Macrophomina phaseolina* under bright field [A]. Mycelia of *M. phaseolina* treated with PAb of the pathogen and labeled with FITC [B - E].

4.8.2.5. Indirect immunofluorescence

Indirect immunoflorescence of hyphae and young sclerotia of *M. phaseolina* were conducted with homologus antibody (PAb of *M. phaseolina*) and reacted with fluorescein isothiocyanate (FITC) labeled antibodies of goat specific for rabbit globulin. Strong apple green fluorescence were evident in both mycelia and scletoria which confirmed the detection of the pathogen (Plate 16, figs A-E). The presence of cross-reactive antigens (CRA) between plant host and parasite that in some instances reflect degrees of compatibility in the parasite association is well known. The unique presence of CRA in hosts and parasites continue to suggest a regulatory role of CRA in host and parasite continues to suggest a regulatory role of CRA in host parasite specificity. Antibody labeling with FITC is known to be one of the powerful techniques to determine the cell or tissue location of CRA shared by host and parasite. Cellular location of major cross reactive antigens shared by *M. phaseolina* in root tissues of *C. reticulata* was determined.

To achieve this antibodies labeled with fluorescein isothiocyanate (FITC) were used to determine location of CRA in cross sections of mandarin roots which provided positive results. Fresh cross sections of healthy roots of *C. reticulata* (Plate 17, figs A-C) were treated with PAb of *M. phaseolina* labeled with FITC conjugate and observed under UV florescent conditions. The root tissues did not exhibit any autofluorescence. On the other hand different tissues of root sections treated with PAb of the pathogen exhibited apple green florescence distributed throughout the root tissue mainly over the outer cortex and the vascular region including pith and pericycle (Plate 17, figs D-F). It appears that CRA may form a continuum between cells of host and pathogen, which favours the growth and establishment in the root tissue.

4.8.3. Molecular detection of *M. phaseolina*

4.8.3.1. ITS-PCR amplification

Genomic DNA of *Macrophomina phaseolina*, *Fusarium solani* and *Fusarium oxysporum* were amplified by mixing the template DNA, with the polymerasè reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. The following primer pairs were used for ITS PCR (Table 15).

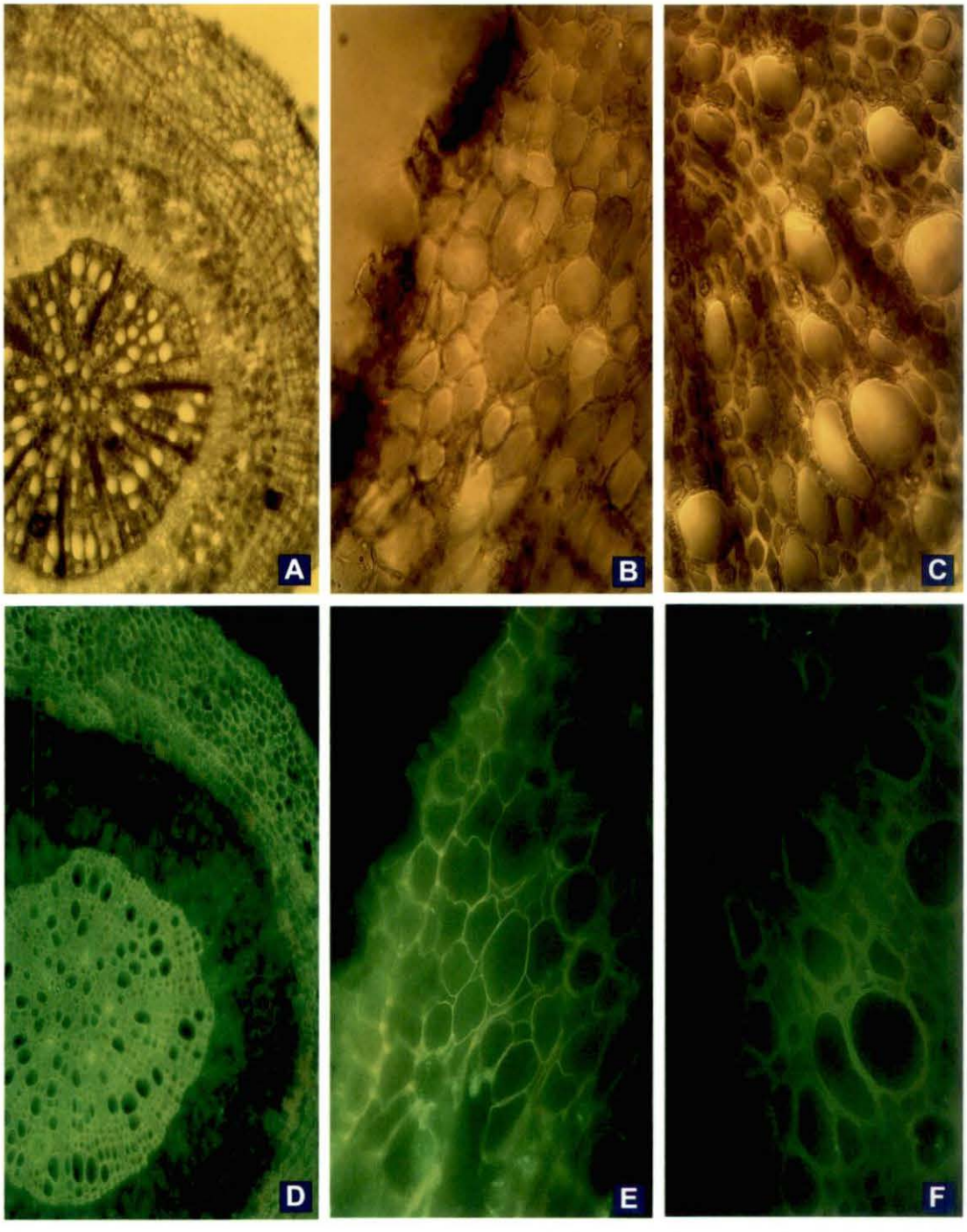


Plate 17 (figs. A-F): Cross section of mandarin root tissue under bright field [A-C]; root tissue treated with PAb of *M. phaseolina* and labeled with FITC.

Table 15 : The nucleotide sequence used for ITS PCR of root pathogens of *C. reticulata*

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC	Amplicaon size (bp)
Macrophomina sp.					
ITS 1	TCCGTAGGTGAACCTGCG	18	61	56%	~620
ITS4	TCCTCCGCTTATTTGATATGC	21	63	59%	
Fusarium sp.					
Fcg17F	TCGATATACCGTGCGATTTCC	21	65	47%	~570
Fcg17R	TACAGACACCGTCAGGGGG	19	66	63%	

PCR was programmed with an initial denaturing at 94°C for 5 min followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec and extension at 70°C for 2 min and the final extension at 72 °C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis.

In the present study, main focus was on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *Macrophomina* and *Fusarium*. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 (for *Macrophomina*) and Fcg17F & Fcg17FR (for *Fusarium*) primers. Amplified products of size in the range of 550-700bp was produced by the all primers. The primer pairs Fcg17F and Fcg17R were found to be highly specific for *Fusarium* genus as reflected in Plate 18 fig.A. (Lanes 9-12), but non specific for *Macrophomina phaseolina,* as no band was detected with this primer pair (Plate 18fig.A, lanes 7&8).

4.8.3.2. DGGE analysis

In the present study *M. phaseolina, F. solani, F. graminearum* and *F. oxysporum* were used for DGGE analysis. For this, 18S rDNA (320 bp with GC clamp) of each isolates were amplified with the forward primer containing GC clamp at NS1 (5'-GTAGTCATATGCTTGTCTC-3') and GCfung (5'-CGCCCGCCGCGCCCCGCGCCC

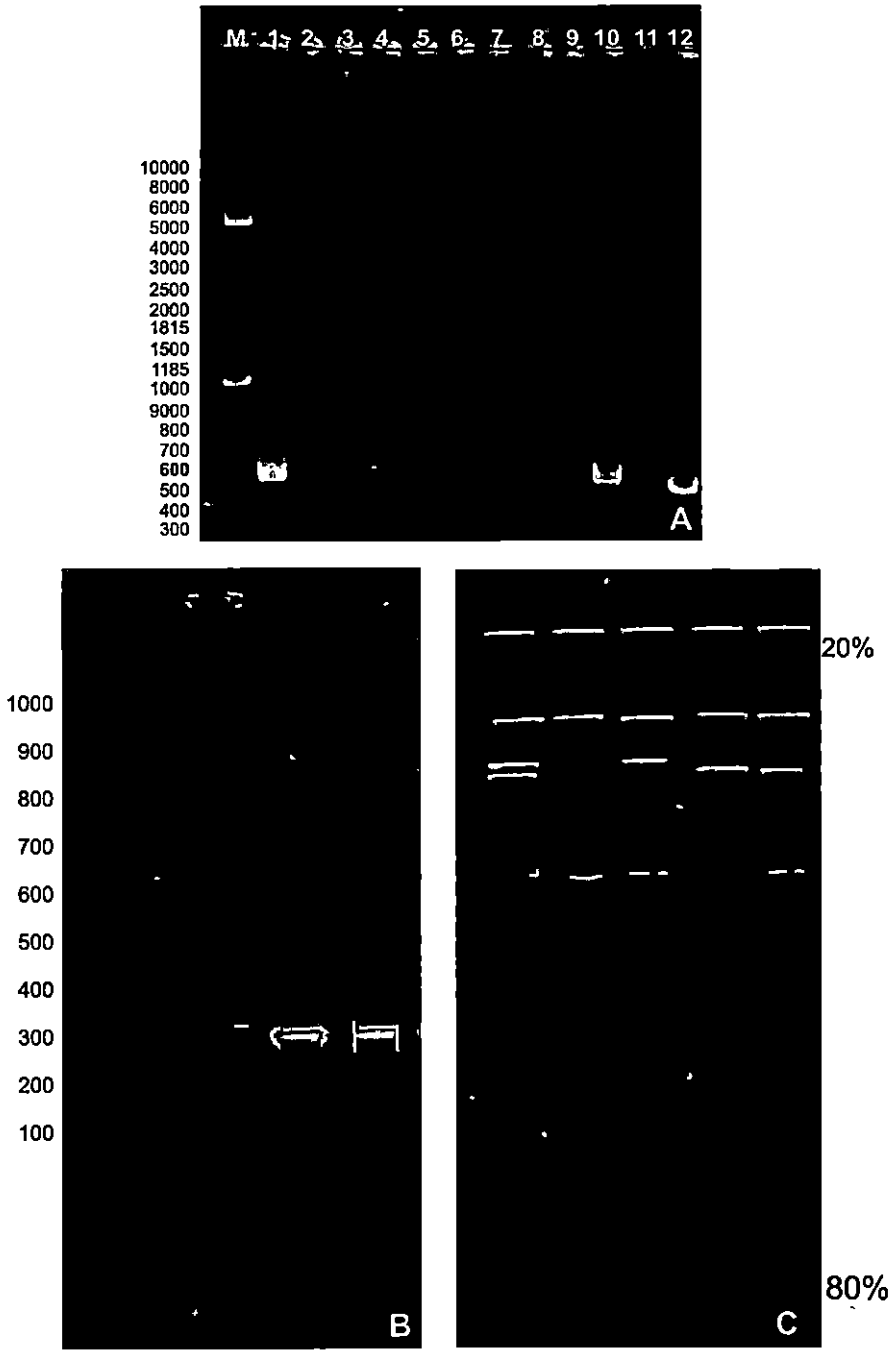
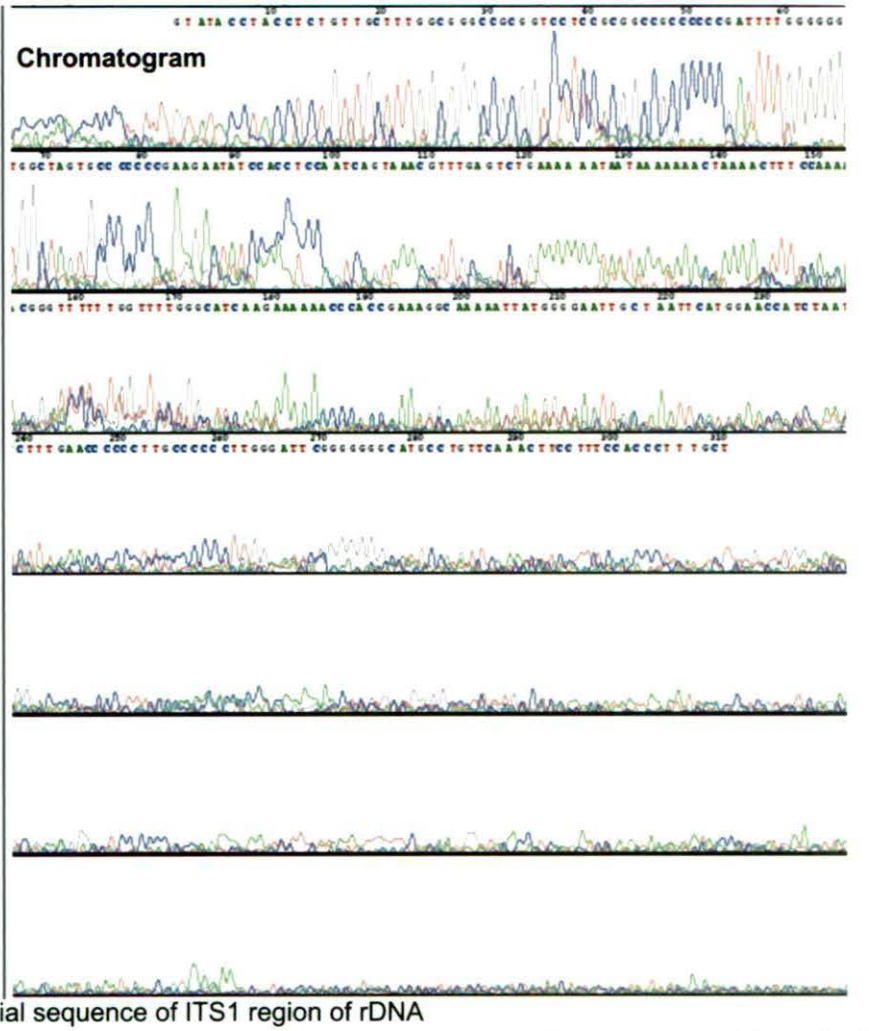


Plate 18 (figs. A-C) : ITS-PCR amplified products of root pathogens [A], Lane M-high range DNA ladder, lanes 1-2,7-8 for *M. phaseolina*, 3-4,9-10 for *F. solani*, 5-6,11-12 for *F. graminearum*. (Primer pairs- Lanes 1-6: ITS1 and ITS4 and Lanes 7-12: Fcg17F and Fcg17R respectively). ITS- PCR amplified products of root pathogens obtained with GC clamp based primer pair [B]. DGGE analysis of root pathogens. Lane 1- Mixture of *M. phaseolina*, *F. solani*, *F. graminearum*, and *F. oxysporum*, lane 2 , *M. phaseolina*, lane 3, *F. solani*, lane 4, *F. graminearum*, and lane 5, *F. oxysporum* [C].

GGCCCG CCGCCCCCGCCCCA TTCCCCGTTAC CCGTTG-3') in 25 µl of reaction mixture containing 1×PCR buffer, 2.5mM $MgCl_2$ (Bangalore Genei, India), 100 ng of the template DNA, 25.0 pmol each of the forward and reverse primers, 250 µM each of dNTPs, and 1 U of *Taq* DNA polymerase. Amplified products of ITS-PCR of root pathogens were resolved in 2% agarose gel (Plate 18, fig.B). The touchdown PCR program was performed which consisted of an initial denaturation at 95°C for 4 min, followed by 35 cycles of 95°C for 1 min, 50°C for 1 min and 10 sec, and 72°C for 2 min then followed by a last extention at 72°C for 8 min. The gels contained 10% (wt/vol) of acrylamide and a range of denaturant concentration from 0% to 100% (formamide and urea). DGGE gels were run at 110 V for 06 hours in 1X TAE buffer (pH 8.0) at 60°C and stained with ethidium bromide. DNA bands on the DGGE gels were excised under UV trans-illumination. The gel photographs were taken and analysed. In this uniform gradient gel of 0% to 100% and shorter run time, individual bands could not separate. However, suitable concentration and running time was optimized using 20 to 80% denaturant and running time 8h at 110V (Plate 18, fig.C). The profile obtained after 8 hours of run time from 20-80 % gradient showed all the bands have co migrated however the profile obtained 12 hours of run time showed a close variation in presence or absence of dominant bands. The DGGE analysis demonstrated that all the corresponding single band on DGGE gels belonged to the isolates of *Macrophomina phaseolina* and other double bands formed for isolates of *Fusarium oxysporum*, *Fusarium solani* and *Fusarium graminearum* gel due to their G+C variation in their ITS region of rDNA . A similar type of distinct band was formed for all selected isolates in the mixture lane but no separate bands were formed in this lane (Plate 18, fig.C lane 1).

4.8.3.3. Sequencing of 18SrDNA region and their phylogenetic analysis

The isolate *Macrophomina phaseolina*, obtained from mandarin root tissue collected from Mirik orchard, the causal organism of root rot of *Citrus reticulata* was finally considered for sequencing of its 18S rDNA region. PCR products produced sequences and chromatogram and 18S rDNA sequence of *M. phaseolina* (Fig 2) that could be aligned and showed satisfactory homology with ex-type strain of *Macrophomina phaseolina* sequences from the NCBI Genbank data base. The priming site of the ITS1 and ITS4 primers were determined in order to confirm that the sequences obtained corresponded to the actual ITS 1 region. ITS1 showed the highest number of nucleotide substitutions, and it was used for the phylogenetic study.



Partial sequence of ITS1 region of rDNA

CGGCGGGCGACGCCTTACGCTCCGAAGCGAGGTGTATTCTACTACGCTTGAGGCA
AGACGCCACCGCCGAGGTCTTTGAGGCGCGCCCGCAAAGGACGGTGCCCAATAC
CAAGCAGAGCTTGAGGGTTGAAATGACGCTCGAACAGGCATGCCCCCCGGAATA
CCAAGGGGCGCAATGTGCGTTCAAAGATTCGATGATTCACTGAATTCTGCAATTC
ACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCAGAACCAAGAGATCCG
TTGTTGAAAGTTTTAGTTTATTTAATATTTTTTTCAGACTGCAACGTTTACTGACTG
GAGTTTGATAGTCCTCTGGCGGGCACTACCCACCCCCCAAAATCGGGGGGCGG

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max Ident	Links
HM990162.1	Macrophomina phaseolina isolate NBEF3 contain 18S ribosomal RNA g	704	704	100%	0.0	99%	
HQ660594.1	Macrophomina phaseolina isolate DTMp3 18S ribosomal RNA gene, pa	704	704	100%	0.0	99%	
HQ660592.1	Macrophomina phaseolina isolate DTMp1 18S ribosomal RNA gene, pa	704	704	100%	0.0	99%	
HQ649832.1	Macrophomina phaseolina isolate r068 18S ribosomal RNA gene, parti	704	704	100%	0.0	99%	
HQ392815.1	Rhizoctonia bataticola clone RB80 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392814.1	Rhizoctonia bataticola clone RB78 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392809.1	Rhizoctonia bataticola clone RB67 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392808.1	Rhizoctonia bataticola clone RB66 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392806.1	Rhizoctonia bataticola clone RB62 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392805.1	Rhizoctonia bataticola clone RB61 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392803.1	Rhizoctonia bataticola clone RB58 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392801.1	Rhizoctonia bataticola clone RB56 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392800.1	Rhizoctonia bataticola clone RB55 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392799.1	Rhizoctonia bataticola clone RB53 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392797.1	Rhizoctonia bataticola clone RB49 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392795.1	Rhizoctonia bataticola clone RB46 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392793.1	Rhizoctonia bataticola clone RB44 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392792.1	Rhizoctonia bataticola clone RB43 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	
HQ392791.1	Rhizoctonia bataticola clone RB39 18S ribosomal RNA gene, partial s	704	704	100%	0.0	99%	

Figure 2: Chromatogram and 18S rDNA sequence of *Macrophomina phaseolina* (RHS/S565) and significant alignments by BLAST analysis.

Table 16 : **Identified *Macrophoina phaseolina* and comparison with referred NCBI GenBank sequences**

Acc. No.	Source	Sequences	Country
HM990163	plant	534 bp	India
HQ660594	plant	583 bp	China
HQ660593	plant	583 bp	China
HQ660592	plant	583 bp	China
HQ660591	plant	583 bp	China
HQ660590	plant	584 bp	China
HQ660589	plant	583 bp	China
JF710587	plant	583 bp	China
HQ380051	Sunflower	685 bp	Turkey
EF446288	corneal scraping	562 bp	India
HQ713771	*Pinus sylvestris*	511 bp	Switzerland
FJ960442	plant	582 bp	China
EU250575	mulberry	582 bp	China
EF570501	plant	642 bp	Canada
DQ314733	*Glycine hispida*	527 bp	India
DQ233666	*Glycine max*	495 bp	India
DQ233664	okra	441 bp	India
DQ233663	cluster bean	519 bp	India
DQ233662	cluster bean	432 bp	India
(RHS/S565)	*Citrus reticulata*	310bp	India

Although studies involving isolates of *Macrophomina phaseolina* revealed that the 5.8S rRNA gene is as variable as ITS1 regions. The sequence information was then analysed through BLASTn program which indicated that the sequences contain the genetic information of internal transcribed spacer region of rDNA gene of *Macrophomina phaseolina* with 100% similarity . This sequence has been deposited to NCBI genebank to get accession number.

Identified *Macrophomina phaseolina* rDNA gene sequences obtained from NCBI genebank (Table 16) of various host plants were selected for comparison with the rDNA gene sequence of *M. phaseolina* (RHS/S565) isolate of mandarin plant. The sequence alignment of the isolate of *M. phaseolina* (RHS/S565) shows variation in this gene . These available sequences of *Macrophomina phaseolina* from NCBI were used in the pair wise and multiple sequence alignment using Bioedit software (Fig 3) for determining the conserved regions of rDNA gene. There were quite a number of gaps that were introduced in the multiple sequence alignment within the ITS region that were closely related and similar sequence indicated. The evolutionary history was inferred using the UPGMA method. The optimal tree with the sum of branch length = 0.56368608 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. 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 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 294 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Fig 4).

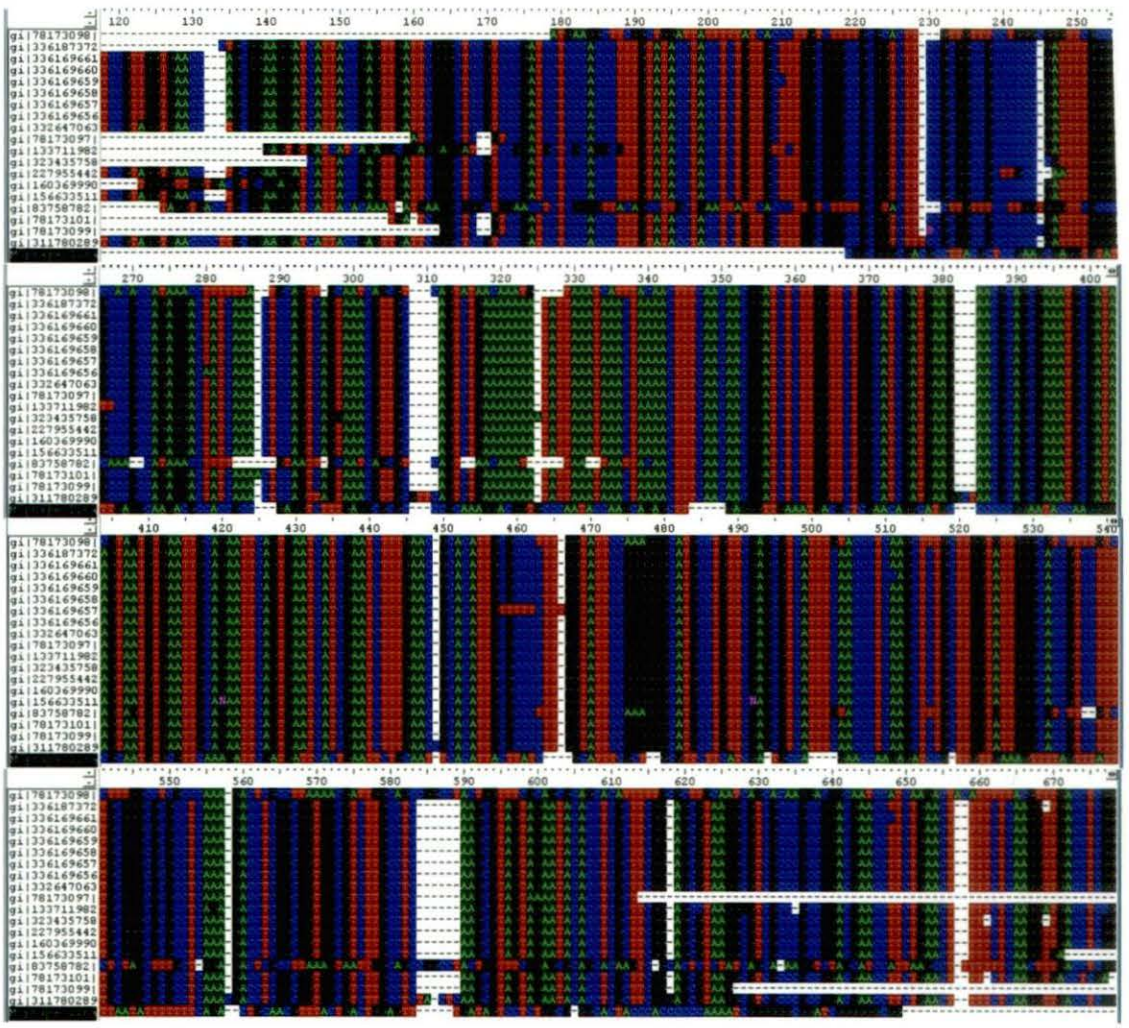


Figure 3: 18S rDNA sequence alignments of *Macrophomina phaseolina* (RHS/S565). Data for other species were gathered from NCBI. The conserved regions of the gene are demonstrated in the different colour.

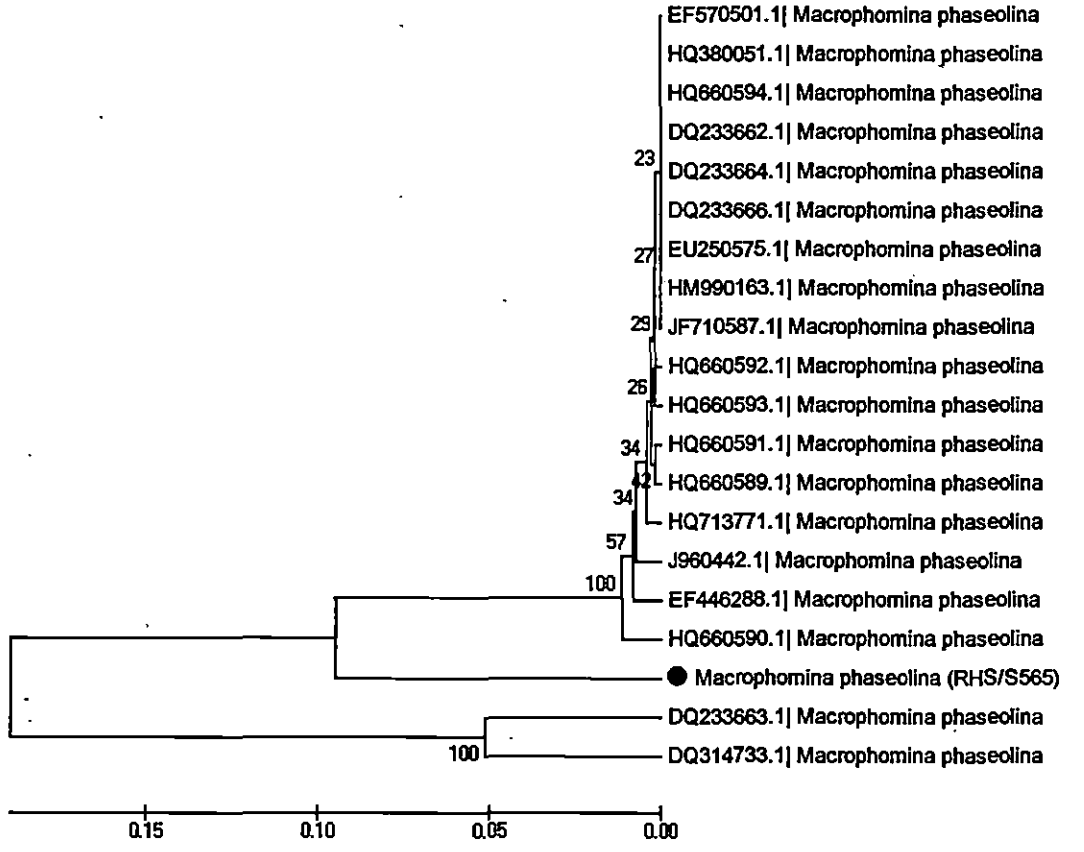


Figure 4: Phylogenetic placement of *Macrophomina phaseolina* (RHS/S565) with extype strains from NCBI genebank

4.9. RAPD-PCR and phylogenetic analysis of microorganisms of mandarin rhizosphere

PCR products of four fungal pathogens of mandarin roots, fungal and bacterial isolates of mandarin rhizosphere using RAPD primers were analysed.

4.9.1. Fungal pathogens of mandarin roots

Four fungal pathogens (*M. phaseolina*, *F. solani*, *F. oxysporum*, and *F. graminearum*) were selected for RAPD-PCR analysis using nine random primers as given in **Table 17**. PCR was programmed with an initial denaturing at 94°C for 4 min. followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 70°C for 90 s and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25 % bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis. RAPD-PCR products using two primers OPA4 and A-05 of the said four root pathogens resolved in agarose gel have been presented in Plate 19 (figs A&B).

Table 17 Analysis of the polymorphism obtained with RAPD markers in *Macrophomina phaseolina*, *Fusarium solani*, *Fusarium graminearum* and *Fusarium oxysporum*

Sl No.	Seq Name	Total no RAPD bands	Approximate band size (bp).		Monomorphic band	Polymorphic bands	Polymorphic (%)
			Min	Max.			
1.	OPA1	11	200	6000	0	11	100
2	OPA-4	17	200	6000	0	17	100
3.	A-05	15	100	6000	0	15	100
4.	A-11	11	100	6000	0	11	100
5.	OPD-6	03	100	6000	0	03	100
6.	AA-4	04	100	6000	0	04	100
7.	OPB-2	03	100	6000	0	03	100
8.	OPB-3	05	100	6000	0	05	100
9.	OPD-5	04	100	6000	0	04	100
	Total	**73**			**0**	**73**	**100**

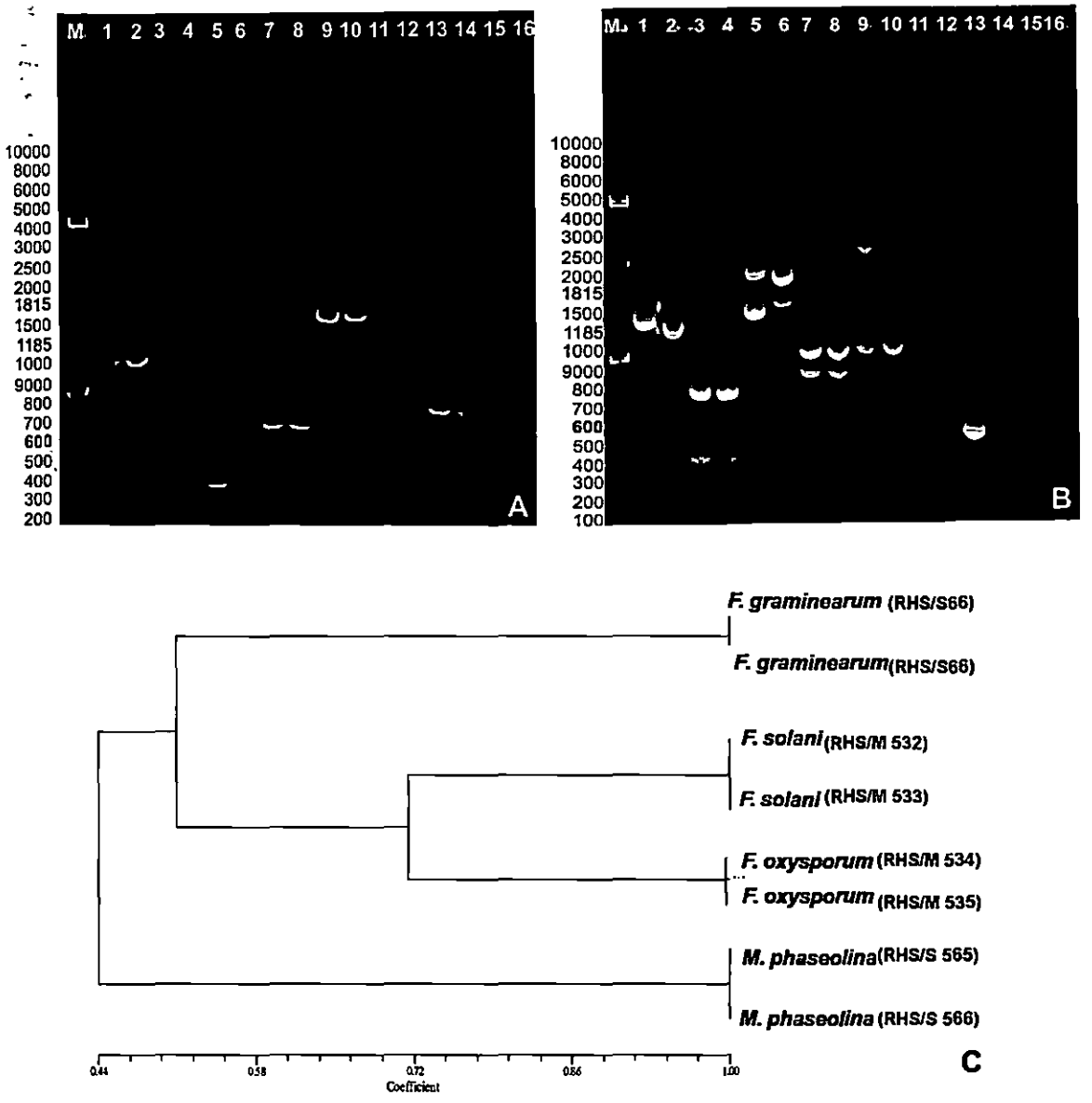


Plate 19 (figs. A-B): RAPD profiles of root pathogens. Lane M-high range DNA ladder, lane 1-2 *M. phaseolina*, 3-4 *F. solani*, 5-6 *F. graminearum*, 7-8 *F. oxysporum* analysed with primer OPA1 & lane 9-10 *M. phaseolina*, 11-12 *F. solani*, 13-14 *F. graminearum*, 15-16 *F. oxysporum* analysed with primer OPA4 [A]; Lane M-high range DNA ladder, lane 1-2 *M. phaseolina*, 3-4 *F. solani*, 5-6 *F. graminearum*, 7-8 *F. oxysporum* analysed with primer A-05 & lane 9-10 *M. phaseolina*, 11-12 *F. solani*, 13-14 *F. graminearum*, 15-16 *F. oxysporum* analysed with primer AA-11; [B] Dendrogram showing the genetic relationships among *Fusarium graminearum*, *F. solani*, *F. oxysporum*, and *Macrophomina phaseolina* based on RAPD analysis using NTSYSPc software [C].

Scoring and data analysis

The image of the gel electrophoresis was documented through Bio-Profil Bio-1D gel documentation system and analysis software. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed *In Silico* into similarity matrix using NTSYSpc (Numerical Taxonomy System Biostastiscs, version 2.11W). The SIMQUAL program was used to calculate the Jaccard's coefficients. The RAPD patterns of each isolate was evaluated, assigning character state "1" to indicate the presence of band in the gel and "0" for its absence in the gel. Thus a data matrix was created which was used to calculate the Jaccard similarity coefficient for each pair wise comparison. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc .

The genetic relatedness among isolates of *F. solani, F. graminearum, F. oxysporum* and *M. phaseolina* 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 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 (Plate 19, fig.C).

4.9.2. Fungal isolates of mandarin rhizosphere

Fifteen fungal isolates obtained from mandarin rhizosphere were further selected for RAPD-PCR analysis using following nine random primers (Table 18). PCR was programmed with an initial denaturing at 94°C for 4 min. followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 70°C for 90 s and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25 % bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by

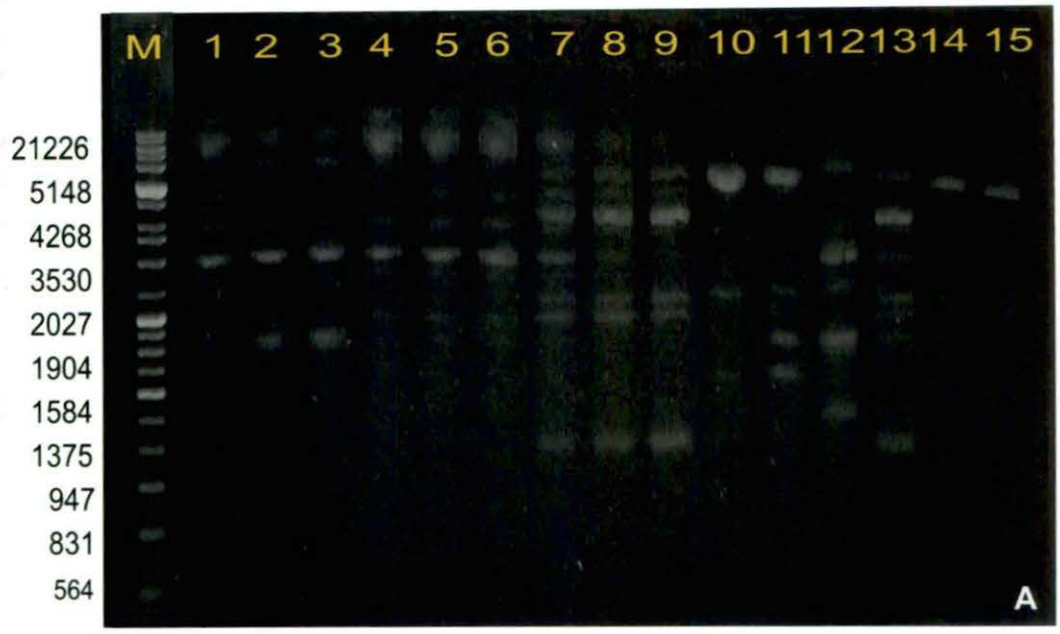
horizontal electrophoresis. RAPD-PCR products using one primer OPA4 of the said fifteen fungal isolates of mandarin rhizosphere resolved in agarose gel have been presented in Plate 20 fig.A.

Table 18 : Analysis of the polymorphism obtained with RAPD markers in fungal isolates of mandarin rhizosphere.

Sl No.	Seq Name	Total no RAPD bands	Approximate band size (bp).		Monomorphic band	Polymorphic bands	Polymorphic (%)
			Min	Max.			
1.	OPA1	04	200	6000	0	04	100
2	OPA-4	06	200	6000	0	06	100
3.	A-5	03	100	6000	0	03	100
4.	A-11	05	100	6000	0	05	100
5.	OPD-6	03	100	6000	0	03	100
6.	AA-4	07	100	6000	0	07	100
7.	OPB-2	05	100	6000	0	05	100
8.	OPB-3	04	100	6000	0	04	100
9.	OPD-5	03	100	6000	0	03	100
	Total	**40**			**0**	**40**	**100**

Scoring and data analysis

The image of the gel electrophoresis was documented through Kodac gel documentation system and analysis software. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed *In Silico* into similarity matrix using NTSYSpc (Numerical Taxonomy System Biostastiscs, version 2.11W). The SIMQUAL program was used to calculate the Jaccard's coefficients. The RAPD patterns of each isolate was evaluated, assigning character state "1" to indicate the presence of band in the gel and "0" for its absence in the gel. Thus a data matrix was created which was used to calculate the Jaccard similarity coefficient for each pair wise comparison. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering programme,



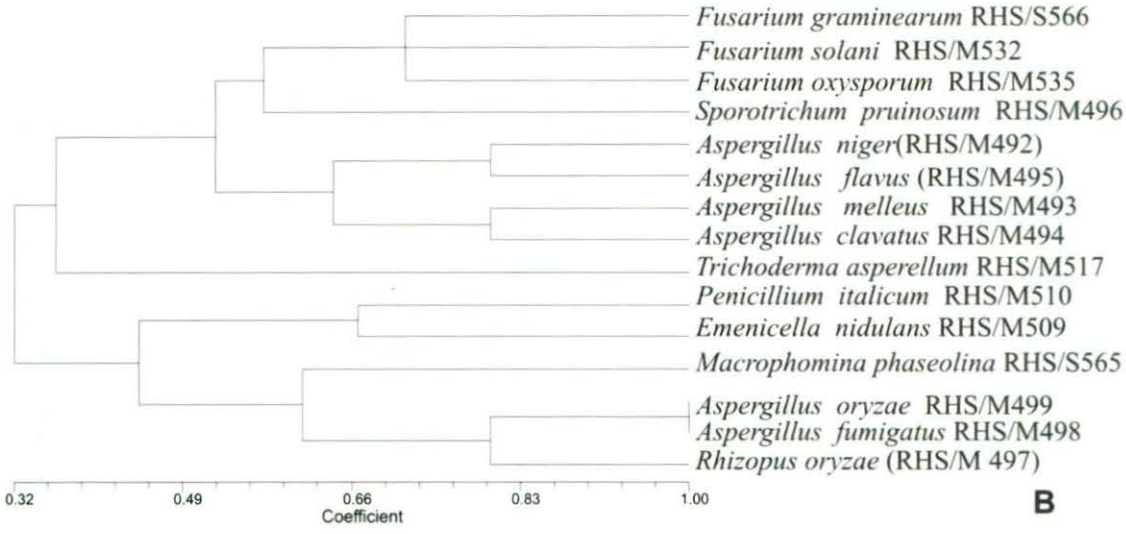


Plate 20 (figs. A-B): RAPD profiles of fungal isolates of mandarin rhizosphere obtained using primer OPA 4 [A] . Lane M-high range DNA ladder, lane 1, *A. niger* RHS/M492, lane 2, *Aspergillus flavus* (RHS/M495), lane 3, *A. mellus* RHS/M493, lane 4, *A. clavatus* RHS/M494, lane 5, *A. fumigatus* RHS/M498, lane 6 , *A. oryzae* RHS/M499, lane 7, *F. graminearum* RHS/S566, lane 8, *F. solani* RHS/M532, lane 9, *F. oxysporum* RHS/M535, lane 10, *Sporotrichum pruinosum* RHS/M496, lane 11, *Macrophomina phaseolina*, RHS/S565, lane 12, *Trichoderma asperellu*m RHS/M517, lane 13, *Penicillium italicum* RHS/M510, lane 14, *Emericella nidulans* RHS/M509, lane 15, *Rhizopus oryzae* (RHS/M 497). Dendogram showing phylogenetic relationship between different fungal isolates based on RAPD banding pattern [B].

selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc .

The genetic relatedness among isolates of rhizopshere of mandarin were analyzed by random primers 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 40 reproducible and scorable polymorphic bands ranging from approximately 200bp to 2000bp were generated. In the RAPD profiles showed that primer AA-4 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 (Plate 20,fig.B).

4.9.3. Bacterial isolates of mandarin rhizosphere

The broth cultures of bacterial isolates (*B. pumilus* . B/RHS/C1, *B. cereus* B/RHS/M2, *B. cereus* B/RHS/M3, *Bacillus sp.* , *Bacillus sp.* B/RHS/M4 B/RHS/M5, *Psedomonus sp.* B/RHS/M6, *Psedomonus sp.* B/RHS/M7, *Psedomonus sp.* B/RHS/M8, *Bacillus sp.* B/RHS/M9, *B. cereus* B/RHS/M10, *Bacillus sp* B/RHS/M11, *Bacillus sp.* B/RHS/M12 and *Bacillus sp.* B/RHS/M13) were centrifuged at 10,000 rpm at 28°C for 5 mins and the pellets were collected by discarding the supernatant. The pellets were washed thrice with distilled water and resuspended in 0.5ml of CTAB buffer was and incubated at 37°C for 3 hrs. Then 10 µl proteinase K solution (20mg/ml) was added and it was allowed to incubate at 65°C for 3min. The lysate was extracted with equal volume of tris water saturated phenol: chloroform: isoamylalcohol (25:24:1) and then centrifuged at 10,000 rpm for 5 min. The aqueous phase was collected in clean tube and 2 volume of chilled absolute ethanol was added to this aqueous phase. The mixture was centrifuged at 10,000 rpm for 5 mins at 4°C, the pellet was air dried and finally dissolved in 40µl TE buffer and stored at 4°C. Genomic DNA was re suspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse . After incubation the sample was re-extracted with PSI (Phenol: Chloroform: Isoamylalcohol) solution and RNA free DNA was precipitated with chilled ethanol. The yield of DNA was determined spectrophotometrically as 24 µg/g of mycelial mat. The purity of DNA genome samples as indicated by A_{260}/A_{280} ratio (Table 19) and DNA quantity was evaluated by 0.8% agarose gel electrophoresis.The quantity and quality of the genomic DNA, isolated from thirty different isolates was checked on 0.8% agarose gel electrophoresis. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

polymerase enzyme, 6µl 2mM dNTPs, 1.5µl of 100mM RAPD primers and 1µl of template

Table 19 : Spectrophotometrical A_{260}/A_{280} ratio of isolated genomic DNA

Organisms	A_{260}	A_{280}	A_{260}/A_{280}	Organisms	A_{260}	A_{280}	A_{260}/A_{280}
B. pumilus. B/RHS/C1	0.276	0.148	1.86	*Psedomonus sp.* B/RHS/M8,	0.379	0.183	2.07
B. cereus B/RHS/M2	0.379	0.183	2.07	*Bacillus sp.* B/RHS/M9,	0.276	0.148	1.86
B. cereus B/RHS/M3	0.276	0.148	1.86	*B. cereus* B/RHS/M10,	0.379	0.183	2.07
Bacillus pumilus. B/RHS/M4	0.197	0.137	1.44	*Bacillus sp* B/RHS/M11,	0.276	0.148	1.86
Bacillus sp. B/RHS/M5	0.276	0.148	1.86	*Bacillus sp.* B/RHS/M12	0.197	0.137	1.44
Psedomonus sp. B/RHS/M6	0.379	0.183	2.07	*Bacillus sp.* B/RHS/M13	0.276	0.148	1.86
Psedomonus sp. B/RHS/M7	0.379	0.183	2.07				

RAPD-PCR

All thirteen bacterial isolates were taken up for RAPD-PCR amplification. Genomic DNA was amplified by mixing the template DNA, with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase chain reaction was performed in a total volume of 100µl, containing 78µl deionized water, 10µl 10X taq polymerase buffer, 1µl of 1U Taq polymerase enzyme, 6µl 2mM dNTPs, 1.5µl of 100mM RAPD primers and 1µl of template DNA. Two random decamers (OPA1 and OPA4) were used to prepare the RAPD profiles of the isolates (Table 20). PCR was programmed with an initial denaturing at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30sec, annealing at 59°C for 30 sec and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. After RAPD-PCR amplifications, all amplified DNA products were resolved by electrophoresis on agarose gel(2%) in TAE(1X) buffer, stained with ethidium bromide and photographed. RAPD-PCR products using two primers OPA4 and OPA1 of the said thirteen bacterial isolates of mandarin rhizosphere resolved in agarose gel have been presented in Plate 21 figs.A&B (Table 21).

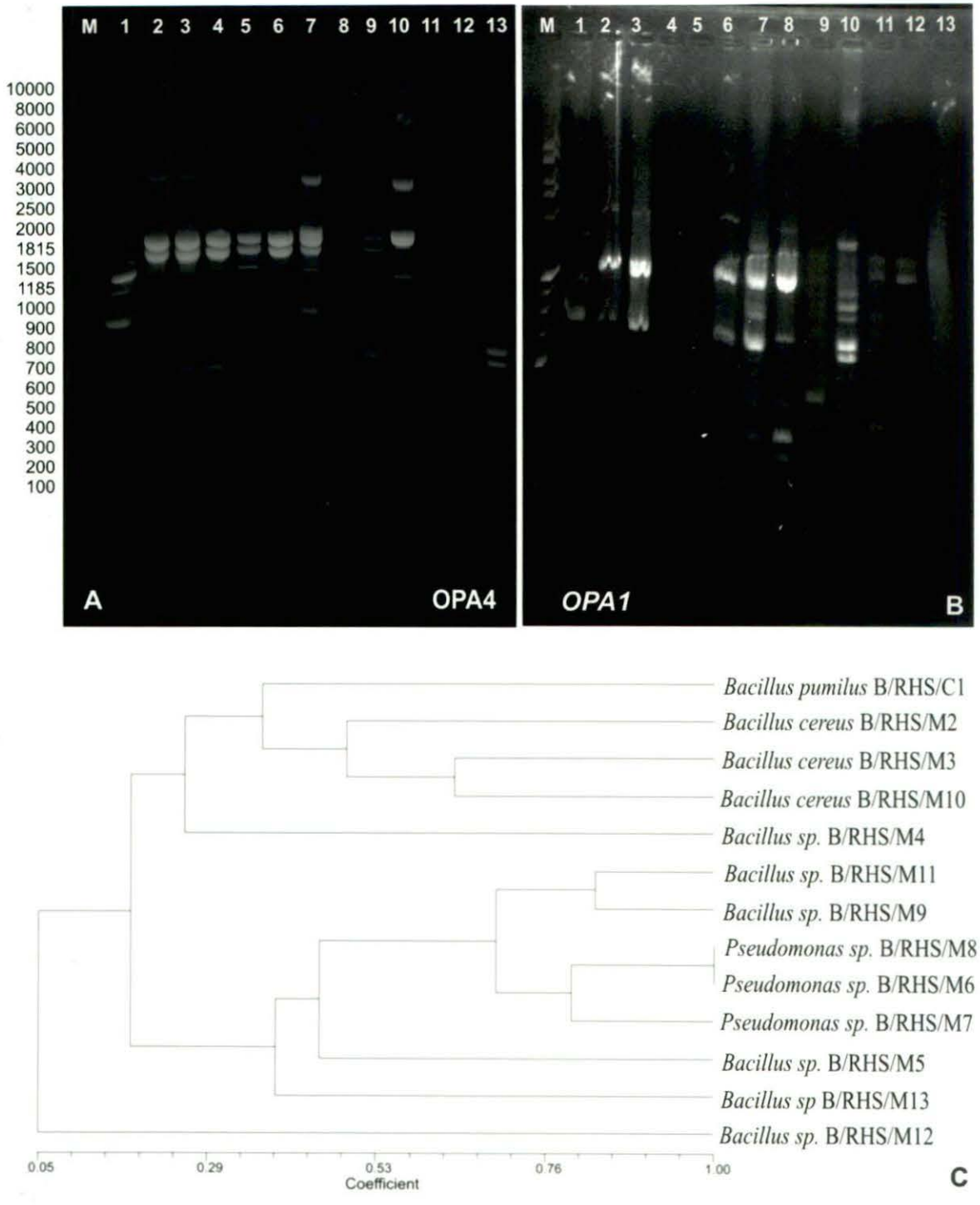


Plate 21 (figs. A-B): RAPD profiles of bacterial isolates obtained using primers OPA 4 [A] and OPA 1[B]. Lane M-high range DNA ladder, lane1, *Bacillus pumilus* B/RHS/C1, lane 2, *Bacillus cereus* B/RHS/M2, lane 3, *Bacillus cereus* B/RHS/M3, lane 4, *Bacillus* sp. B/RHS/M4, lane 5, *Bacillus* sp. B/RHS/M5, lane 6, *Pseudomonas* sp. B/RHS/M6, lane 7, *Pseudomonas* sp. B/RHS/M7 lane 8, *Pseudomonas* sp. B/RHS/M8, lane 9, *Bacillus* sp. B/RHS/M9 lane 10, *Bacillus cereus* B/RHS/M10, lane 11, *Bacillus* sp. B/RHS/M11, lane 12, *Bacillus* sp. B/RHS/M12, lane 13, *Bacillus* sp B/RHS/M13. Dendogram showing phylogenetic relationship between different bacterial isolates based on RAPD banding pattern [C].

Table 20 : The nucleotide sequence used for RAPD PCR

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC
OPA1	CAGGCCCTTC	10	38.2	70%
OPA-4	AATCGGGCTG	10	39.3	60%

Table 21 Analysis of the polymorphism obtained with RAPD markers

Sl No.	Seq Name	Total no RAPD bands	Approximate band size (bp).		Monomorphic band	Polymorphic bands	Polymorphic (%)
			Min	Max.			
1.	OPA1	04	100	2000	0	3	100
2	OPA-4	05	100	1000	0	4	100

Phylogenetic analysis

The genetic relatedness among isolated thirty phosphate solubilizer bacterial isolates were analysed by two random primers (OPA1 and OPA4) to generate reproducible polymorphisms. All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicate the genetic diversity of isolates. RAPD profiles showed that primer OPA4 scored highest bands 7 (Table 21). 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 arithmetric mean (UPGMA) using NTSYSpc software (Plate 21 fig.C). Similarity co-efficient ranged from 0.65-1.0. Based on the results obtained all nine isolates can be grouped into five main clusters grouped into *Bacillus pumilus*, *Bacillus cereus*, *Bacillus sp* and *Pseudomonas sp*.

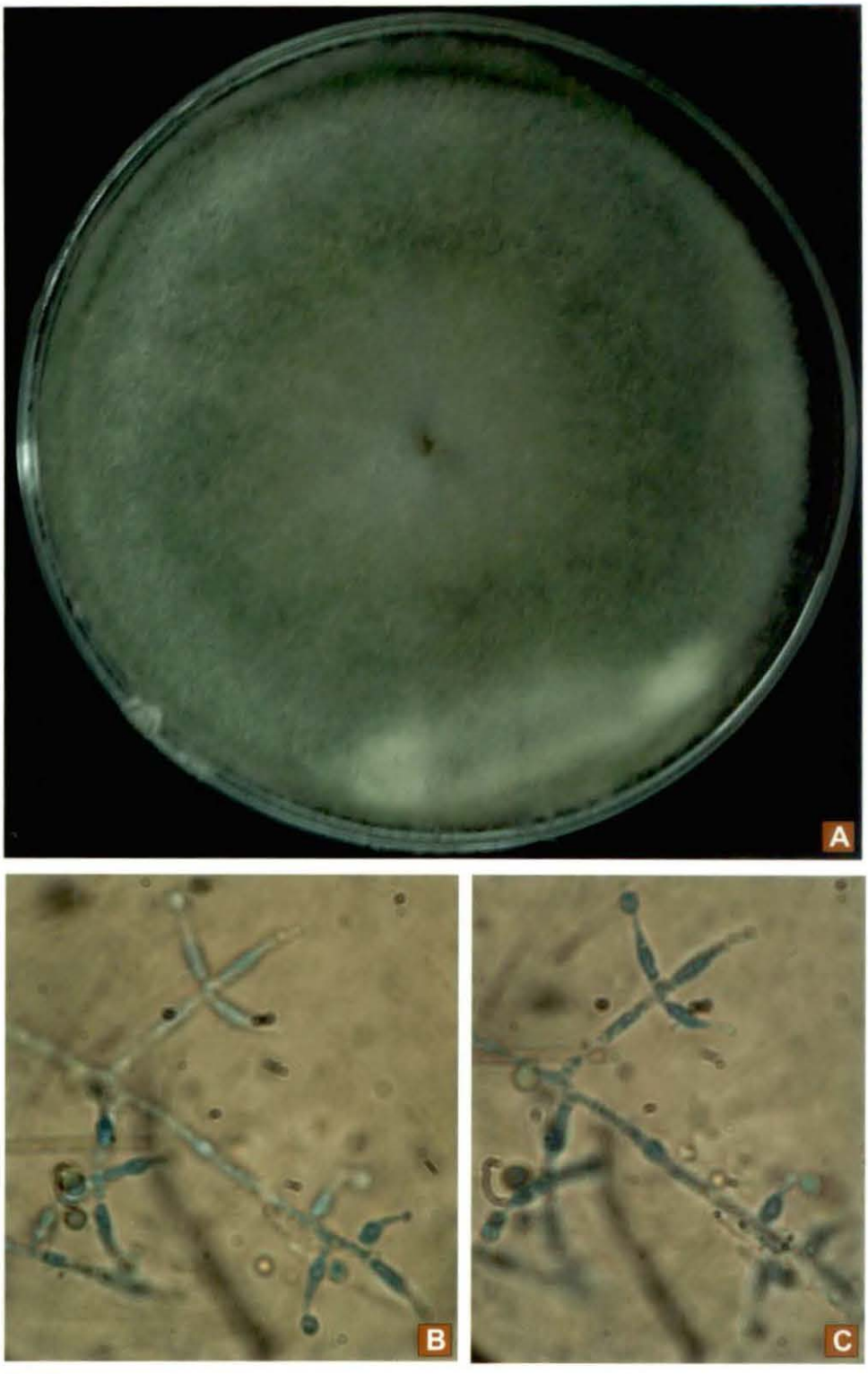


Plate 22 (figs. A-C): Radial growth of *Trichoderma asperellum* [A] and microscopic observation showing phialides and hyphae [B & C].

4.10. Morphological and molecular identification of a potential BCA isolate

4.10.1. Scanning electron microscopy

Isolate of *Trichoderma asperellum* (Plate 22 fig.A) which showed *in vitro* antagonistic activity against *Macrophomina phaseolina, Fusarium solani, Fusarium oxysporum* and *Fusarium graminearum* were selected as potential BCA isolate for SEM study. Before this, the isolate of *T. asperellum* was also taken for determining their phialide and conidia structures. Drops of spore suspension were placed on clean grease free glass- slides, mounted with lacto phenol cotton blue, covered with cover slip and sealed. The slides were then observed under the microscope following which spore characteristics were determined and size of spore measured. Microscopic observations under bright field of all these isolates have been presented in Plate 22 (figs.B&C). Detailed informations on conidiophore, conidia and phialides of these isolates have been presented in Table 20.

Table 20 Morphological characteristics of different isolates of *Trichoderma asperellum*

Morphological characteristics	*Trichoderma asperellum* (RHS/M 517)
Conidiophores Central axis (µm)	1.9 X 4.2
Conidia (µm)	2.0x 1.5
Phialide dimensions (µm)	
[width where arising from a cell]	
length	9.8
width	3.8
widest point	4.1
width at base	4.1

Scanning electron microscopic observations of the conidia of *T. asperellum* was also made. Photographic presentations of *T. asperellum* has been presented in (figs.A-C). Results revealed that isolate had smooth conidial surfaces. The conidia were an irregular pyramidal shape with a diameter within the 150 to 250 nm size range. The conidia of different isolates varied in shape from globose to sub globose and in size, with diameters ranging from 4.0 to 4.5 µm. Fragments of what appeared to be a thin layer of tissue were observed on and around the conidia in most conidial preparations.

Plate 23 (figs. A-C): Scanning Electron Micrograph of *Trichoderma asperellum*.

4.10.2. 18S rDNA sequencing

Genomic DNA was prepared from *T. asperellum* isolate -RHS/M517 using lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH8.0), 100 mM NaCl and 2% SDS,following the method as described in detail in Materials and Methods. DNA was precipitated with chilled ehanol (100%), pelleted by centrifuging at 12000 rpm for 15 min, washed in 70% ethanol by centrifugation and finally the pellets were air dried and suspended in TE buffer (pH 8.0). Genomic DNA was resuspended in 100 µl 1 X TE buffer and incubated at 37°C for 30 min with RNAse (60µg). The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel which produced clear sharp bands, indicating good quality of DNA. The result revealed that RNA free DNA was yielded and the size of DNA of each isolates ranging from 1.5-1.8 kb. Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. For amplification of the ITS1–5.8S–ITS2 region of *T. asperellum*, the primer pair T/ITS1 TCTGTAGGTGAACCTGCGG and T/ITS4 TCCTCCGCTTATTGATATGC was used.

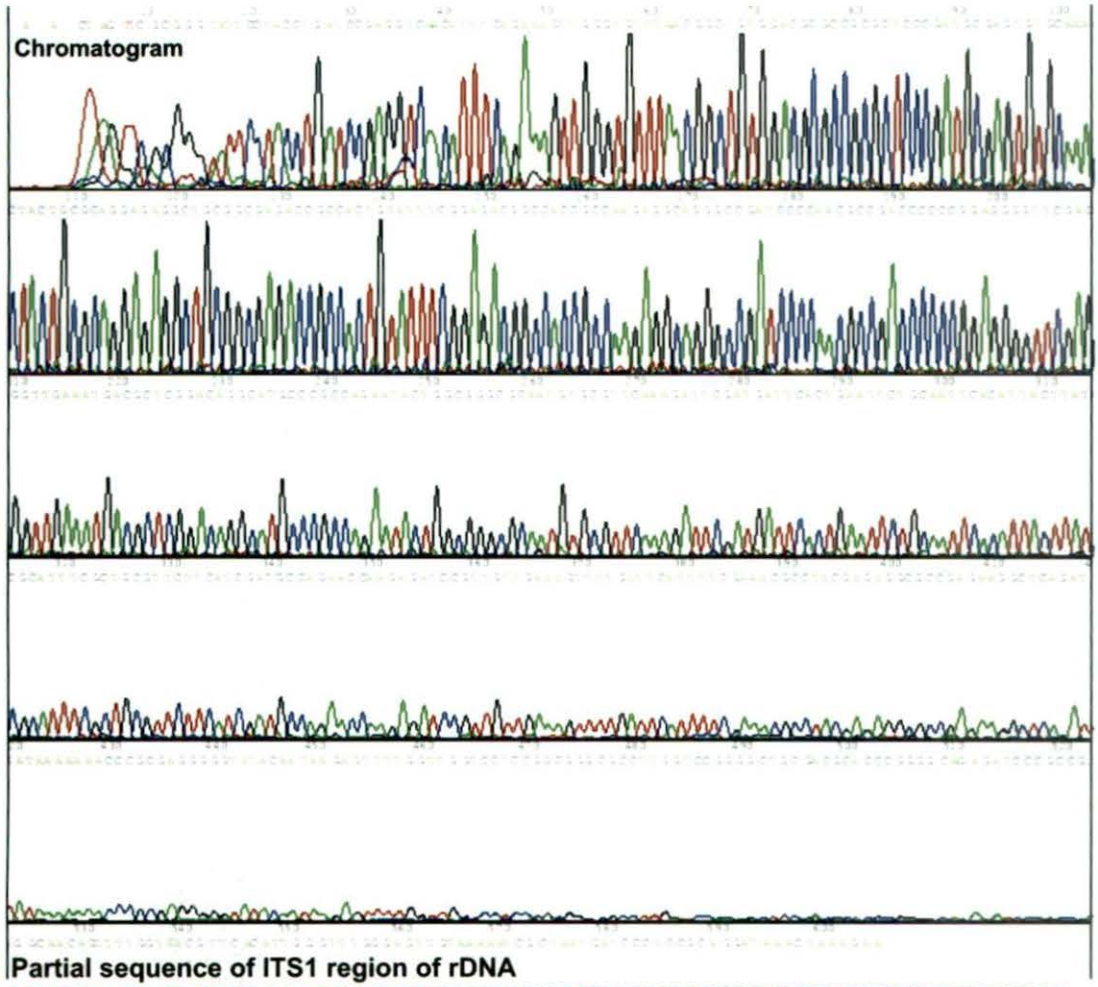
PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 30 cycles of denaturation at 94 °C for 30 sec, annealing at 59 °C for 30 sec and extension at 70 °C for 2 min and the final extension at 72 °C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1 % ethidium bromide for examining by with horizontal electrophoresis. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 primers. Amplified products of size in the range of 600bp was produced by the primers. A single distinct DNA bands was

observered on the gel for each isolates. Midium range of DNA rular (Genei, Bangalore) was used in the marker lane. The purified PCR products of *T. asperellum* were sequenced bidirectionally in Applied Biosystems by Bangalore Genei. Partial sequence of ITS region of rDNA of *T. asperellum* have been presented in Figure 5.

4.10.3. Analyses of rDNA gene sequences

After direct sequencing of the PCR product of *T. asperellum* which showed satisfactory homology with ex-type strain of *T. asperlleum* sequences from the NCBI Genbank data base, sequence was submitted to NCBI Genbank (HQ334994). The priming site of the ITS1 and ITS4 primers were determined in order to confirm that the sequences obtained corresponded to the actual ITS 4 region. A multiple sequence alignment was carried out that included the ITS 1 region, including gaps and the complete sequences align. There were quite a number of gaps that were introduced in the multiple sequence alignment within the ITS-4 region that were closely related and similar sequence indicated.

Identified *Trichoderma asperellum* rDNA gene sequences obtained from NCBI genebank (Table 21) of various locations were selected for comparison with the rDNA gene sequence of *T. asperellum* (RHS/M517) isolate of mandarin rhizosphere. The sequence alignment of the isolate of *T. asperellum* (RHS/M517) shows variation in this gene . These available sequences of *T. asperellum* from NCBI were used in the pair wise and multiple sequence alignment using Bioedit software (Fig 6) for determining the conserved regions of rDNA gene. The ITS PCR has helped to detect polymorphism at ITS region of rDNA among the *Trichoderma asperellum* isolates. The evolutionary history was inferred using the UPGMA and Neibourhood-Joining (N J)method. The optimal tree with the sum of branch length = 1.84709756 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances



Partial sequence of ITS1 region of rDNA

ATGTGTAACGTTACCAAACTGTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCGCAGCCCCGGAAC
CAGGCGCCCGCCGGAGGAACCAACCAAACTCTTTCTGTAGTCCCCTCGCGGACGTATTTCTTACAG
CTCTGAGCAAAAATTCAAAATGAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATG
AAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA

Sequence Deposited: NCBI

Title : *Trichoderma asperellum* isolate RHS/M 517 18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S

ACCESSION: HQ334994

VERSION: HQ334994.1

GI:315112485

DNA linear : 544 bp

```
  1 atgtgtaacg ttaccaaact gttgcctcgg cggggtcacg ccccgggtgc gtcgcagccc
 61 cggaaccagg cgcccgccgg aggaaccaac caaactcttt ctgtagtccc ctcgcggacg
121 tatttcttac agctctgagc aaaaattcaa aatgaatcaa aactttcaac aacggatctc
181 ttggttctgg catcgatgaa gaacgcagcg aaatgcgata agtaatgtga attgcagaat
241 tcagtgaatc atcgaatctt tgaacgcaca ttgcgcccgc cagtattctg gcgggcatgc
301 ctgtccgagc gtcatttcaa ccctcgaacc cctccggggg atcggcgttg gggatcggga
361 cccctcacac gggtgccggc cccggaatac agtggcggtc tcgccgcagc ctctcctgcg
421 agtaggttgc acaactcgca ccgggagcgc gggcgtccac gtccgtaaac agccactttc
481 tgaaatggcg acctggatca ggtaggaata cccgcgaaat aaagctatca taggcgggga
541 gaaa
```

Figure 5: Chromatogram and 18S rDNA sequence of *Trichoderma asperellum* (RHS/M517) and significant alignments by BLAST analysis.

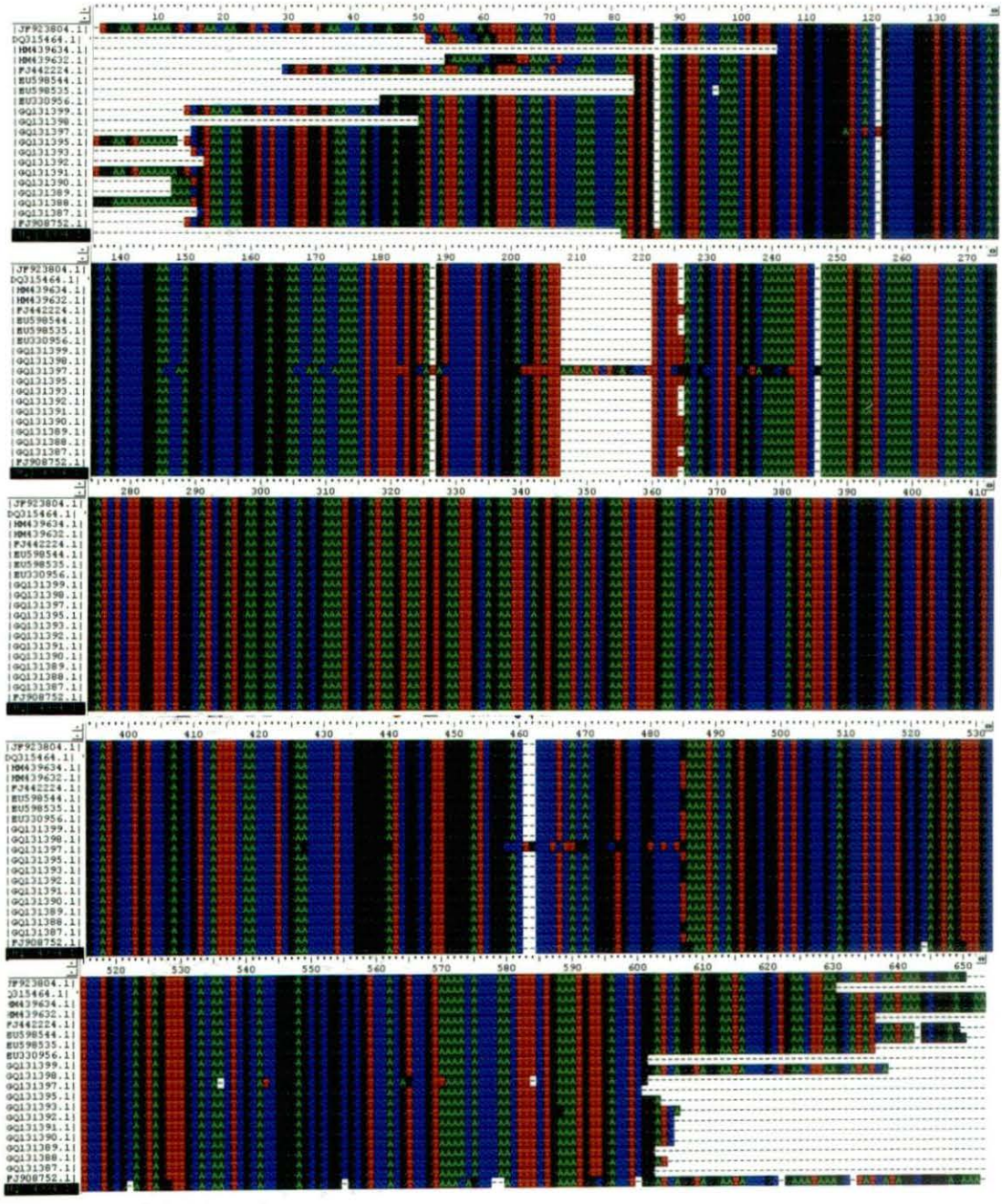


Figure 6: 18S rDNA sequence alignments of *Trichoderma asperellum* (RHS/M517). Data for other species were gathered from NCBI. The conserved regions of the gene are demonstrated in the different colour.

used to infer the phylogenetic tree (Fig 7 & 8). The evolutionary distances were computed using the Kimura 2-parameter method 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 189 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. An additional standard error test was performed with the data set using the same characters in order to evaluate the statistical confidence of the inferred phylogeny.

Table 21 : **Identified *Trichoderma harzianum* and comparison with referred NCBI GenBank**

Accession No.	Strain No	rDNA Sequence	Country	Organisms
JF923804,	NRCfBA-40	628 bp	India	*Trichoderma asperellum*
HQ334994,	**RHS/M 517**	**544 bp**	**India**	***Trichoderma asperellum***
HM439634,	IARI Mycology PP 15	528 bp	India	*Trichoderma asperellum*
HM439632,	IARI Mycology PP 13	578 bp	India	*Trichoderma asperellum*
FJ442224,	GJS 91-162	587 bp	India	*Trichoderma asperellum*
EU598544,	CNRA 361	544 bp	Africa	*Trichoderma asperellum*
EU598535,	CNRA 338	544 bp	Africa	*Trichoderma asperellum*
EU330956,	GJS 90-7	572 bp	USA	*Trichoderma asperellum*
GQ131399,	D24	567 bp	China	*Trichoderma asperellum*
GQ131398,	D21	568 bp	China	*Trichoderma asperellum*
GQ131397,	D19	583 bp	China	*Trichoderma asperellum*
GQ131395,	D3	579 bp	China	*Trichoderma asperellum*
GQ131393,	B51	567 bp	China	*Trichoderma asperellum*
GQ131392,	B10	568 bp	China	*Trichoderma asperellum*

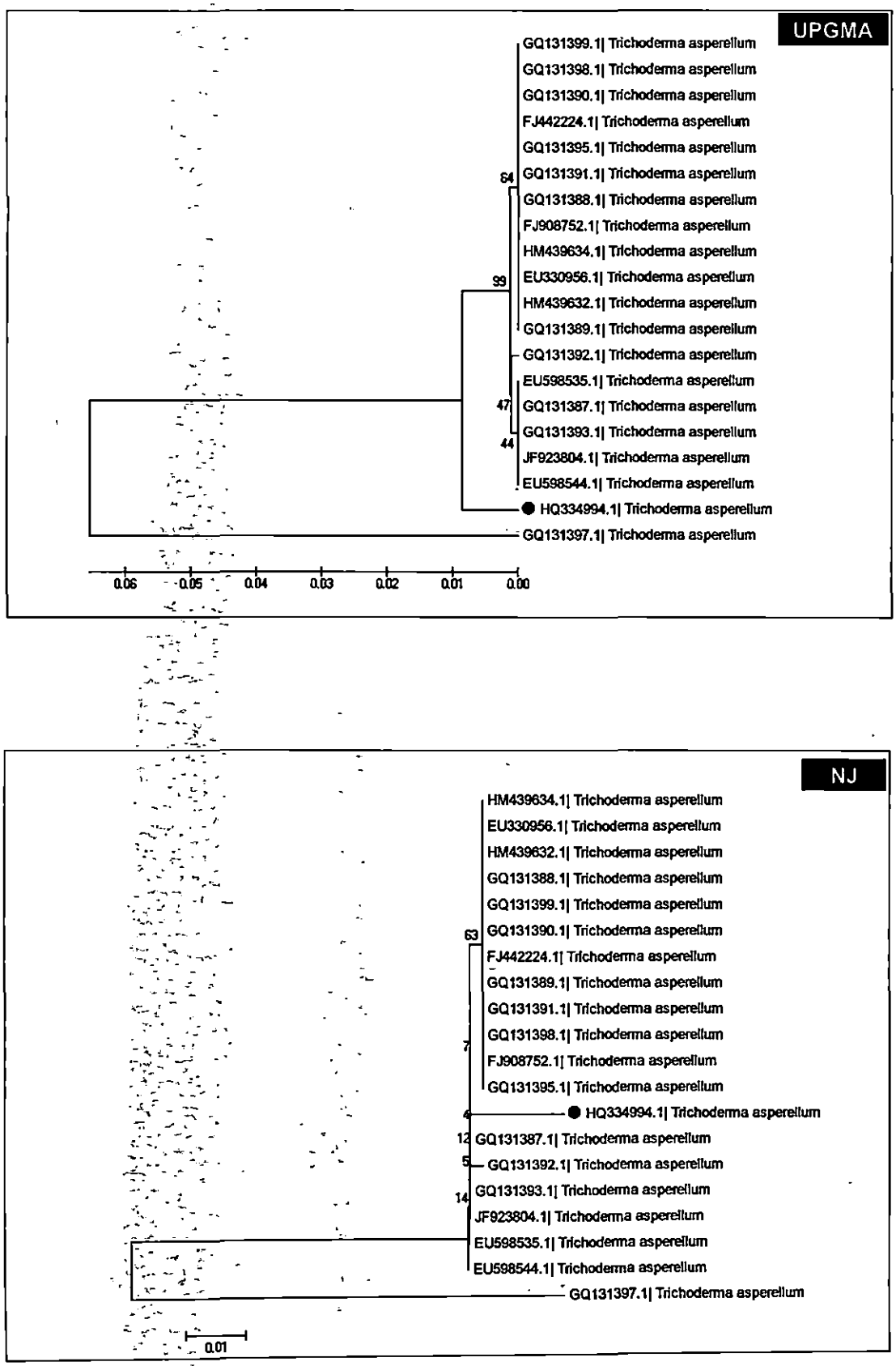


Figure 7: Phenogram of *Trichoderma asperellum* (RHS/M517) by UPGMA and NJ method

Accession No.	Strain No	rDNA Sequence	Country	Organisms
GQ131391,	A60	584 bp	China	*Trichoderma asperellum*
GQ131390,	A51	573 bp	China	*Trichoderma asperellum*
GQ131389,	A44	573 bp	China	*Trichoderma asperellum*
GQ131388,	A15	582 bp	China	*Trichoderma asperellum*
GQ131387,	A4	567 bp	China	*Trichoderma asperellum*
FJ908752	D20	568 bp	China	*Trichoderma asperellum*
HQ647325	F1002	560 bp	China	*Trichoderma harzianum*
HQ845040	T-14	582 bp	China	*Trichoderma harzianum*
JF923807	NRCfBA-46	636 bp	India	*Trichoderma harzianum*
JF923806	NRCfBA-45	643 bp	India	*Trichoderma harzianum*
JF923805	NRCfBA-44	642 bp	India	*Trichoderma harzianum*
JF923802	NRCfBA-37	642 bp	India	*Trichoderma harzianum*
JF923801	NRCfBA-31	624 bp	India	*Trichoderma harzianum*
JF923799	NRCfBA-25	637 bp	India	*Trichoderma harzianum*
JF923798	JF923798	628 bp	India	*Trichoderma harzianum*
HQ637340	JZ-179	593 bp	China	*Trichoderma harzianum*
HQ637339	JZ-77	580 bp	China	*Trichoderma harzianum*
EU718085	DMC 794b	593 bp	Germany	*Trichoderma harzianum*
GU048860	S17TH	976 bp	India	*Trichoderma viride*
|GU048859	GITXKohli-2	634 bp	India	*Trichoderma jecorina*
GU048858	GITXKohli-1	672 bp	India	*Trichoderma jecorina*
GU048857.	GITXPanog-I	898 bp	India	*Trichoderma longibrachiatum*
GU048856	GITXPanog-C	644 bp	India	*Trichoderma saturnisporum*
GU048855	S17TH	803 bp	India	*Trichoderma harzianum*
FJ442681	DIS 110A	598 bp	USA	*Trichoderma harzianum*
FJ442679	GJS 05-107	599	Italy	*Trichoderma harzianum*

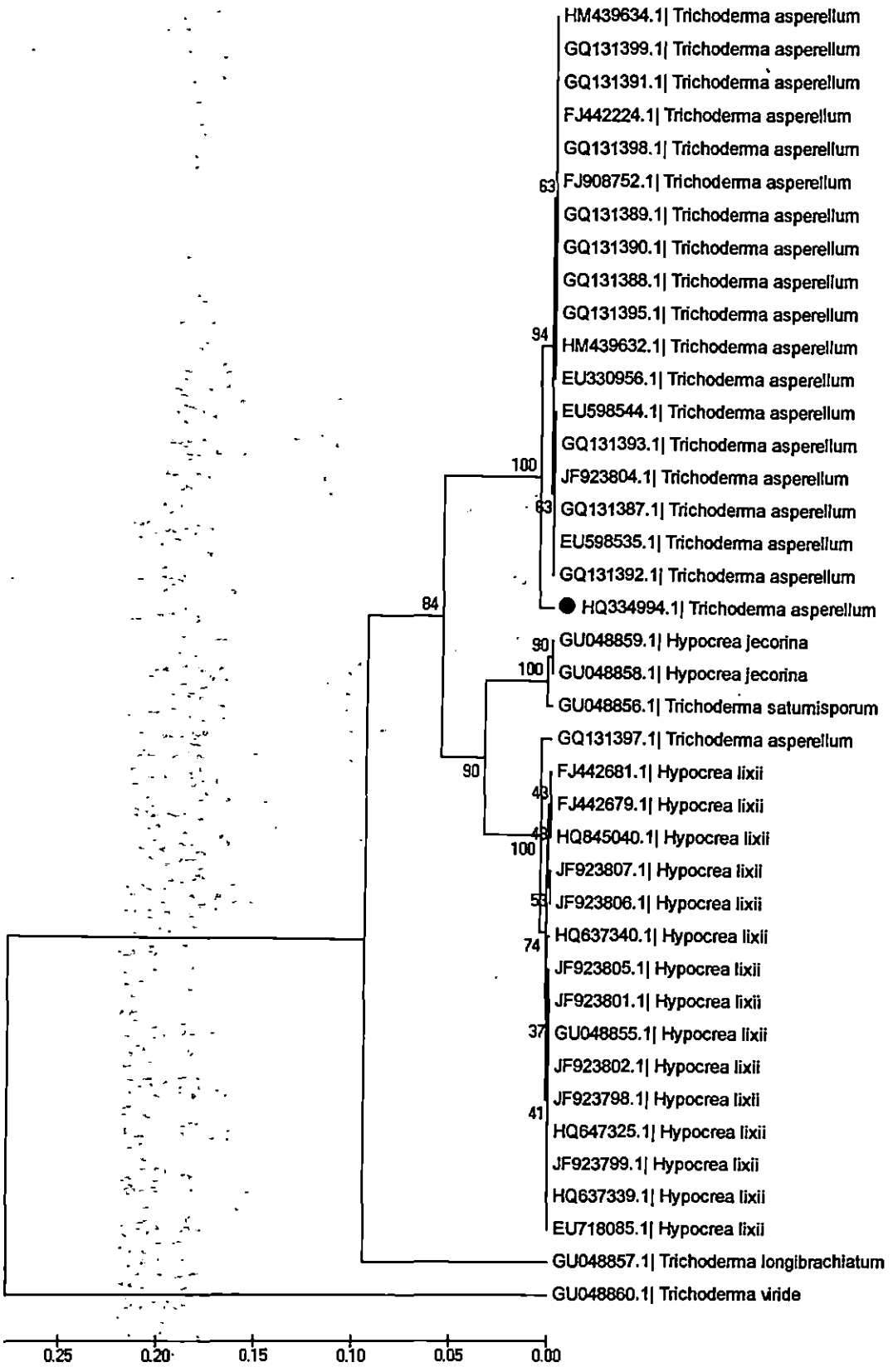


Figure 8: Phylogenetic placement of *Trichoderma asperellum* (RHS/M517) with extype strains from NCBI genebank

4. 11 Morphological and molecular identification of a potential PGPR isolate

4.11.1. Scanning electron microscopy

Isolate of *Bacillus pumilus* - B/RHS/C1(Plate24, fig.A) which showed *in vitro* antagonistic activity against *Macrophomina phaseolina, Rhizoctonia solani, Fusarium solani, Fusarium oxysporum* and *Fusarium graminearum* were selected as potential PGPR isolate for SEM study. Microscopic observation under bright field of this bacterial isolate has been presented in Plate 24 (fig.B). Scanning electron microscopic observations of *B. pumilus* isolate-B/RHS/C1 has been presented in Plate 24 (fig.C).

4.11.2. 16S rDNA sequencing

Genomic DNA was prepared from *B. pumilus* (B/RHS/C1) using lysis buffer (100mM Tris Hcl, pH 7.5, 20mM EDTA, 250mM NaCl, 2% SDS, 1mg/ml lysozyme) followed by RNase and proteinase K treatments. The lysate was extracted with equal volume of tris water saturated phenol: chloroform: isoamylalcohol (25:24:1) and then centrifuged at 10,000 rpm for 5 min. The aqueous phase was collected in clean tube, chilled with absolute ethanol, centrifuged and the pellet was air dried and finally dissolved in 40µl TE buffer and stored at 4°C. The yield of DNA was determined spectrophotometrically (A_{260}/A_{280} ratio) and the purity of DNA sample was evaluated by 0.8% agarose gel electrophoresis which produced clear sharp bands, indicating good quality of DNA. Genomic DNA of *Bacillus pumilus* (B/RHS/C1) was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. For amplification of the rDNA region of *B. pumilus* isolate B/RHS/C1) the primer pairs 5'AGAGTRTGTCMTYGCTWAC 3' and 5'CGYTAMCTTWTTWTTACGGRCT 3' were used.

PCR was programmed with an initial denaturing at 94°C for 5 min. followed by 35 cycles of denaturation at 94 °C for 30 sec, annealing at 55 °C for 30 sec and extension at 70 °C for 2 min and the final extension at 72 °C for 5 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1

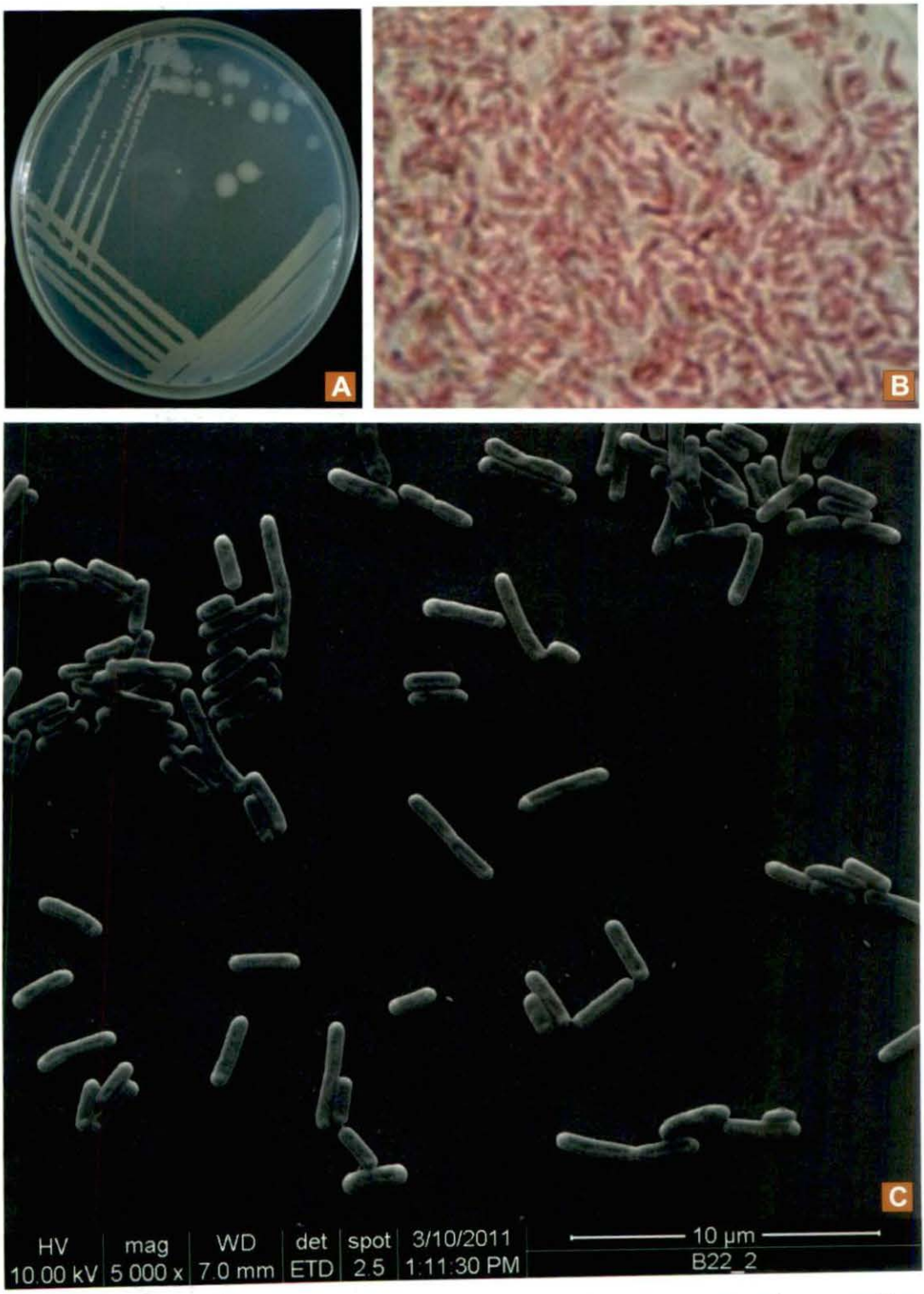
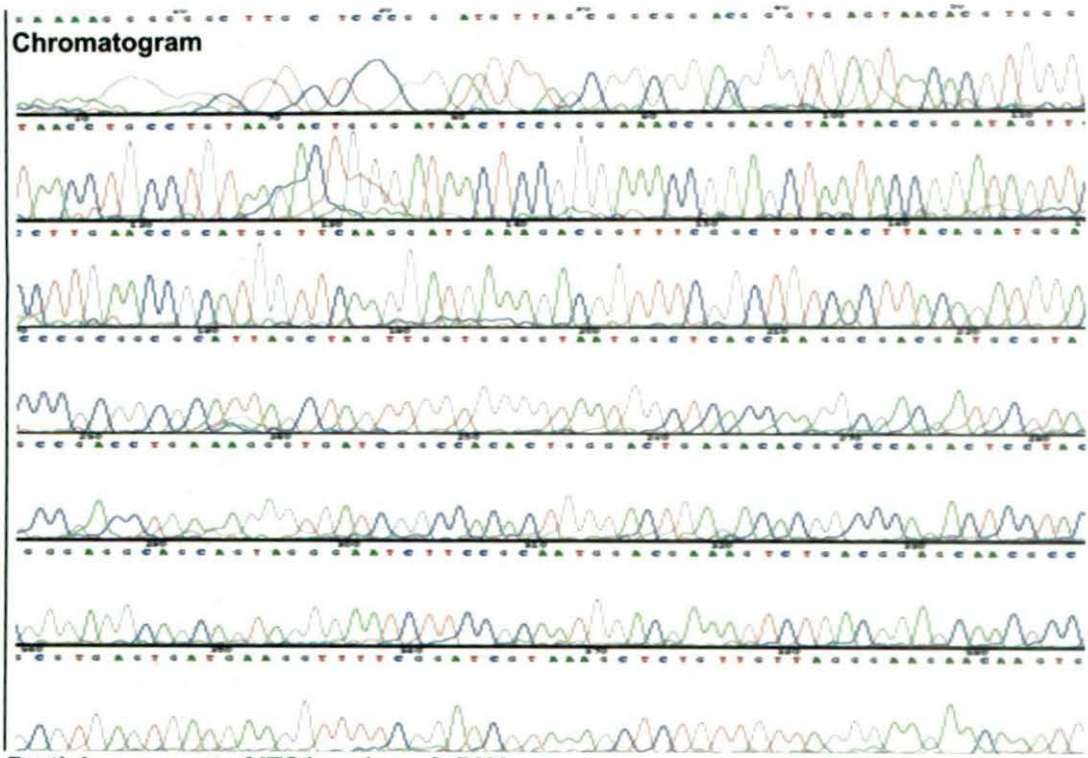


Plate 24 (figs. A-C): Phosphate solubilising bacterium *Bacillus pumilus*. Streaking NA medium [A]. Microscopic views after Gram staining [B] and Scanning Electron Microscopic view [C].

% ethidium bromide for examining by with horizontal electrophoresis. Region of rDNA was amplified using genus specific primers. Amplified products of size in the range of 1.3kbp was produced by the primers. A single distinct DNA bands was observered on the gel. The purified PCR product of *B. pumilus* (B/RHS/C1) was sequenced bidirectional primer walking method in Applied Biosystems by Chromous Biotech, Bangalore. Partial sequence of rDNA region of above mentioned bacterial isolate of *B. pumilus* has been presented in Figure 9.

4.11.3. Analyses of rDNA gene sequences

Identified *B. pumilus* rDNA gene sequences obtained from NCBI genebank (Table 22) of various host plants were selected for comparison with the rDNA gene sequence of *B. pumilus* , isolate B/RHS/C1 of mandarin plant. The sequence alignment of the isolate of *B. pumilus* (B/RHS/C1) shows variation in this gene . These available sequences of *B. pumilus* from NCBI were used in the pair wise and multiple sequence alignment using Bioedit software (Fig.10) for determining the conserved regions of rDNA gene. The evolutionary history was inferred using the UPGMA method . The optimal tree with the sum of branch length = 0.10748173 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. 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 (Fig.11). The evolutionary distances were computed using the Maximum Composite Likelihood method 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 385 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4. An additional standard error test was performed with the data set using the same characters in order to evaluate the statistical confidence of the inferred phylogeny.



Partial sequence of ITS1 region of rDNA

GAAAAGGGGGGCTTGCTCCCGGATGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGC
CTGTAAGACTGGGATAACTCCGGGAAACCGGAGCTAATACCGGATAGTTCCTTGAACCGCATG
GTTCAAGGATGAAAGACGGTTTCGGCTGTCACTTACAGATGGACCCGCGGCGCATTAGCTAGTT
GGTGGGGTAATGGCTCACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACAC
TGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC
GAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCGTAAAGCTCTGTTGTT
AGGGAAGAACAAGTGCGAGAGTAACTGCTCGCACCTTGACGGTACCTAACCAGAAAGCCACGG
CTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCG
TAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCTACCGGGGAGGGTC
ATTGGAAACTGGGAAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAAT
GCGTAGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAG
GAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAG
TGCTAAGTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGG
GGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCGGTGGAGC
ATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGTCTTGACATCCTCTGACAACCCTA
GAGATAGGGCTTTCCCTTCGGGGACAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTC
GTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCAGCATTTAGTT
GGGCACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATCATCA
TGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACAGAACAAAGGGCTGCGAGACCGC
AAGGTTTAGCCAATCCCATAAATCTGTTCTCATTTCGATCGCAGTCTGCAATCGACTGCGTGAA
GCTGGAACGCTAGAATCGCGAACAGCATGCCGCGGTGATTACGTTCCGGGCCTGTCCCCACGCC
GTACACCCGAAAGTTTGTACCCCGAGTCGGTAGTGACCTTTTGACTCCCCCCAAC

Sequences producing significant alignments:

Accession	Description	Max score	Total score	Query coverage	E value	Max ident
AB576891.1	Bacillus sp. CSL09-1 gene for 16S rRNA, partial sequence	2396	2396	98%	0.0	98%
EU308307.1	Bacillus sp. F1A07 16S ribosomal RNA gene, partial sequence	2396	2396	98%	0.0	98%
EU869263.1	Bacillus pumilus strain BG-B79 16S ribosomal RNA (rrs) gene, partial seque	2396	2396	98%	0.0	98%
EU379253.1	Bacillus pumilus strain 1RN-3E 16S ribosomal RNA gene, partial sequence	2396	2396	98%	0.0	98%
EU379249.1	Bacillus pumilus strain 1RN-3B 16S ribosomal RNA gene, partial sequence	2396	2396	98%	0.0	98%
CP000813.1	Bacillus pumilus SAFR-032, complete genome	2396	1.677e+04	98%	0.0	98%
AM237370.1	Bacillus pumilus partial 16S rRNA gene, isolate OS-70	2396	2396	98%	0.0	98%
AY690701.1	Bacillus sp. MH07 16S ribosomal RNA gene, partial sequence	2396	2396	98%	0.0	98%
AY690700.1	Bacillus sp. MH06 16S ribosomal RNA gene, partial sequence	2396	2396	98%	0.0	98%
AY167862.1	Bacillus pumilus strain SAFN-034 16S ribosomal RNA gene, partial sequenc	2396	2396	98%	0.0	98%
AB020208.1	Bacillus pumilus gene for 16S ribosomal RNA, strain: OM-F6	2396	2396	98%	0.0	98%
AB374310.1	Bacillus sp. RV103 gene for 16S rRNA, partial sequence	2392	2392	97%	0.0	98%
FJ237280.1	Bacillus pumilus strain XJAS-ZB-14 16S ribosomal RNA gene, partial seque	2392	2392	98%	0.0	98%
FJ237277.1	Bacillus pumilus strain XJAS-ZB-28 16S ribosomal RNA gene, partial seque	2392	2392	98%	0.0	98%
DQ180948.1	Bacillus sp. MI-23a1 16S ribosomal RNA gene, partial sequence	2392	2392	97%	0.0	98%
HQ891969.1	Bacillus sp. MM110(2011) 16S ribosomal RNA gene, partial sequence	2390	2390	98%	0.0	98%
GU968462.1	Bacillus sp. RS114(2010) 16S ribosomal RNA gene, partial sequence	2390	2390	97%	0.0	98%
HM585071.1	Bacillus pumilus strain AUEC29 16S ribosomal RNA gene, partial sequence	2390	2390	98%	0.0	98%
HM585070.1	Bacillus pumilus strain AUEM12 16S ribosomal RNA gene, partial sequence	2390	2390	98%	0.0	98%
HM585069.1	Bacillus pumilus strain AUEM104 16S ribosomal RNA gene, partial sequenc	2390	2390	98%	0.0	98%
HM585067.1	Bacillus pumilus strain AUES82 16S ribosomal RNA gene, partial sequence	2390	2390	98%	0.0	97%

Figure 9: Chromatogram and 18S rDNA sequence of *Bacillus pumilus* (B/RHS/C1) and significant alignments by BLAST analysis

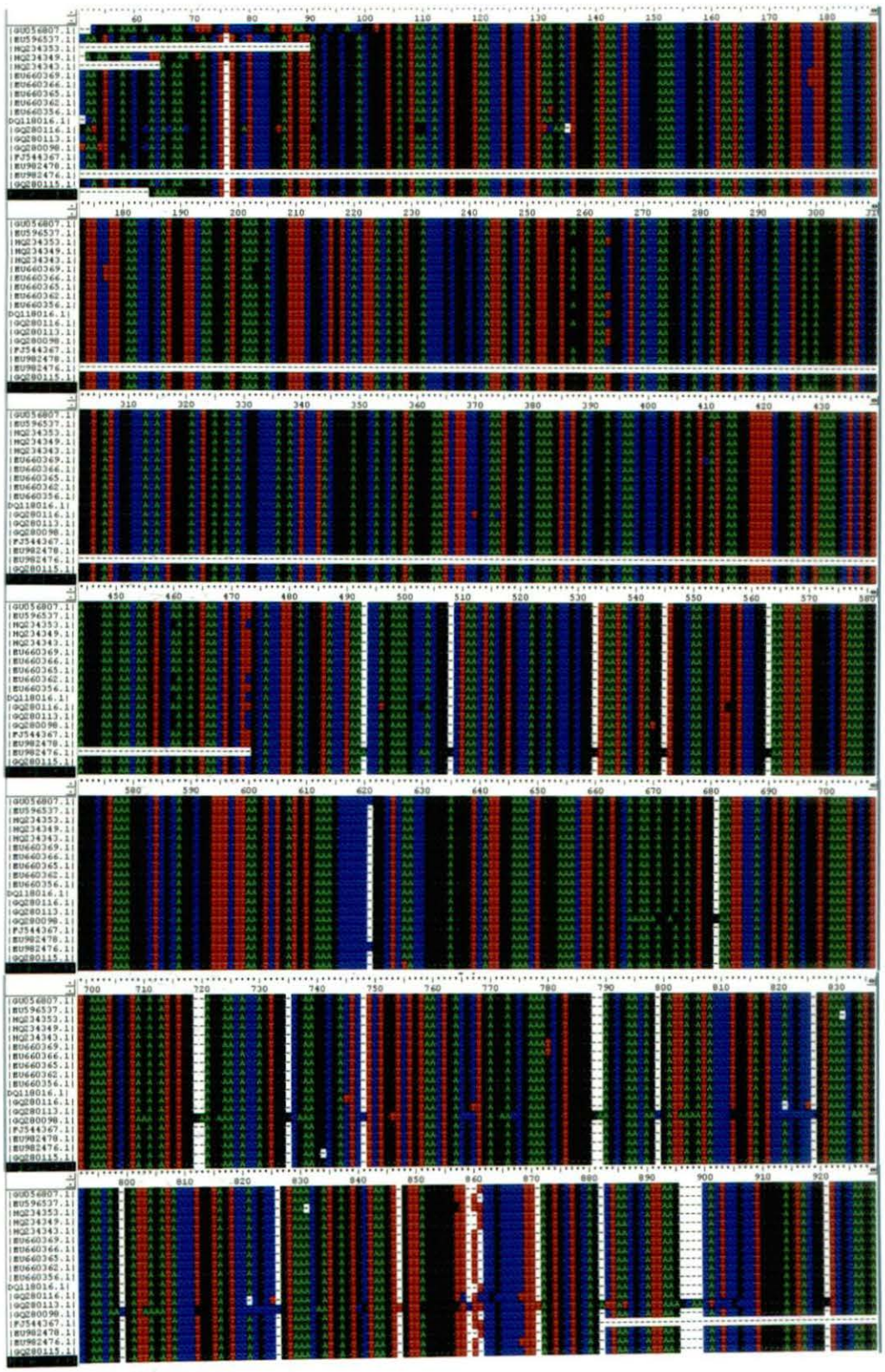


Figure 10: 18S rDNA sequence alignments of *Bacillus pumilus* (B/RHS/C1). Data for other species were gathered from NCBI. The conserved regions of the gene are demonstrated in the different colour.

Table 22: Identified *Bacillus pumilus* and comparison with referred NCBI GenBank sequences

Accession No.	Strain No	rDNA Sequence	Country	Organisms
GU056807	SRS-3	1470 bp	India	*Bacillus pumilus*
EU596537	HN005	1457 bp	China	*Bacillus pumilus*
HQ234353	AK39885	1337 bp	India	*Bacillus pumilus*
HQ234349	AK39674	1385 bp	India	*Bacillus pumilus*
HQ234343	AK39651	1385 bp	India	*Bacillus pumilus*
EU660369	CT19	1505 bp	India	*Bacillus pumilus*
EU660366	CT14	1513 bp	India	*Bacillus pumilus*
EU660365	CT13	1511 bp	India	*Bacillus pumilus*
EU660362	CT10	1512 bp	India	*Bacillus pumilus*
EU660356	CT3	1512 bp	India	*Bacillus pumilus*
DQ118016	RGR7	1369 bp	India	*Bacillus pumilus*
GQ280116	JS-46	1413 bp	India	*Bacillus pumilus*
GQ280113	JS-43	1424 bp	India	*Bacillus pumilus*
GQ280098	JS-28	1445 bp	India	*Bacillus pumilus*
FJ544367	st9	1449 bp	China	*Bacillus pumilus*
EU982478	5	814 bp	China	*Bacillus pumilus*
EU982476	4-1-14-c	994 bp	China	*Bacillus pumilus*
GQ280115	JS-45	1424 bp	India	*Bacillus pumilus*

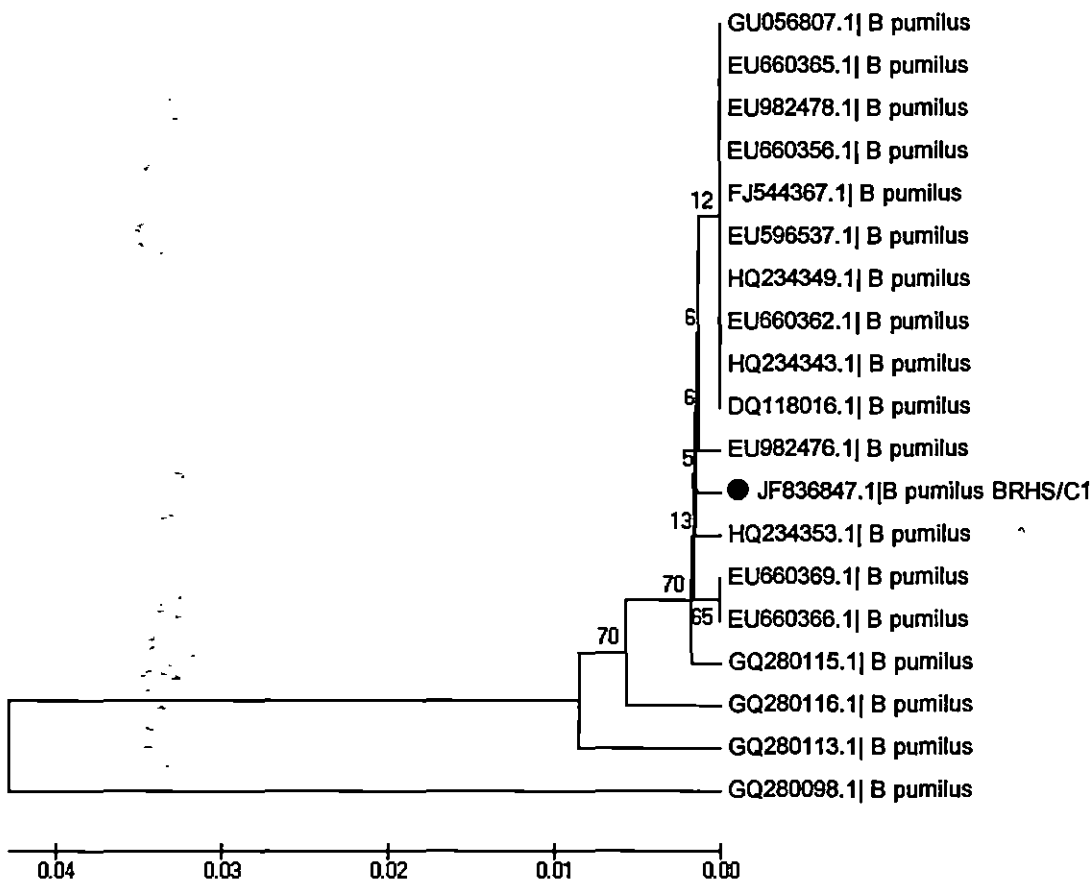


Figure 11 : Phylogenetic placement of *Bacillus pumilus* (B/RHS/C1) with extype strains from NCBI genebank

4.12. Effect of bioinoculants on suppression of charcoal rot disease of *Citrus reticulata*

Based on the screening of association of arbuscular mycorrhizal fungi (AMF) with mandarin roots in four locations of Darjeeling hills and percentage colonization behavior established by artificial inoculation of mandarin seedlings, predominant AMF *Glomus mosseae* and *Glomus fasciculatum* were selected for application in mandarin plants. Total phosphate content of soil was determined after application of *G. mosseae* and *G. fasciculatum* singly or jointly. Results revealed that soil P content had decreased due to application of AMF indicating that the plant could uptake phosphorus which had been solubilized by AMF (Table 23).

Table 23 . Soil phosphate content in rhizosphere of mandarin plants following root colonization with *G. mosseae* and *G. fasciculatum*

Treatment	Soil phosphate (µg/g tissue)
Control	48.25^{a}±1.12
G. mosseae	31.12^{b}±0.57
G.fasciculatum	35.25^{b}±2.10
G. mosseae + *G.fasciculatum*	30.75^{c}±1.23

Average of 3 replicates
±= Standard Error
Difference between values significant at P=0.01where superscript is different; not significant where superscript is same

Glomus mosseae was further selected as one of the important bioinoculants in this experiment. for mass multiplication in sorghum and maize seedlings (Plate 25, figs B&C), and root colonization was confirmed after three weeks (Plate 25, figs D&E).In order to develop pure cell line culture of *G. mosseae* experimental setup was made using sterilized pre-soaked sorghum seeds in sterile petriplate. After isolation of spores from the soil by wet sieving and decanting process, *G. mosseae* was carefully selected and placed in sorghum roots which were grown in aseptical conditions (Plate 25, fig.A). The same experiment was done in 3 months old mandarin seedlings. The seeds were germinated in aseptical conditions. *G. mosseae* was carefully selected and inoculated in the root of the mandarin plant and grown in

Plate 25 (figs. A-E): Experimental setup for raising single spore in sorghum seedlings[A]; mass multiplication of AMF spores in *Sorghum vulgare* [B] and *Zea mays* [C]; root colonization of sorghum grown in sterile soil [D] and maize [E] with AMF (*G. mosseae*).

pots having sterile sand. Establishment of the AM spore was confirmed a month after inoculation (Plate 26,figs.A-E). 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 (Plate 27).

The present work was aimed at developing a management strategy to control root rot of mandarin plants by biological means. Antibiosis to *M. phaseolina* by biocontrol agent (*Trichoderma asperellum*) was evaluated in *vitro* and *in vivo* condition. The application of *T. asperellum* to the soil as a biocontrol agent, in glasshouse conditions, not only resulted in reduced disease severity but also enhanced plant growth (Table 24). Joint inoculation with both the microorganisms (G. *mosseae* and *T. asperellum*) gave most significant results (Plate 28, fig.A-D). Marked reduction in disease development was evident following dual inoculations of G. *mosseae* and *T. asperellum* (Table 25)

Table 24 : Effect of application of *G. mosseae* and *T. asperellum* on growth of citrus seedlings: % increase in height and leaves no. After 1 and 2 months. (* mo- month)

Treatments	% increase in height		% increase in leaves no.	
	1 mo*	2 mo*	1 mo*	2 mo*
Control	8.5	12.5	21.0	36.0
T.asperellum	16.0	22.0	40.0	47.0
G.mosseae	21.0	28.5	38.0	55.0
T.asperellum+G.mosseae	35.0	41.0	69.0	80.0

Table 25 : Effect of application of *G. mosseae* and *T. asperellum* on development of root rot of *Citrus reticulata*

Treatments	**Root Rot Index** (Days after inoculation)		
	15	30	45
M. phaseolina	2.0	3.2	5.8
M. phaseolina+T. asperellum	0.5	1.0	2.6
M. phaseolina+G. mosseae	0.9	2.1	3.0
M.phaseolina+T.asperellum+G.mosseae	0.3	0.9	1.8

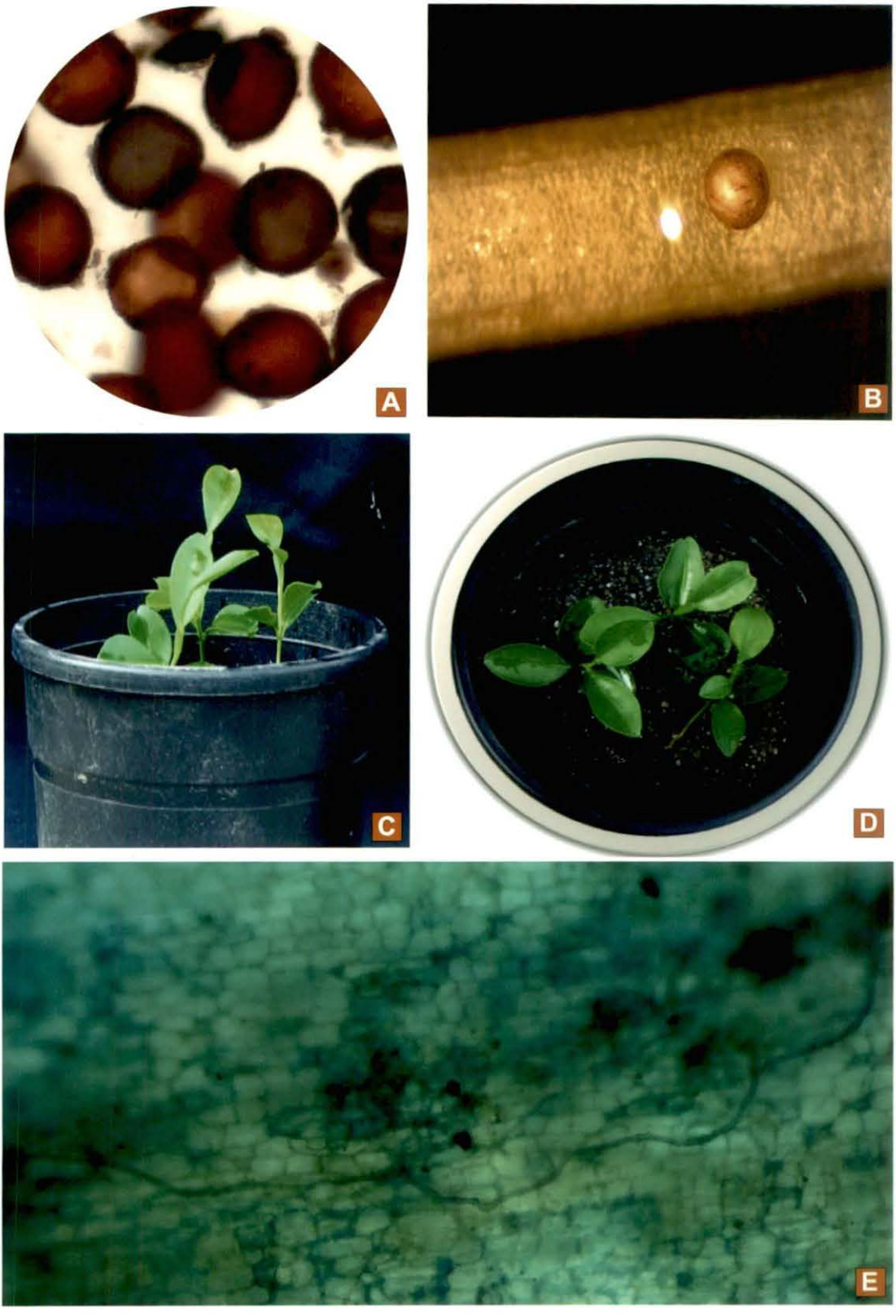


Plate 26 (figs.A-E): *Glomus mosseae* spores mass multiplied in maize roots [A]. Single spore inoculation of *G. mosseae* in mandarin root [B]. Single spore inoculated mandarin seedlings grown in sterile sand [C & D] and maintained in glass house. Mycorrhizal association observed in mandarin roots after 30 days of inoculation [E].

Plate 27 (figs. A): Joint inoculation of mandarin seedlings with AMF (*G. mosseae*) and PGPR (*B. pumilus*) and maintained in nurser .

Plate 28 (figs. A-D): Effect of *Glomus mosseae, Trichoderma asperellum* and coinoculation of *G. mosseae* and *T. asperellum* [A], *B. pumilus* alone and in combination with *T. asperellum* [B], on growth of 2 yr old potted mandarin plant and inoculated with *M. phaseolina* [C & D].

In vitro studies also confirmed PGPR activities of *Bacillus pumilus*. *B.pumilus* which produced a clear halo zone in Pikovskaya's medium indicating that it could solubilize phosphate. Similarly, production of siderophore as well as secretion of IAA into the medium were confirmed. Besides, significant inhibition of growth of root pathogens was also observed. *In vivo* application of *B.pumilus* to the rhizosphere of two year old mandarin plants grown in earthenware pots in the glass house condition resulted in an increase in growth in terms of increase in height, leaf number and number of branches (Plate 29figs.A&B) . Increase of height in seedlings was evident even one months after application. Application of *B. pumilus* and *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 gave most significant results (Figure 12). Total phosphate content of soil was determined after application of the microorganisms. Results revealed that soil P content had decreased due to application indicating that the plant could uptake phosphorus which had been solubilized by the microorganisms. Both acid and alkaline phosphatase activities in rhizosphere soil of mandarin plants were enhanced following application of *B. pumilus*. In rhizosphere soil of untreated plants, alkaline phosphatase activity was higher than acid phosphatase, but after treatment with *B. pumilus*, acid phosphate showed a greater increment of activity. Rhizosphere of mandarin was inoculated by *B.pumilus* and *G.mosseae* prior to challenge inoculation with *M. phaseolina*. Development of root rot was determined after 15, 30 and 45 days of inoculation. *B. pumilus* and *G. mosseae* could reduce root rot, but maximum suppression of disease was due to joint inoculation (Figure 13).

Plate 29 (figs. A-B): Control [A] and *Bacillus pumilus* treated mandarin seedlings [B].

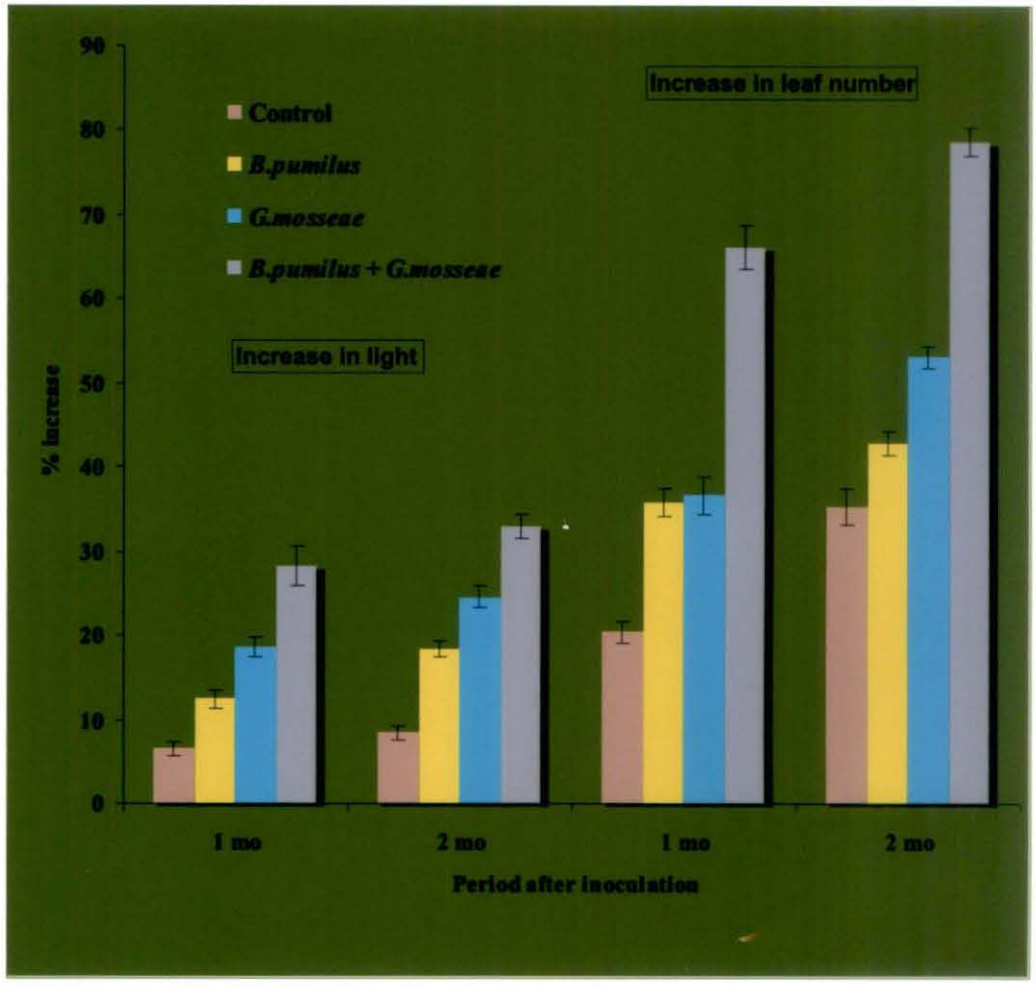


Figure 12: Effect of application of *B.pumilus* and *G.mosseae* on growth of citrus seedlings in terms of increase in height and number of leaves

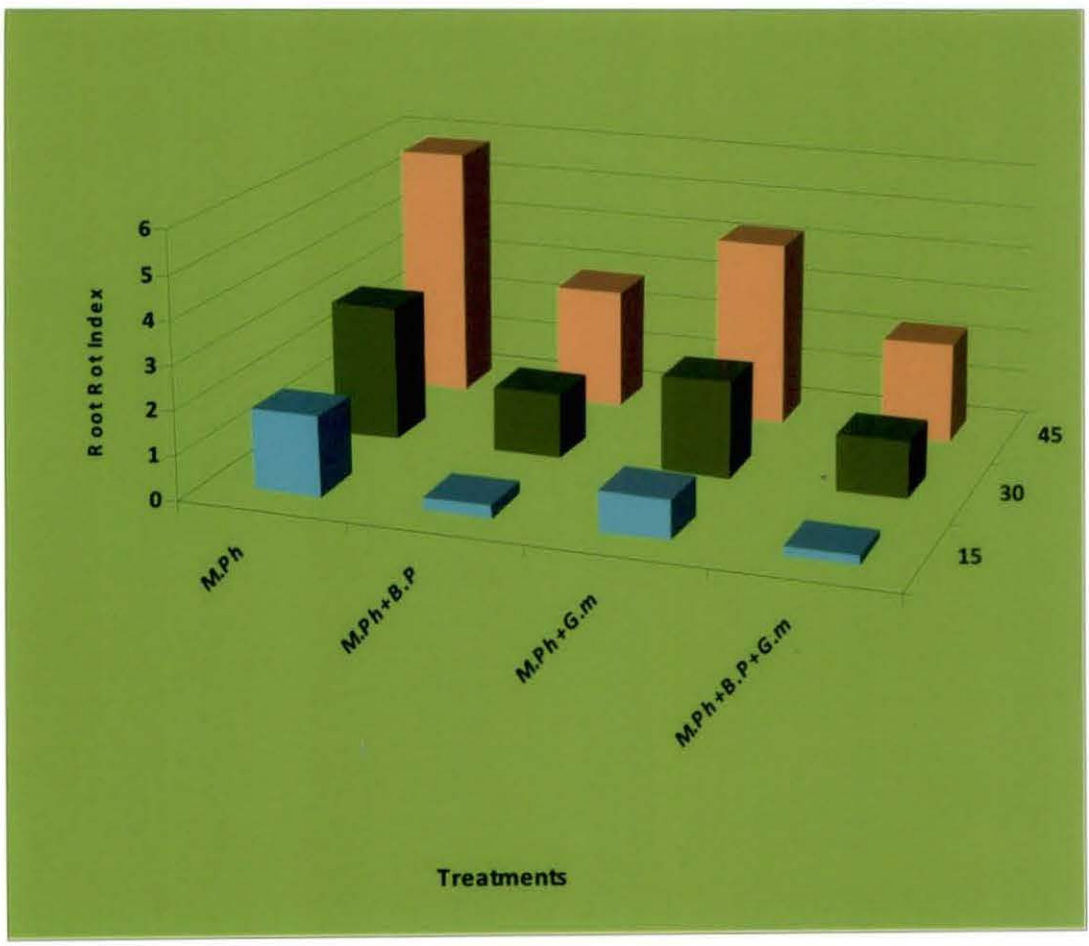


Figure 13: Influence of *B. pumilus* and *G. mosseae* on root rot caused by *M. phaseolina*

4.13. Activation of defense response of *Citrus reticulata* following application of bioinoculants against *M. phaseolina* and associated changes in defense enzymes

Defense responses of mandarin plants against root rot pathogen (*M. phaseolina*) were demonstrated during early stages of root colonization by *G. mosseae* and *T. asperellum* along with application of *B. pumilus.* Activities of 3 major defense enzymes- β 1,3 - glucanase, chitinase and peroxidase were assayed in roots and leaves and of mandarin seedlings subjected to various treatments- ie., *G. mosseae*, *B. pumilus*, *T. asperellum*, *M. phaseolina*, *B. pumilus* + *G. mosseae*, *B. pumilus* + *M. phaseolina*, *G. mosseae* +*M. phaseolina*, *T. asperellum*+ *G. mosseae*, *B. pumilus* + *G. mosseae* +*M. phaseolina*, *T. asperellum*+ *G. mosseae*+*M. phaseolina* . Activities of all 3 enzymes, in both leaves and roots, were significantly enhanced due to the various treatments (Table 26, 27, and 28; Figures 14,15 & 16). In most of the treatments, results were significantly higher than control at P=0.01 as tested by Student's't' test. Peroxidase activity was more than double in leaves compared to the roots, whereas chitinase was more or less similar.

Table 26 : Changes in chitinase activities in citrus seedlings following single as well as dual application of *T. asperellum* and *G. mosseae* following inoculation with *M. phaseolina*.

Treatments	Chitinase (µgGlc-Nac/min/gtissue)	
	Leaf	Root
Control	87	82
T.asperellum	91	85
G.mosseae	110	92
T.asperellum+G.mosseae	135	96
M. phaseolina	95	95
M.phaseolina+T.asperellum	104	100
M.phaseolina+G.mosseae	125	115
M.phaseolina+T.asperellum+G. mosseae	168	155

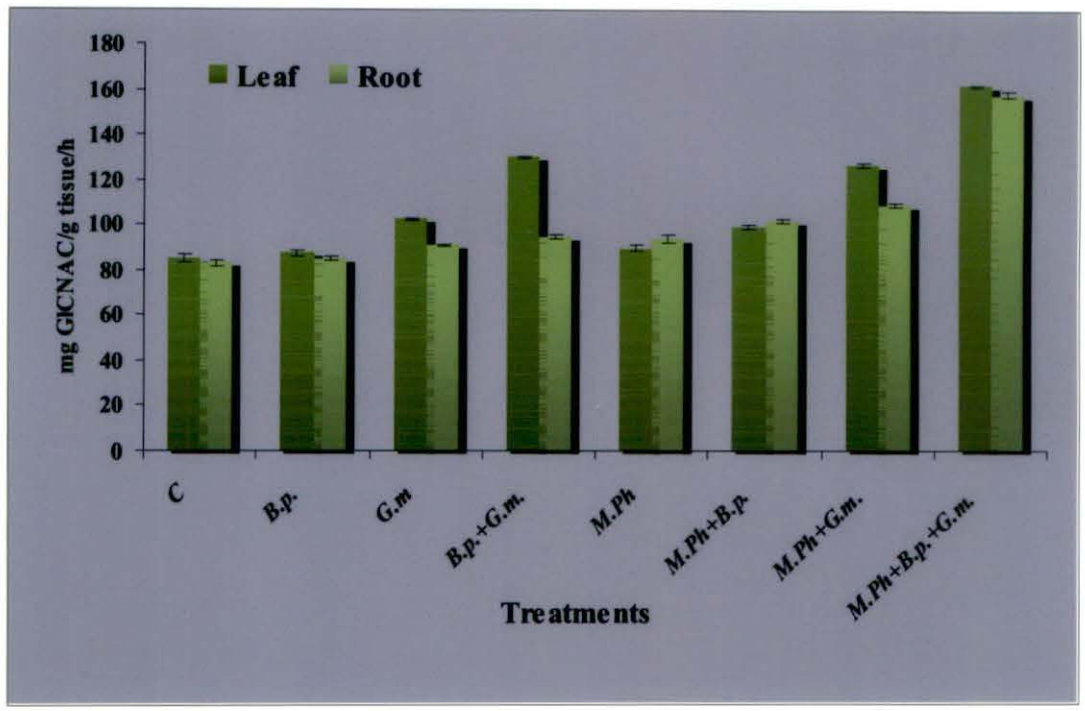


Figure 14: Activities of chitinase in roots and leaves of mandarin following application of *B. pumilus* and *G .mosseae* and inoculated with *M. phaseolina*

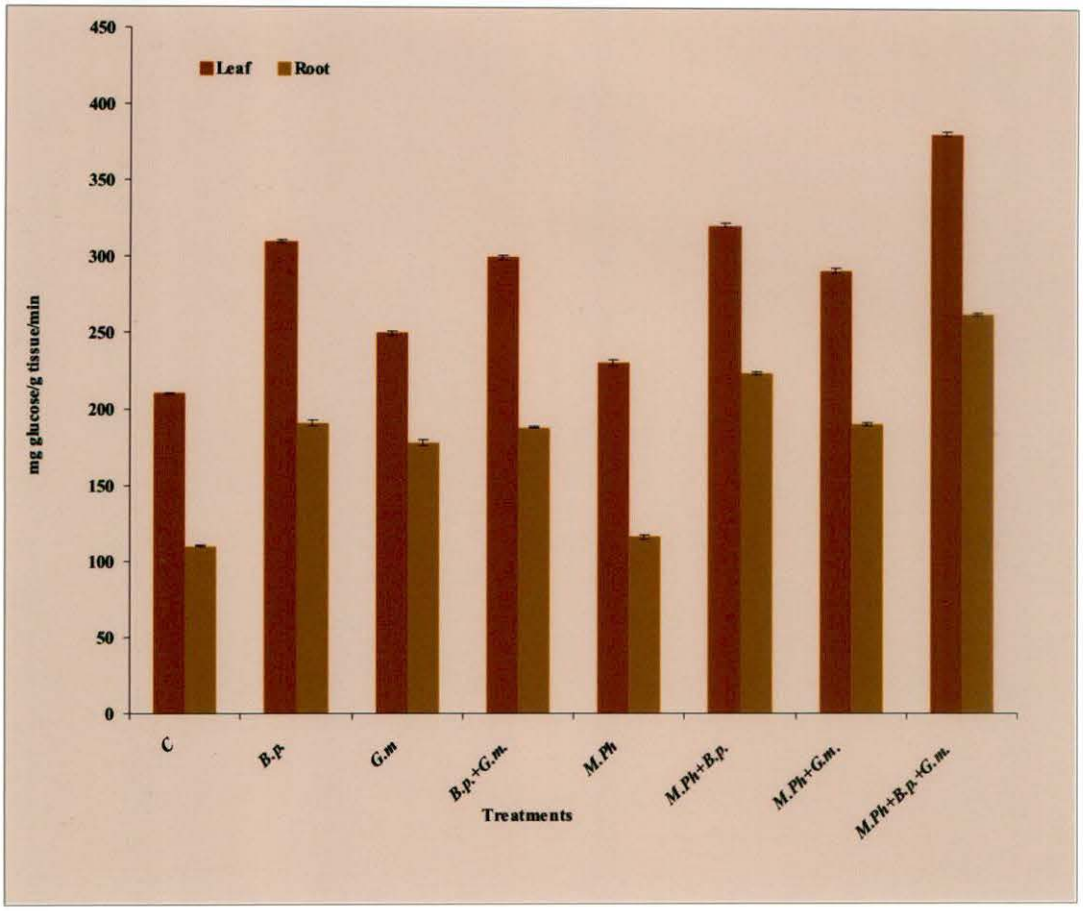


Figure 15: Activities of β1,3 glucanase in roots and leaves of mandarin following application of *B. pumilus* and *G. mosseae* and inoculated with *M. phaseolina*

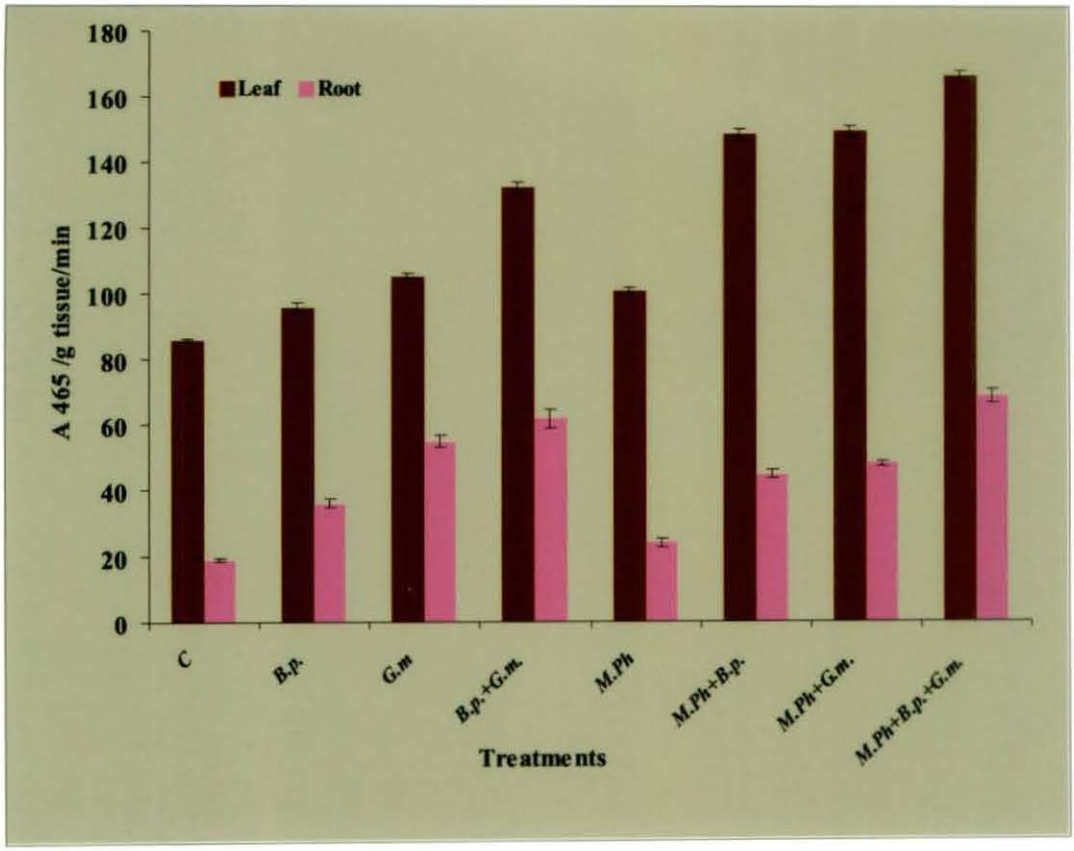


Figure 16: Activities of peroxidase in roots and leaves of mandarin following application of *B. pumilus* and *G. mosseae* and inoculated with *M. phaseolina*

Table 27: Effect on β-1,3 glucanase activities in citrus seedlings by application of *T. asperellum* and *G. mosseae* following inoculation with *M. phaseolina*

Treatments	β-1,3 glucanase (µg glucose/min/gtissue)	
	Leaf	Root
Control	211	115
T.asperellum	315	191
G.mosseae	255	181
T.asperellum+G.mosseae	303	189
M. phaseolina	230	118
M.phaseolina+T.asperellum	320	221
M.phaseolina+G.mosseae	296	193
M.phaseolina+T.asperellum+G. mosseae	387	260

Table 28: Effect of application of *T. asperellum* and *G. mosseae* on peroxidise activities of citrus seedland following inoculation with *M. phaseolina*

Treatments	Peroxidase (ΔA465/min/gtissue)	
	Leaf	Root
Control	88	27
T.asperellum	108	48
G.mosseae	114	68
T.asperellum+G.mosseae	138	72
M. phaseolina	107	39
M.phaseolina+T.asperellum	157	51
M.phaseolina+G.mosseae	159	55
M.phaseolina+T.asperellum+G. mosseae	170	72

Reduction of root rot in *Citrus reticulata* following application of bioinoculants (*G. mosseae*, *T. asperellum* and *B. pumilus*) were evident both singly or jointly. However, joint inoculation with both AMF (*G. mosseae*) and PGPR (*B. pumilus*) as well as AMF (*G. mosseae*) and BCA (*T. asperellum*) reduced disease markedly. These observed root rot reduction following separate and dual application of bioinoculants may be correlated with increased accumulation of defense enzymes such as β- 1, 3- glucanase (Fig.17A) chitinase (Fig.17B), and peroxidase (Fig.17C).

In order to confirm the induction of enhanced activities of defense enzymes due to treatment with *B.pumilus*, *G.mosseae* and *T. asperellum* or both, immunological tests were done using PAbs raised against two enzymes- chitinase and β 1,3 glucanase.

Enzyme extracts were used as antigens and PTA-ELISA and Dot immunobinding assays were carried out. Results (Table 29) revealed that ELISA values of reaction of PAbs of chitinase and β 1,3-glucanase with enzyme extracts from leaves of mandarin plants grown in treated soil were higher than the control values. Similarly, in Dot-Blot, more intense dots were observed in treated plants.

Table 29: . PTA-ELISA and Dot-Blot values of reactions between PAbs of defense enzymes and enzyme extracts from mandarin plants inoculated with *G. mosseae* and *T. asperellum* challenge inoculation with *M. phaseolina*

Antigen source*	PAb of chitinase		PAb of β 1,3-glucanase	
	A 405 ELISA	Colour intensity# Dot-Blot	A 405 ELISA	Colour intensity# Dot-Blot
Control	0.034	+	0.049	+
M.phaseolina inoculated	0.036	+	0.052	+
G.mosseae treated +*M.phaseolina* inoculated	0.520	+++	0.368	++
B.pumilus treated +*M.phaseolina* inoculated	0.426	++	0.345	++
T. asperellum treated +*M.phaseolina* inoculated	0.768	++	0.445	++
G.mosseae + *T.asperellum* treated +*M.phaseolina* inoculated	0.982	++++	0.865	+++
G.mosseae+ *B.pumilus* treated +*M.phaseolina* inoculated	0.954	++++	0.741	+++

* Enzyme extracts from leaves of plants treated as mentioned;
# Colour Intensity - += Light pink; ++ = Dark pink; +++ = Deep purplish

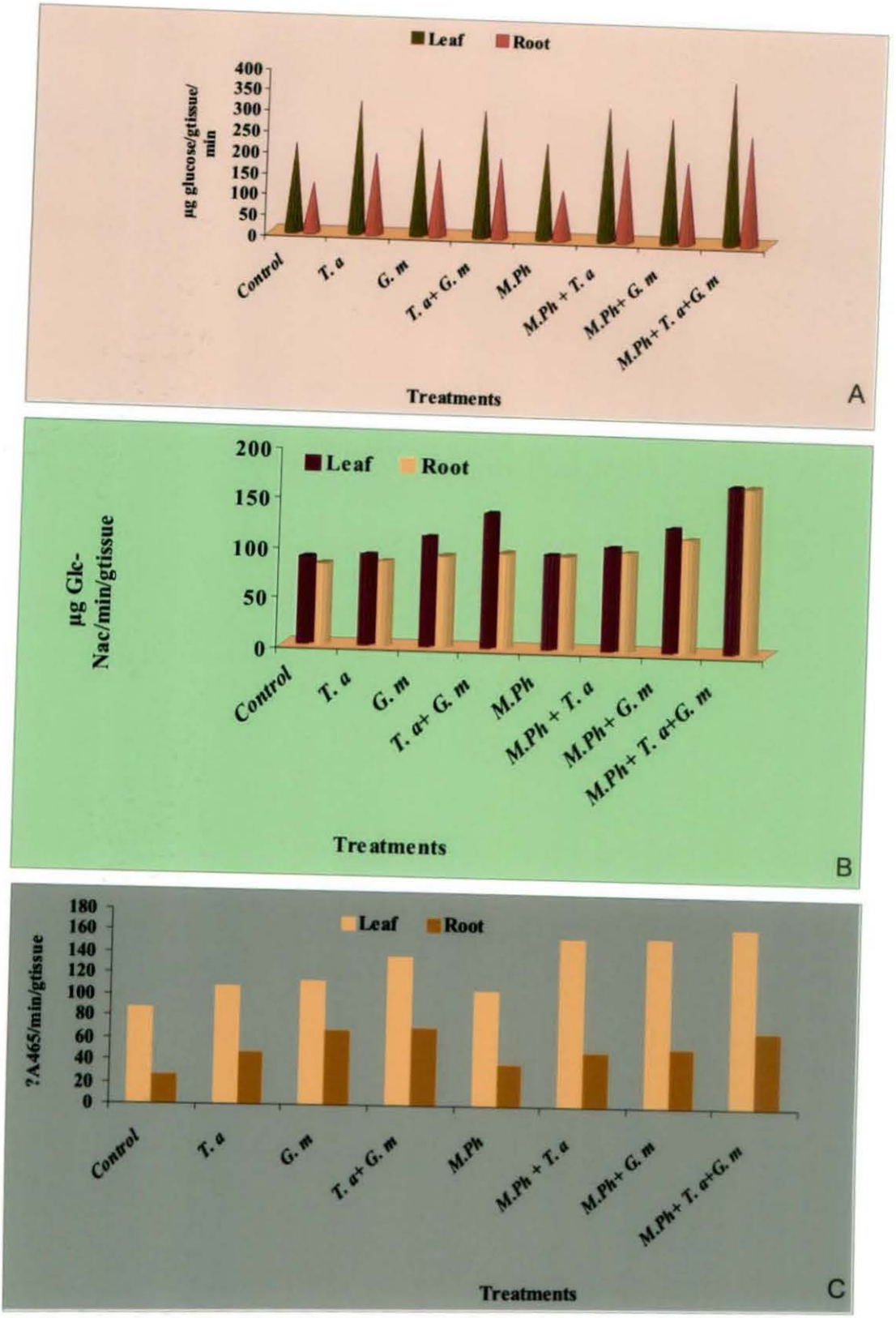


Figure 17: Changes in defense enzyme activities in citrus seedlings following single as well as dual application of *T. asperellum* and *G. mosseae* and challenged inoculation with *M. phaseolina*. (A- β-1,3 glucanase, B- chitinase, C- Peroxidase)

4.14 Cellular location of Chitinase in root and leaf tissues of *Citrus reticulata* following induction of resistance

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 assays. Accumulation of both the defense enzymes (β, 1.3- glucanase and chitinase) in leaf tissues were always higher than the root tissues as evident in PTA-ELISA reaction using PAbs of Chitinase and β, 1.3- Glucanase. Enhanced activities were noted in bioinoculant(s) treated (singly or jointly) mandarin plants following challenge inoculation with the pathogen (*M. phaseolina*)

Keeping this in mind root and leaf samples were collected from the bioinoculant(s) treated plants. Similarly root and leaf samples were collected from healthy plants for immunoflorescence study. Main objective was to localize chitinase (PR-3) at the cellular level in the root and leaf tissues of *Citrus reticulata* following induction of resistance.

Cross sections of the root and leaf tissues (Plate 30,fig.A&C) were treated separately with normal antiserum and PAb of Chitinase and labeled with FITC as mentioned in materials and methods. Leaf tissues exhibited a natural autoflorescence under UV- Light but was not characteristic of FITC fluorescence. Observations in the treatement with the normal antiserum was the same. When the cross sections of the untreated and treated root and leaf tissues were incubated with PAb- chitinase and labeled with FITC, fluorescence was observed in the treated root (Plate 30, fig.B) and leaf tissues (Plate 30, fig.D). Bright apple green fluorescence was evident in the epidermal and homogenously in the mesophyll tissues (Plate 30, fig.E-J). Similarly bright fluorescence was also observed in the cortical tissues of the treated root sections. So strong reaction with FITC in plant tissues gave indication of the induction of chitinase (PR-3) in *Cirtus reticulata* plants following induction of resistance with bioinoculants

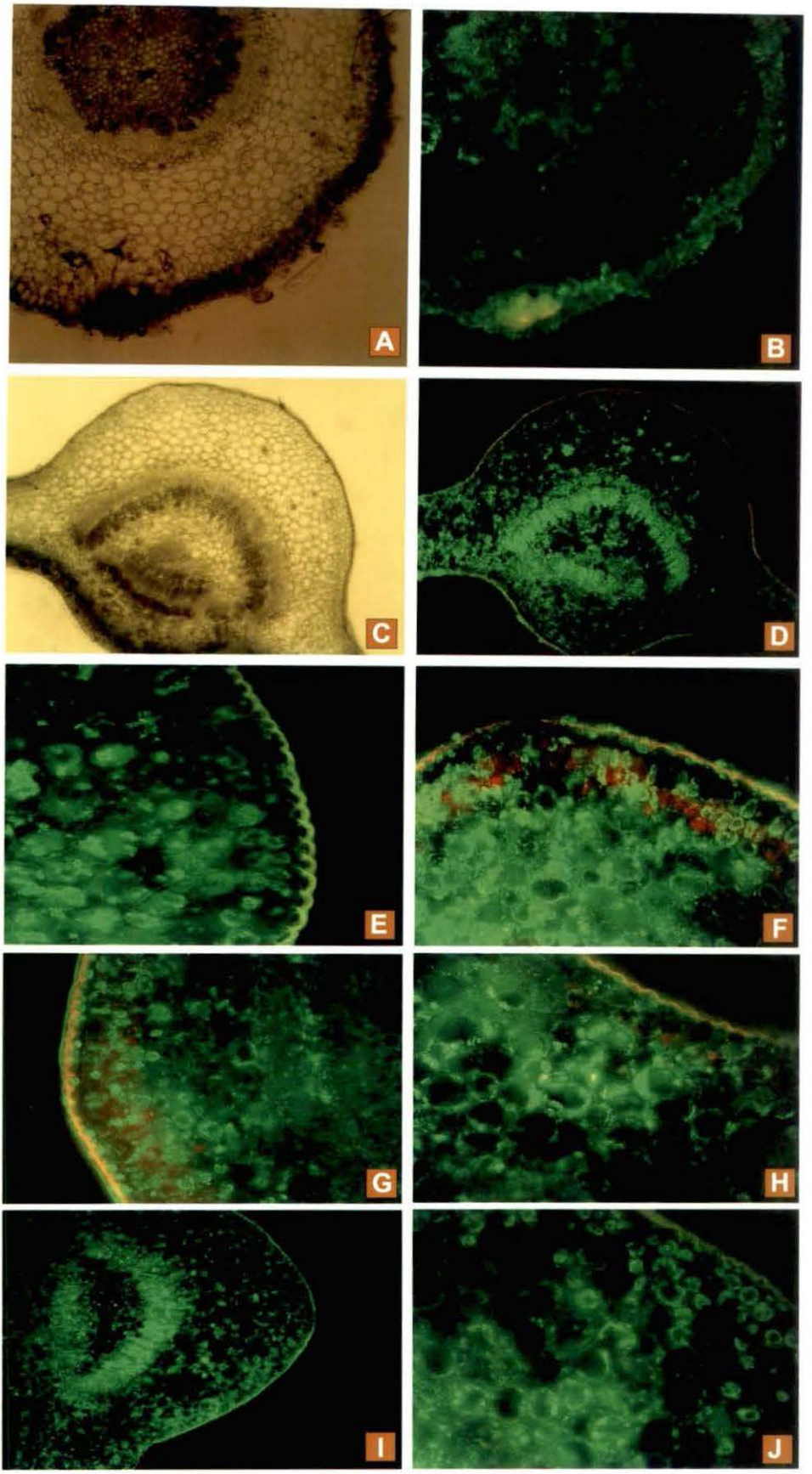


Plate 30 (figs. A-J): Cross section of mandarin root [A & B] and leaf [C-J] following inoculation with *G. mosseae*, and treated with *T. asperellum* and *B. pumilus* under bright field [A & C] and probed with PAb-chitinase and labeled with FITC conjugates.

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.

Summary

A review of literature has been presented to focus the microbial resources available in the rhizosphere of various plants with special references to the colonization of roots with Arbuscular Mycorrhizal fungi (AMF), as well as involvement of plant growth promoting Rhizobacteria (PGPR) for the improvement of plant health status. Materials used and methods followed have been presented in Materials and Methods.

Initially survey was conducted in Darjeeling Hills to record prevalent diseases of mandarin (*Citrus reticulata*) caused by pest and pathogens and an inventory was outlined. Among the root diseases caused by fungal pathogens, *Macrophomina phaseolina*, *Fusarium solani* and *Fusarium oxysporum* were mostly recorded in various mandarin orchards. One of the predominant root rot disease caused by *Macrophomina phaseolina* was selected for its management using bioinoculants available in the rhizosphere of mandarin plants.

Charcoal root rot pathogen (*Macrophomina phaseolina*) isolated from mandarin orchards of Darjeeling hill was used for present study after completion of Koch's postulate. Development of root rot disease of *Citrus reticulata* was studied using mandarin seedlings from eight different locations, viz. Rangali Rangliot, Bijanbari, Sukhia Pokhari, Kurseong, Mirik, Kalimpong Block I, Kalimpong Block II and Gorubahan. The root rot index as well as percentage loss in dry weight of roots were found very low at the initial stage of infection which increased significantly with time in compatible interaction. Mandarin seedlings of three locations (Mirik, Kalmpong Block-I and Sukia Pokhri) were found to be highly susceptible. Cultural conditions affecting growth of the pathogen were studied *in vitro*.

The soil samples collected from eight different locations were analysed before the isolation of microorganisms. Moisture content, pH, soil type, soil texture, carbon and nitrogen ratio, available K and P were determined. Rhizosphere microflora of mandarin plants were studied with special reference to their growth and sporulation behaviour. It was found that most of the fungal isolates belonged to the genera *Aspergillus*, *Fusarium*, *Penicillium*, *Sporotrichum*, *Rhizopus*, *Macrophomina*, *Emenicella* and *Trichoderma*. Bacterial isolated were characterized based on morphological and biochemical studies following Bergey's manual of Systematic Bacteriology. Isolates were characterized for H_2S production, phosphate solubilization, starch hydrolysis, casein hydrolysis, chitin degrading, siderophore production, catalase production, protease production, urase production, cellulase production

and indole production. Overall, *Bacillus pumilus, Bacillus cereus, Bacillus sp.*, and *Pseudomonas sp.*, were found to be more abundant. Among these *Bacillus pumilus* was found to be phosphate solubilizer and also antagonistic against root pathogens (*M. phaseolina, F. oxysporum, F. solani, F. graminearum* and *Rhizoctonia solani*)

Screening of arbuscular mycorrhizal fungi (AMF) from rhizosphere of mandarin plants grown in Darjeeling hill yielded *Glomus mosseae, G. fasciculatum, G. intraradices, G. versiforme, Gigaspora margarita, G. rosea, G. gigantea, Acaulospora spinosa, A. bireticulata, Scutellospora* sp. Some of the characteristic features were considered for identification of all those isolates which were found as consistent association and maximum colonization with mandarin roots. Scanning electron microscopic observation of three important genera *Glomus, Gigaspora* and *Acaulospora* were made. *Glomus mosseae* and *G. fasciculatum* were found to be dominant. However, based on maximum percent root colonization of mandarin seedlings *Glomus mossese* was selected for mass multiplication in sorghum and maize plants.

Polyclonal antibodies (PAbs) were raised against mycelial antigens of *M. phaseolina* . IgG were purified and further packaged into immunological formats using PTA-ELISA, dot blot, western blot and immunofluorescence for quick and accurate detection of pathogens from soil and mandarin root tissue. Indirect staining of mycelia and sclerotia of *M. phaseolina* with homologous PAb and labeling with goat antirabbit IgG conjugated with FITC developed strong fluorescence in young hyphal tips, conidia and sclerotia of pathogens. Major cross reactive antigens shared by root and the pathogen was detected using PTA-ELISA format. Cellular location of CRA in mandarin root tissue was confirmed using PAb of the pathogen and FITC conjugates.

Besides, PCR based molecular detection of pathogens have also been developed. Genomic DNA from *M. phaseolina, F. solani, F. oxysporum and F. graminearum* (among root pathogens), biocontrol agent (*Trichoderma asperellum*) and a selective PGPR (*Bacillus pumilus*) were prepared, purified. PCR amplifications of 18S rDNA were done for the root pathogens and BCA isolate using ITS specific primer pairs. The product size was approximately 570 bp for *Fusarium*, 620 bp for *Macrophomina* and 600 bp for *Trichoderma* with the size variation across the isolates. RAPD profile was also obtained for fungal and bacterial isolates using random decamer primers from Operon technology kit. All

reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed into similarity matrix using NTSYS computer programme. A dendogram was made with similarity coefficient ranged from 0.67 to 0.95.

In vitro screening of *Trichoderma asperellum* was made for evaluation of their antagonistic activity against *M. phaseolina* and *F. solani* and further evaluated *in vivo* on the development of root rot disease of mandarin under green house as well as field conditions. Based on the screening of association of arbuscular mycorrhizal fungi (AMF) with mandarin roots in four locations of Darjeeling hills, percentage colonization behavior established by artificial inoculation of mandarin seedlings, predominant AMF *Glomus mosseae* and *Glomus fasciculatum* were selected for application in mandarin plants. Total phosphate content of soil was determined after application of *G. mosseae* and *G. fasciculatum* singly or jointly. Results revealed that soil P content had decreased due to application of AMF indicating that the plant could uptake phosphorus which had been solubilized by AMF. Application of *G. mosseae* in the mandarin saplings exhibited marked increase in growth of the plants. Application of *G. mosseae* and *T. asperellum* singly or jointly suppressed root rot of mandarin caused by *F. solani* was suppressed to certain extent by *G. mosseae*. Reduction of root rot in *Citrus reticulata* following application of bioinoculants (*G. mosseae*, *T. asperellum* and *B. pumilus*) were evident both singly or jointly. However, joint inoculation with both AMF (*G. mosseae*) and PGPR (*B. pumilus*) as well as AMF (*G. mosseae*) and BCA (*T. asperellum*) reduced disease markedly.

Defense responses of mandarin plants against root rot pathogen (*M. phaseolina*) were demonstrated during early stages of root colonization by *G. mosseae* and *T. asperellum* following application of *B. pumilus.* Activities of β 1,3 - glucanase, chitinase and peroxidase were assayed in roots and leaves and of mandarin seedlings subjected to various treatments- ie., *G. mosseae*, *B. pumilus*, *T. asperellum*, *M. phaseolina*, *B. pumilus* + *G. mosseae*, *B. pumilus* + *M. phaseolina*, *G. mosseae* +*M. phaseolina*, *T. asperellum*+ *G. mosseae*, *B. pumilus* + *G. mosseae* +*M. phaseolina*,*T. asperellum*+ *G. mosseae*+*M. phaseolina* . Activities of all 3 enzymes, in both leaves and roots, were significantly enhanced which was also confirmed by immunological assays. Cellular location of Chitinase in root and leaf tissues of *Citrus reticulata* following induction of resistance was also demonstrated using PAb of Chitinase and FITC conjugates.

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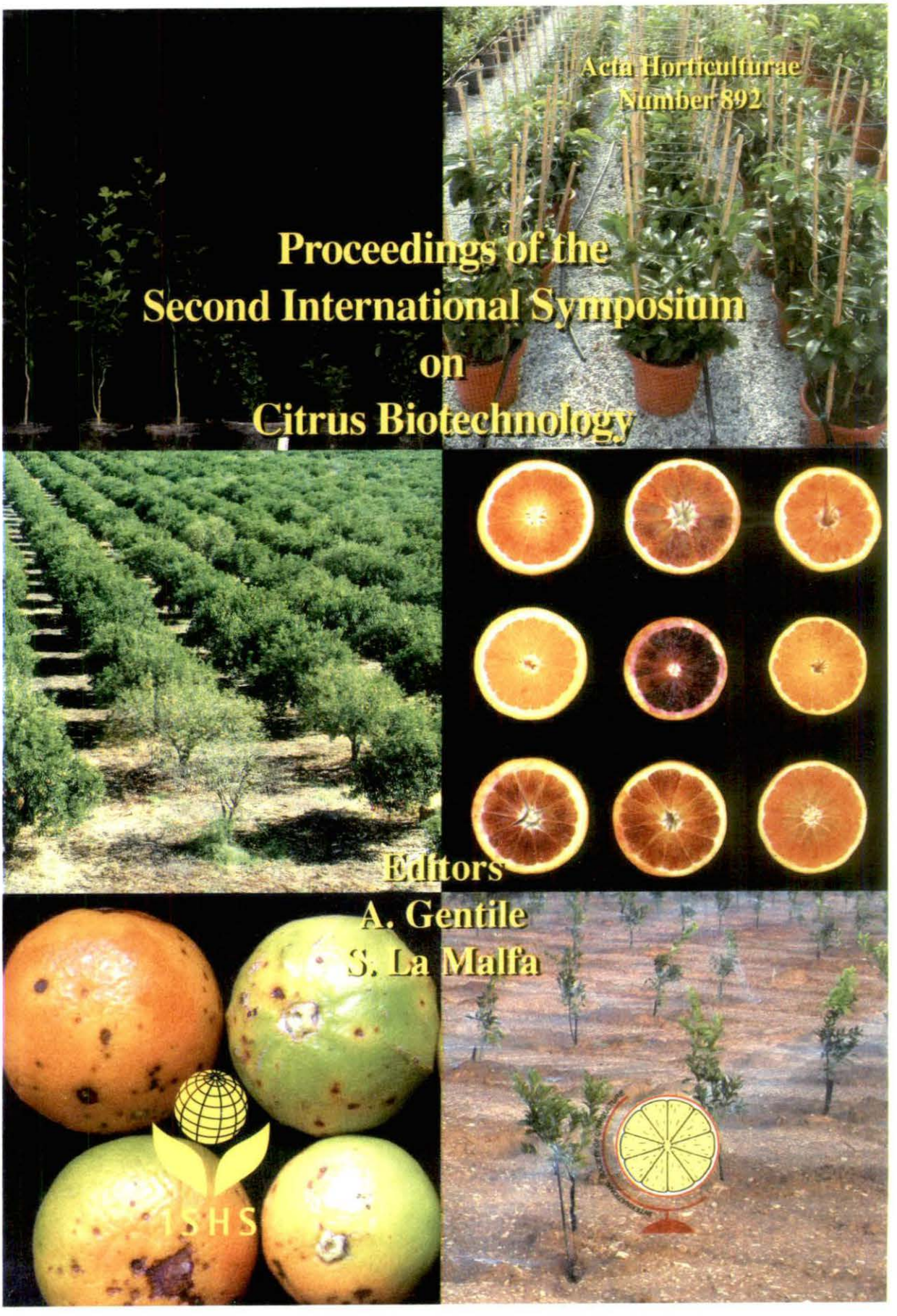
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Molecular Detection of Fungal Pathogens of *Citrus reticulata* Grown in Darjeeling Hills and Their Management

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Abstract

Mandarin orchards in Darjeeling hills are recently showing severe signs of citrus decline caused by *Macrophomina phaseolina* and *Fusarium solani* resulting in decreased fruit production. Polyclonal antibodies (PAbs) were raised against mycelial antigens of *M. phaseolina* and *F. solani*; IgG were purified and further packaged into immunological formats using PTA-ELISA, dot blot, western blot and immunofluorescence for quick and accurate detection of pathogens from soil and mandarin root tissue. Indirect staining of mycelia and sclerotia of *M. phaseolina* as well as conidia of *F. solani* with homologous PAb and labeling with goat antirabbit IgG conjugated with FITC developed strong fluorescence in young hyphal tips, conidia and sclerotia of pathogens. Besides, PCR based molecular detection of pathogens have also been developed. Genomic DNA from *M. phaseolina*, *F. solani* and twenty five *Trichoderma* isolates were prepared, purified and PCR amplification of 18S rDNA were done using ITS region specific primer pair keeping annealing temperature of 59°C with good product yield and minimum nonspecific amplification. The product size was approximately 570 bp for *Fusarium*, 620 bp for *Macrophomina* and 600 bp for *Trichoderma* with the size variation across the isolates. RAPD profile was obtained using six random decamers from Operon technology kit [OPA1, OPA4, OPB2, OPB3, OPB6 and OPD5] for *Fusarium* and *Macrophomina*, while A-5, AA-11 and AA-04 random primers specifically for *Trichoderma*. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed into similarity matrix using NTSYS computer programme. A dendogram was made with similarity coefficient ranged from 0.67 to 0.95. In vitro screening of all these *Trichoderma* isolates were made for evaluation of their antagonistic activity against *M. phaseolina* and *F. solani* and further evaluated in vivo on the development of root rot disease of mandarin under green house as well as field conditions.

INTRODUCTION

Citrus is the second priority fruit crop in the world after grapes. The most widely cultivated cultivar in India is the mandarin (*Citrus reticulata* Blanco) followed by sweet orange (*Citrus sinensis* Osbeck) and acid lime (*Citrus aurantifolia* Swingle), sharing 65, 25 and 10% of total production respectively but still remains unexplored for systemic collection, evaluation and characterization to shortlist them in order to identify active germplasm. There are four different strains of mandarin cultivated in India viz. Khasi mandarin grown in north-eastern states, Darjeeling mandarin grown in the hills of Darjeeling and Sikkim, Nagpur mandarin grown in Maharashtra, Coorg mandarin grown in south India. In addition to the above, the Kinnow mandarin is grown in north and north-western states of India. The eastern Himalaya and the north-eastern states are considered as the original homeland of citrus in India it is also true in case of Darjeeling and Sikkim (Mukhopadhyay and Thapa, 2001). Although different species of citrus are grown in the hills, mandarin (*Citrus reticulata*) is the principal crop which dominates

more than 90% of the area under citrus cultivation. It is an indigenous economic crop of Darjeeling-Sikkim hills of the eastern Himalayas. The hills of Darjeeling grow mandarin in the time immemorial and its ethno botanical records are reflected in the local folklore of highlanders (Thapa, 2007). The cultivation practices in these areas are very traditional and production is also low. Current area of production in Darjeeling hills is 2090 ha with an annual production of 22000 tons and an average productivity of 8.11 tons/ha (Mukhopadhyay et al., 1996). The mandarin cultivation in Darjeeling has a massive decline due to various pathological, entomological and nutritional stresses. *Macrophomina phaseolina*, *Fusarium solani* and *Fusarium oxysporum* infects roots of both nursery grown and field grown mandarin plants. Outbreaks are common in fields where mandarin is grown under water stress.

The use of immunological assays for both detection and diagnosis of plant diseases have increased rapidly (Chakraborty and Chakraborty, 2003; Gawande et al., 2006). It has long been known that most plant pathogens possess as part of their structures, specific antigenic determinants or recognition factors in the form of proteins, glycoproteins, complex carbohydrate polymers or other complex molecules (DeVay and Adler, 1976; Chakraborty, 1988). On the other hand, the ribosomal RNA genes (rDNA) possess characteristics that are suitable for the detection of pathogens at the species level. These rDNA are highly stable and exhibit a mosaic of conserved and diverse regions with the genome (Hibbet, 1992). Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan et al., 1995). Therefore we focused on the ITS regions of ribosomal genes for the construction of primers that can also be used to identify root pathogens (*Fusarium* and *Macrophomina* spp.) and also biocontrol agent (*Trichoderma* spp.) isolated from the rhizosphere of mandarin plants.

The objectives of our investigation was to develop a reliable and sensitive serological as well as PCR assays for the selective detection of pathogenic *Fusarium* and *Macrophomina* species associated with root damage of *C. reticulata* as well as for one of the potential biocontrol agents *Trichoderma* species in order to develop the management strategies of root diseases of mandarin plant.

MATERIALS AND METHODS

Fungal Culture

Isolates of root pathogens (*Macrophomina phaseolina*, *Fusarium solani*, *Fusarium oxysporum*) and biocontrol fungi (*Trichoderma harzianum* and *Trichoderma viride*) were obtained from the culture collection of Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal and were maintained by regular sub-culturing in PDA. These isolates were originally collected from various locations of Darjeeling hills as enlisted in Table 1.

Preparation of Inocula and Inoculation Techniques

1. Fungal Pathogens. *Fusarium solani* was grown in sand, maize meal medium (maize meal:sand:water 1:9:1.5 w:w:v) in autoclavable plastic bags (sterilized at 20 lbs. pressure for 20 min) for a period of three weeks at 28°C until the mycelia completely covered the substrate. Nursery grown mandarin seedlings were inoculated by adding 100g of previously prepared inoculum of *F. solani* to the rhizosphere soil. Disease assessment was done based on percentage loss in dry mass of inoculated roots as well as on the colour intensity of infected roots, 28 day after inoculation in relation to the control.

M. phaseolina was grown on PDA in Erlenmeyer flasks (250 ml) for 2 days. Subsequently 30 sterilized mandarin stem pieces (one inch long) were transferred to each flask and incubated for 15 days. Five such pieces containing the mycelia and sclerotia were inserted in the rhizosphere of each plant. The inoculated plants were examined at an interval of 7 days up to a period of 28 days. Each time, the plans were uprooted, washed and symptoms noted. Finally roots were dried at 60°C for 96 h and weighed. Root rot

index was calculated on the basis of percentage root area affected and they were graded into 6 groups and a value was assigned to each group (viz. no. root rot = 0; up to 10% root area affected = 0.10; 11-25% = 0.25; 26-50% = 0.50; 51-75% = 0.75; 76-100% = 1.0). The root rot index in each case was the quotient of the total values of the replicate roots and the number of roots (i.e. number of plants).

2. Biocontrol Fungi. Inocula of biocontrol fungi (*Trichoderma harzianum* and *Trichoderma viride*) were prepared separately by inoculating wheat bran (sterilized) with 5 mm disc of 7 day old fungal culture and incubated at 28°C for 10 days. To each pot, 10 g of the wheat bran colonized by *T. harzianum* or *T. viride* was mixed to give a concentration of 10^5 cfu/g of soil at least 10 days prior to inoculation with fungal pathogen(s).

Preparation of Antigen

Antigens were prepared from mycelia of *M. phaseolina* and *F. solani* as well as from healthy and artificially inoculated (separately with the pathogen) root tissue of mandarin plants following the methods as described by Chakraborty and Purkayastha (1983). They were stored at -20°C and used as mycelial and root antigens.

Production and Purification of Polyclonal Antibody

New Zealand white male rabbits were used to raise polyclonal antibodies against mycelial antigens of *M. phaseolina* and *F. solani* following the method of Chakraborty and Purkayastha (1983). Normal sera were collected from the rabbit by ears vein puncture before immunization. The antigen emulsified with an equal volume of Freund's complete/incomplete adjuvant was injected subcutaneously at weekly interval for six consecutive weeks. The blood samples were collected after six weeks following injection and kept for 1h at 30°C. The clots were loosened and stored at 4°C. The antisera were then clarified by centrifugation and stored at -20°C until required. IgGs were purified by DEAE-Sephadex column chromatography following the protocol of Clausen (1988).

Immunodiffusion

Agar gel double diffusion tests were performed using PAb raised against *M. phaseolina* and *F. solani* following the method of Ouchterlony (1967).

PTA-ELISA

Plate trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA) was performed essentially as described by Chakraborty and Sharma (2007). Antigens from fungal pathogen and host roots were diluted with coating buffer and IgGs were diluted to 1:125 with PBS-Tween containing 0.5% BSA. Goat antirabbit IgG (whole molecule) alkaline phosphatase (Sigma) conjugate and 4-nitrophenyl phosphate (pNPP) as enzyme-substrate, were used for ELISA tests. Absorbance values were measured at 405 nm in an ELISA reader (Multiskan EX, Labsystems). Absorbance values in wells not coated with antigens were considered as blanks.

Dot Immunobinding Assay

Mycelial antigens prepared from root pathogens of mandarin (*M. phaseolina* and *F. solani*), non pathogens (*Curvularia lunata*, *Sphaerostilbe repens*), healthy and artificially inoculated (separately with *M. phaseolina* and *F. solani*) roots of *C. reticulata* were loaded on nitrocellulose membrane filters using Bio-Dot apparatus (Bio-Rad). Dot immunobinding assay was performed using PAb of *M. phaseolina* and *F. solani* as outlined by Lange et al. (1989).

Western Blotting

Protein samples were electrophoresed on 10% SDS-PAGE gels as suggested by Laemmli (1970) and electrotransferred to NCM using semi-dry Trans-blot unit (BioRad) and probed with PAbs of *M. phaseolina* and *F. solani* following the method of Wakeham

and White (1996). Hybridization was done using alkaline phosphatase conjugate and 5-bromo-4-choloro-3-indolylphosphate (NBT-BCIP) as substrate. Immunoreactivity of the proteins was visualized as violet coloured bands on the NCM.

Immunofluorescence

PAbs of *M. phaseolina* and *F. solani* and goat antisera specific to rabbit globulins conjugated with Fluorescein isothiocyanate (FITC) were used for indirect immunofluorescence study to detect the pathogen in root tissues as well as to determine the cellular location of major cross reactive antigens (CRA) shared by the pathogens in healthy root tissues following the method of Chakraborty et al. (1995). Observations were made using a Biomed microscope (Leitz) equipped with an I3 filter block ideal for FITC fluorescence under UV light in the dark. Photographs were taken by Leica Wild MPS 48 camera on Kodak 800 ASA film.

Preparation of Genomic DNA

Extraction of genomic DNA from *F. solani*, *F. oxysporum*, *F. graminearum*, *M. phaseolina* and isolates of *Trichoderma* was done. Fungal mycelia (3-4 day-old) was crushed with liquid nitrogen and incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8.0), 100 mM NaCl and 2% SDS, for 1 hr at 65°C followed by centrifugation at 12,000 rpm for 15 min. The supernatant was extracted with equal volume of water saturated phenol, centrifuged at 12,000 rpm for 15 min, and further extracted with equal volume of phenol: chloroform: isoamyl alcohol (25:24:1) by centrifugation at 12000 rpm for 15 min; the aqueous phase was transferred in a fresh tube and chloroform (1:4 v/v) was added followed by 0.5 M Na-acetate (1:10 v/v). Next isopropanol was added to the above mixture (0.7 times the final volume) and centrifuged. DNA was precipitated from the aqueous phase with chilled ethanol (100%) and pelleted by centrifuging at 12000 rpm for 15 min followed by washing in 70% ethanol and centrifugation. The pellets were air dried and suspended in Tris-EDTA (TE-1 X) buffer pH 8.0.

Qualitative and Quantitative Estimation of DNA

Total genomic DNA extracted from isolates of *Fusarium*, *Macrophomina* and *Trichoderma* was resuspended in 100 µl 1 X TE buffer, treated with RNAse and incubated at 37°C for 30 min. After incubation the sample was re-extracted with PCI (Phenol:Chloroform:Isoamylalcohol 25:24:1) solution and RNA free DNA was precipitated with chilled ethanol as described earlier. The quality and quantity of DNA was analyzed both spectrophotometrically and in 0.8% agarose gel. Spectrophotometrical analysis for pure isolated $DNA_{A260/280}$ was ~1.8. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

ITS-PCR Amplification

All isolates of *Fusarium*, *Macrophomina* and *Trichoderma* were taken up for ITS-PCR amplification (Table 2). Genomic DNA was amplified by mixing the template DNA, with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase chain reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. The following primer pairs were used for ITS PCR.

PCR was programmed with an initial denaturing at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 59°C for 30 s and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40% w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination with horizontal electrophoresis.

RAPD-PCR

For RAPD-PCR, nine random primers were selected (Table 3). PCR was programmed with an initial denaturing at 94°C for 4 min. followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min and extension at 70°C for 90 s and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µl) containing 0.25% bromophenol blue, 40% w/v sucrose in water, and then loaded in 2% Agarose gel with 0.1% ethidium bromide for examination by horizontal electrophoresis.

Scoring and Data Analysis

The image of the gel electrophoresis was documented through Bio-Profil Bio-1D gel documentation system and analysis software. All reproducible polymorphic bands were scored and analysed following UPGMA cluster analysis protocol and computed in silico into similarity matrix using NTSYSpc (Numerical Taxonomy System Biostastisics, version 2.11W) (Rohlf, 1993). The SIMQUAL program was used to calculate the Jaccard's coefficients. The RAPD patterns of each isolate was evaluated, assigning character state "1" to indicate the presence of band in the gel and "0" for its absence in the gel. Thus a data matrix was created which was used to calculate the Jaccard similarity coefficient for each pair wise comparison. Jaccard coefficients were clustered to generate dendograms using the SHAN clustering programme, selecting the unweighted pair-group methods with arithmetic average (UPGMA) algorithm in NTSYSpc.

RESULTS AND DISCUSSION

Root samples collected from eight different locations of mandarin orchards were evaluated for resistance to *F. solani* and *M. phaseolina* separately. Disease assessment was based on percentage loss in dry mass of inoculated roots as well as on the colour intensity of infected roots, 15 days after inoculation in relation to control. Two locations, Mirik and Kalimpong where disease was prevalent showed highest population when isolation was made. Trend was also same in the artificial inoculation test (Table 4). Similar results were obtained when the plants were inoculated with *M phaseolina*. Root rot index was highest in three locations and their colour intensity of infected roots turned into black after 28 days of inoculation (Table 5).

PAbs raised against *F. solani* and *M. phaseolina* were tested with homologous and heterologous antigens of mandarin roots. Strong precipitation reactions occurred in homologous reactions in immunodiffusion test (Figs. 1A and B). Among the root antigens of mandarin plants of eight different locations tested against PAbs of *F. solani* and *M. phaseolina*, strong and positive reactions were noticed in root antigens of four specific locations (Table 6). Previous studies have also suggested that common antigens may be indicators of plant host-parasite compatibility (Chakraborty, 1988).

Optimization of ELISA was done by considering two variables, dilution of the antigen extract and dilution of the antiserum to obtain maximum sensitivity. Purified IgG were tested against homologous and heterologous antigens at 25 µg/L concentrations. Doubling dilutions of *F. solani* and *M. phaseolina* ranging from 1:125 to 1:4000 were initially tested. ELISA values decreased with PAb dilution. Finally, 1:125 dilution of PAb was selected for further assay. Dilutions of antigen concentration in two-fold series ranging from 25 to 1600 µg/L were tested against two antiserum dilutions (1:125 and 1:250). ELISA values increased with a concomitant increase of antigens concentrations. Concentrations as low as 25 µg/L could be easily detected by ELISA in both antisera dilutions. PTA-ELISA could readily detect reaction between root antigen and PAb of pathogens (*F. solani* and *M. phaseolina*). Antigens extracted from healthy and artificially inoculated with *F. solani* and *M. phaseolina* were tested against PAbs of the pathogens separately (Table 6). In time course experiment involving artificial inoculation of roots with the fungal pathogen(s),infection could be detected from 20 h onwards in ELISA on the basis of significantly higher (p=0.01) absorbance values of infected root extracts in comparison with healthy root extracts. Absorbance values in PTA-ELISA were also

significantly higher for infected root extracts than for healthy controls up to a concentration of 2 mg/L. Kitagawa et al. (1989) successfully used a competitive ELISA technique to develop an assay to identify *F. oxysporum* f. sp. *cucumerinum* among other fusaria.

Mycelial antigen of both the pathogens were analysed on SDS-PAGE and then western blot analyses were done using homologous PAb (Figs. 1C, D, F and G). Antibody labeling with fluorescein isothiocyanate (FITC) is known to be one of the powerful techniques to determine the cell or tissue location of major cross reactive antigens (CRA) shared by host and parasite. Specific detection of cross reactive antigens was confirmed as bright green fluorescence in young hyphal tip as well as in conidia of the pathogen (Figs. 1E and H).

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, we focused on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *Fusarium*, *Macrophomina* and *Trichoderma* spp. ITS region of rDNA was amplified using genus specific ITS-1 & ITS4 (for *Macrophomina*); T/ITS1 &T/ITS4 (for *Trichoderma*) and Fcg17F & Fcg17FR (for *Fusarium*) primers. Amplified products of size in the range of 550-700 bp was produced by the all primers (Fig. 2A). The primer pairs Fcg17F and Fcg17R were found to be highly specific for *Fusarium* genus as reflected in Figure 2A (Lanes 9-12), but non specific for *Macrophomina phaseolina*, as no band was detected with this primer pair (Fig. 2A, lanes 7&8).

Trichoderma produced a single amplified product ranging from 600-620 bp (Fig. 2B) 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* (Lieckfiledt et al., 1999; Muthumeenakshi et al., 1994; Ospina et al., 1999; Venkateswarlu et al., 2008).

Among nineteen isolates of *Trichoderma* sp. eleven isolates were identified as *T. viride* and eights isolates were *T. harzianum*. The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. PCR products 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 of our isolates of *T. harzianum* (RHS/M511) obtained from mandarin rhizosphere, 18S rRNA gene sequence which has been submitted to GenBank databases (Acc. No. GQ995194.1)

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. The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973) (Fig. 3). 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 $1^{st}+2^{nd}+3^{rd}+$ 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 *F. solani*, *F. graminearum*, *F. oxysporum*, *M. phaseolina* and *Trichoderma* spp. 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 93 reproducible and scorable polymorphic bands ranging from approximately 100 to 2000 bp were generated with primers among the nineteen *Trichoderma* isolates (Table 8, Figs. 2E and F) and 73 reproducible and scorable polymorphic bands ranging from approximately 200 to 6000 bp 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 200 to 6000 bp. Relationships among the isolates was evaluated by cluster analysis of the data based on the similarity matrix (Table. 9, Figs. 2C and D). The Dendogram was generated by unweighted pair-group methods with arithmetic mean (UPGMA) using NTSYSpc software (Fig. 4). Based on the results obtained all the nineteen isolates can be grouped into two main clusters. One cluster represents *T. viride* and other *T. harzianum*. Again the *T. viride* cluster may be sub grouped into three, first subgroup with four isolates (TVFS/20, TVFS/C90, TV/RHS/T455, TV/FS458) second one with five isolates (TNRHS/T460, TNRHS/T472, TVRS/S473, FS/S474, TNRHS/T 463) and third one with two isolates (TV/FS/S475, TV/FS/S478). The second cluster of *T. harzianum* could be further divided into two different groups containing different isolates. The first group consists of four isolates (THAG/S471, THAG/S479, THAG/S476, THFS/S477) and second group consists of another four isolates of *T. harzianum* (THRHS/AC480, THRHS/AC481, THRHS/AC482, THRHS/AC483). These results are in agreement with those of Latha et al. (2002), Muthumeenakshi (1994) and Venkateswarlu (2008) who studied genetic variability among the isolates of *Trichoderma* by RAPD using random primers. 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) (Fig. 5).

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 (*Gossypium hirsutum* L.) (Su et al., 2001). Isolates were clearly grouped according to the host origin. Additionally, the authors did not observe any molecular variation 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 and wilt complex of mandarin plants by biological means. Antibiosis to *F. solani* and *M. phaseolina* by biocontrol agents (*T. harzianum* and *T. viride*) were evaluated in vitro and in vivo condition. Both the biocontrol agents tested in vitro were effective in causing significant suppression of growth of *M. phaseolina* and *F. solani* (Fig. 6). After 3-4 days of incubation *T. harzianum* and *T. viride* over grew the pathogen. But in control plate pathogen grew characteristically. In order to find out the efficacy of these biocontrol fungi 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 biocontrol fungi in soil was done at least 10 days before inoculation with pathogen. Results revealed that both *T. harzianum* and *T. viride* were found to be very effective in reducing root rot disease (Table 10). *Trichoderma* sp. thus has the potential for use in management of root rot complex in the field. 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.

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Tables

Table 1. Isolates of root pathogens and biocontrol fungi.

Isolates	Code	GPS location	
		Latitude	Longitude
Root pathogens			
Fusarium graminearum	RHS/S66	N 23°01'12.13"	N 23°01'12.13"
F. graminearum	RHS/S66	N 26°48'18.68"	N 26°48'18.68"
F solani	RHS/M 532	N 26°48'18.64"	N 26°48'18.64"
F solani	RHS/M 533	N 26°48'18.64"	N 26°48'18.64"
F. oxysporum	RHS/M 534	N 26°48'18.68"	N 26°48'18.68"
F. oxysporum	RHS/M 535	N 26°48'18.68"	N 26°48'18.68"
Macrophomina phaseolina	RHS/S 565	N 26°42'42.56"	N 26°42'42.56"
M. phaseolina	RHS/S 566	N 26°42'42.56"	N 26°42'42.56"
Biocontrol fungi			
Trichoderma viride	FS/S-477	N 26°48'18.64"	N 26°48'18.64"
T. viride	FS/S-478	N 26°48'18.68"	N 26°48'18.68"
T. viride	RHS/T- 460	N 26°45.11.75"	N 26°45.11.75"
T. viride	RHS/T- 463	N 26°48'18.68"	N 26°48'18.68"
T. viride	RHS/T- 472	N 26°45'11.75"	N 26°45'11.75"
T. harzianum	RHS/AC480	N 26°42'42.56"	N 26°42'42.56"
T. harzianum	RHS/AC481	N 26°42'42.56"	N 26°42'42.56"
T. harzianum	RHS/AC482	N 26°42'42.56"	N 26°42'42.56"
T. harzianum	RHS/AC483	N 26°42'42.56"	N 26°42'42.56"
T. harzianum	Ag/S476	N 25°01'13.13"	N 25°01'13.13"
T. harzianum	Ag/S471	N 25°01'13.13"	N 25°01'13.13"
T. harzianum	Ag/S479	N 25°01'11.13"	N 25°01'11.13"
T. harzianum	RHS/M511	N 26°42'42.56"	N 26°42'42.56"

Table 2. The nucleotide sequence used for ITS PCR.

Seq name	Primer seq 5'-3'	Mer	TM	% GC	Amplicaon size (bp)	References
***Macrophomina* sp.**						
ITS 1	TCCGTAGGTGAACCTGCG	18	61	56	~620	White et al.
ITS4	TCCTCCGCTTATTTGATATGC	21	63	59		(1990)
***Fusarium* sp.**						
Fcg17F	TCGATATACCGTGCGATTTCC	21	65	47	~570	Nicholson
Fcg17R	TACAGACACCGTCAGGGGG	19	66	63		et al. (1998)
***Trichoderma* sp.**						
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57	~600	White
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45		et al. (1990)

Table 3. The nucleotide sequence used for RAPD.

Seq name	Primer seq 5'-3'	Mer	TM	% GC	References
OPA-1	CAGGCCCTTC	10	38.2	70	White et al.
OPA-4	AATCGGGCTG	10	39.3	60	(1990)
OPD-2	TGATCCCTGG	10	34	60	
OPB-3	GATCCCCTG	10	37	70	
OPB-6	TGCTCTGCCC	10	40	70	
OPD-5	TGAGCGGACA	10	37	60	
A-5	AGGGGTCTTG	10	31.8	73	
AA-11	AGACGGCTCC	10	37.1	70	
AA-04	AGGACTGCTC	10	25.8	60	

Table 4. Pathogenicity test of *Fusarium solani* on different root samples of *Citrus reticulata*.

Locality of *C. reticulata* saplings	*Loss in dry mass of roots (%)	**Colour intensity of infected roots
Rangli Rangliot	28.5	+
Bijanbari	42.5	++
Sukhia Pokhari	78.6	++++
Kurseong	39.7	++
Mirik	82.0	++++
Kalimpong Block I	87.5	++++
Kalimpong Block II	79.6	++++
Gorubathan	56.8	+++

*In relation to control on the basis of 3 separate trials of 50 plants each.
**+ Light brown, ++ deep brown, +++ blackish brown, ++++ black.

Table 5. Pathogenicity test of *Macrophomina phaseolina* on different root samples of *Citrus reticulate*.

Locality of *C. reticulata* saplings	*Root rot index	**Colour intensity
Rangli Rangliot	0.10	+
Bijanbari	0.25	++
Sukhia Pokhari	0.75	++++
Kurseong	0.25	++
Mirik	0.75	++++
Kalimpong Block I	0.75	++++
Kalimpong Block II	0.50	+++
Gorubathan	0.50	+++

*On the basis of root area affected: 0-10% (0.10); 11-25% (0.25); 26-50% (0.50); 51-75% (0.75); 76-100% (1.0).

**+ Light brown, ++ deep brown , +++ blackish brown, ++++ black.

Table 6. Detection of cross reactive antigens among *C. reticulata* and *F. solani* and *M. phaseolina* using agar gel double diffusion.

Root antigens of *C. reticulata*	PAb	
	F. solani	*M. phaseolina*
Rangli Rangliot	-	±
Bijanbari	-	±
Sukhia Pokhari	+	+
Kurseong	±	±
Mirik	+	+
Kalimpong Block I	+	+
Kalimpong Block II	+	+
Gorubathan	±	±

Common precipitin band (+) present, (-) absent, (±) weak.

Table 7. PTA-ELISA values showing reaction of PAbs of *F. solani* and *M. phaseolina* with antigens of healthy and inoculated roots of *C. reticulata* (absorbance at 405 nm).

Citrus saplings locality	Antigen concentration (40 mg/L)		
	Healthy	Inoculated	
		F. solani[a]	*M. phaseolina*[b]
Rangli Rangliot	0.812	1.182	1.264
Bijanbari	0.890	1.139	1.149
Sukhia Pokhari	1.115	1.345	1.774
Kurseong	0.972	1.265	1.880
Mirik	1.064	1.876	1.993
Kalimpong Block I	1.007	1.766	1.887
Kalimpong Block II	1.187	1.980	1.932
Gorubathan	0.938	1.765	1.872

PAb of *F. solani* and *M. phaseolina* were used at 1:125 dilution.

[a]3 days after inoculation.

[b]7 days after inoculation.

Table 8. Analysis of the polymorphism obtained with RAPD markers in 19 isolates of *Trichoderma* species.

Sl no.	Seq name	Total no RAPD bands	Approximate band size (bp)		Monomorphic band	Polymorphic bands	Polymorphic (%)
			Min	Max			
1.	AA-11	09	100	2000	0	09	100
2	OPA-4	12	100	1000	0	12	100
3.	A-5	18	100	2000	0	18	100
4.	OPD-6	14	100	1000	0	14	100
5.	AA-04	11	100	1000	0	11	100
6.	OPA1	09	100	1000	0	09	100
7.	OPB2	07	100	2000	0	07	100
8.	OPB3	08	100	1000	0	08	100
9.	OPD5	05	100	2000	0	05	100
Total		93			0	93	100

Table 9. Analysis of the polymorphism obtained with RAPD markers in *Macrophomina phaseolina* and *Fusarium solani*, *Fusarium graminearum*, *Fusarium oxysporum* isolates.

Sl no.	Seq name	Total no RAPD bands	Approximate band size (bp)		Monomorphic band	Polymorphic bands	Polymorphic (%)
			Min	Max			
1.	OPA1	11	200	6000	0	11	100
2	OPA-4	17	200	6000	0	17	100
3.	A-5	15	100	6000	0	15	100
4.	A-11	11	100	6000	0	11	100
5.	OPD-6	03	100	6000	0	03	100
6.	AA-4	04	100	6000	0	04	100
7.	OPB-2	03	100	6000	0	03	100
8.	OPB-3	05	100	6000	0	05	100
9.	OPD-5	04	100	6000	0	04	100
Total		73			0	73	100

Table 10. Effect of *Trichoderma harzianum* and *Trichoderma viride* on the development of root rot disease of *Citrus reticulata*.

Locality of *C. reticulata* saplings	Treatments	*Loss in dry mass of roots (%)	**Colour intensity	Treatments	***Root rot index	**Colour intensity
Mirik	Fs	80.0	++++	Mp	0.75	++++
	Fs + Th	12.8	+	Mp + Th	0.10	+
	Fs + Tv	20.5	+	Mp + Tv	0.25	+
Kalimpong Block I	Fs	91.6	++++	Mp	1.00	++++
	Fs + Th	16.5	+	Mp + Th	0.25	+
	Fs + Tv	24.0	+	Mp + Tv	0.25	+

*In relation to control on the basis of 3 separate trials of 50 plants of each treatments.
**Light brown (+), deep brown (++), blackish brown (+++), black (++++).
***On the basis of root area affected; 0-10% (0.10); 11-25% (0.25); 26-50% (0.50); 51-75% (0.75); 76-100% (1.0).
Fs = *Fusarium solani*, Mp = *Macrophomina phaseolina*, Th = *Trichoderma harzianum*, Tv = *Trichoderma viride*.

Figures

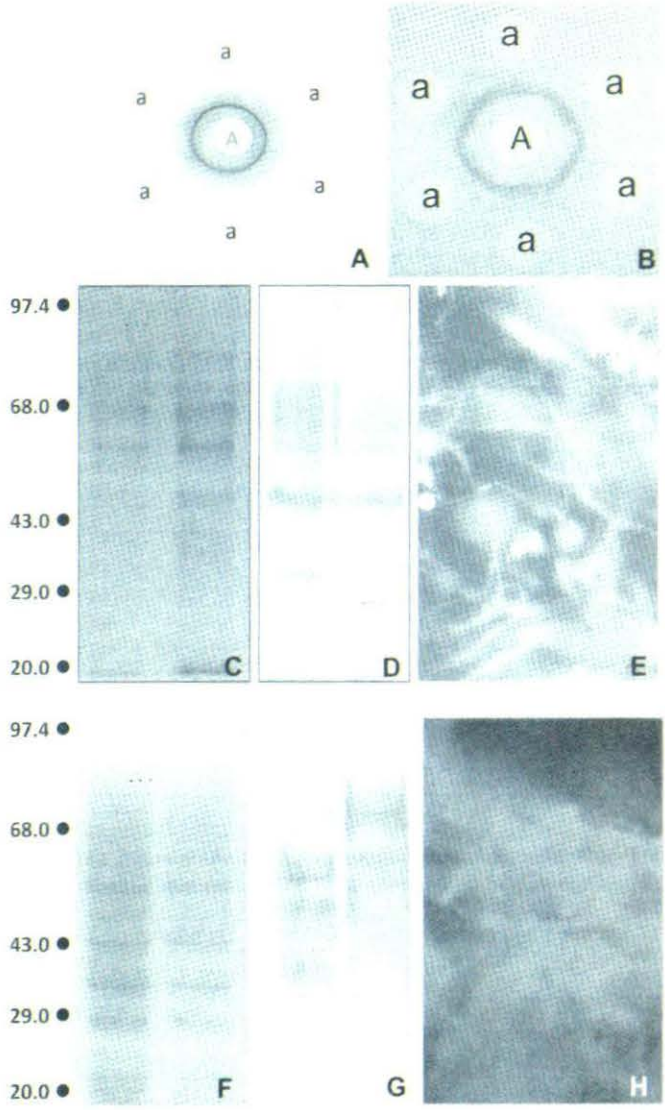

Fig. 1. **A.** Immunodiffusion of antigens of *F. solani* (peripheral wells) treated with homologous PAb (central well); **B.** Immunodiffusion of antigens of *M. phaseolina* (peripheral wells) treated with homologous PAb (central well); C. SDS PAGE of protein profile of *F. solani*; **D.** Western Blot of *F. solani*; **E.** Immunofluorescence of hyphae of *F. solani* labeled with FITC-conjugate; **F.** SDS PAGE of protein profile of *M. phaseolina*; **G.** Western Blot of *M. phaseolina*; and **H.** Immunofluorescence of hyphae of *M. phaseolina* labeled with FITC-conjugate.

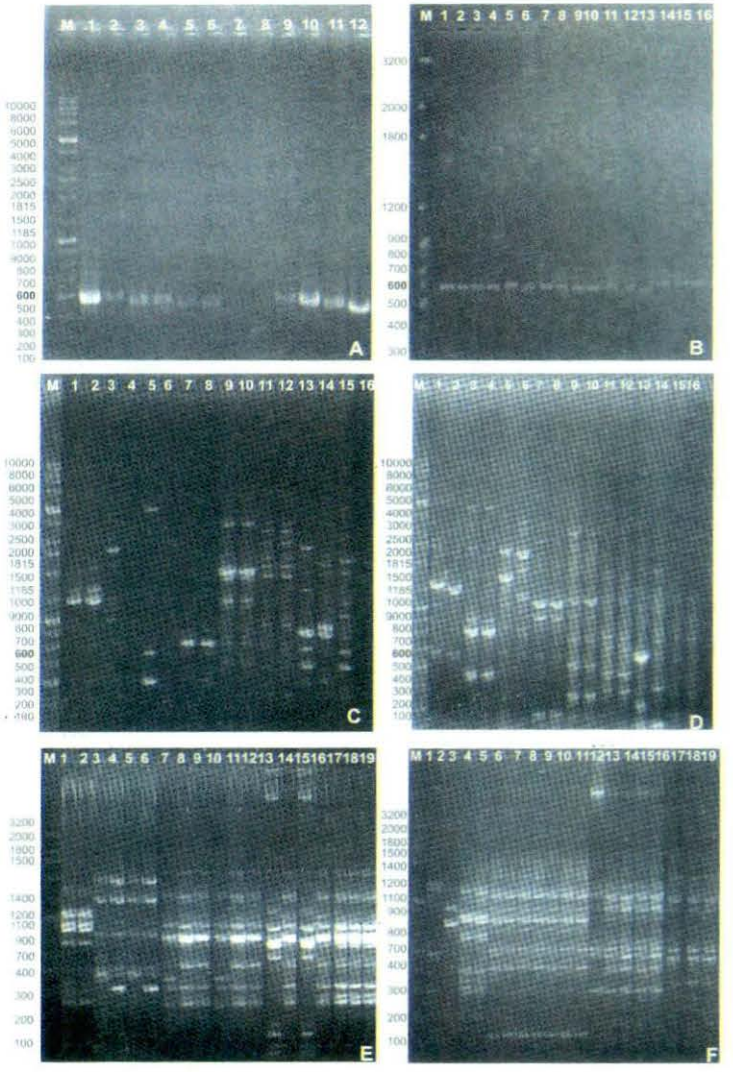

Fig. 2. A&B: Agarose gel (2%) electrophoresis of ITS region. A) Lane M-high range DNA ladder, lane 1-2,7-8 for *M. phaseolina*, 3-4,9-10 for *F. solani*, 5-6,11-12 for *F. graminearum* (lanes 1-6; ITS1 & ITS4 and lanes 7-12; Fcg17F and Fcg17R); B) M-medium range DNA ladder, lane 1-8 for *T. viride* and 9-16 for *T. harzianum* (lanes 1-16; T/ITS1 & T/ITS4); C-F: Agarose gel (2%) electrophoresis of RAPD. C) Lane M-high range DNA ladder, lane 1-2 *M. phaseolina*, 3-4 *F. solani*, 5-6 *F. graminearum*, 7-8 *F. oxysporum* analysed with primer OPA1 & lane 9-10 *M. phaseolina*, 11-12 *F. solani*, 13-14 *F. graminearum*, 15-16 *F. oxysporum* analysed with primer OPA4; D) Lane M-high range DNA ladder, lane 1-2 *M. phaseolina*, 3-4 *F. solani*, 5-6 *F. graminearum*, 7-8 *F. oxysporum* analysed with primer A-05 & lane 9-10 *M. phaseolina*, 11-12 *F. solani*, 13-14 *F. graminearum*, 15-16 *F. oxysporum* analysed with primer AA-11; E&F: Lane M-High range DNA ladder, lane 1-11 for *T. viride* & lane 12-19 *T. harzianum* with Primer AA-04 (E) & A-05 (F) respectively.

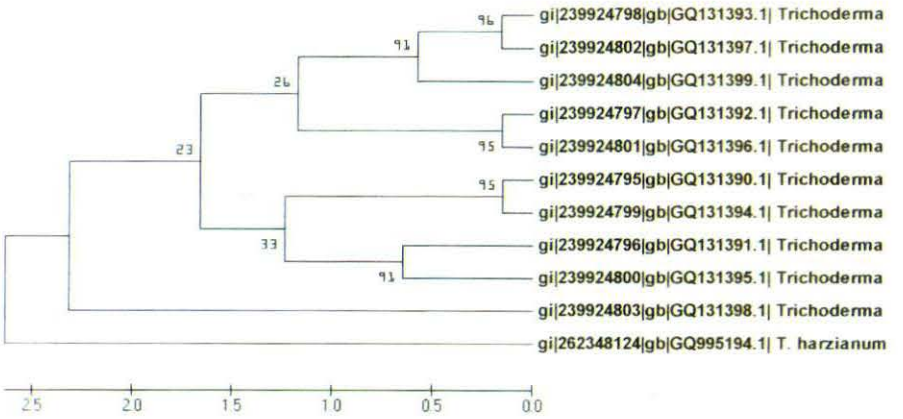

Fig. 3. Dendogram of evolutionary relationships of 11 taxa using MEGA4 software.

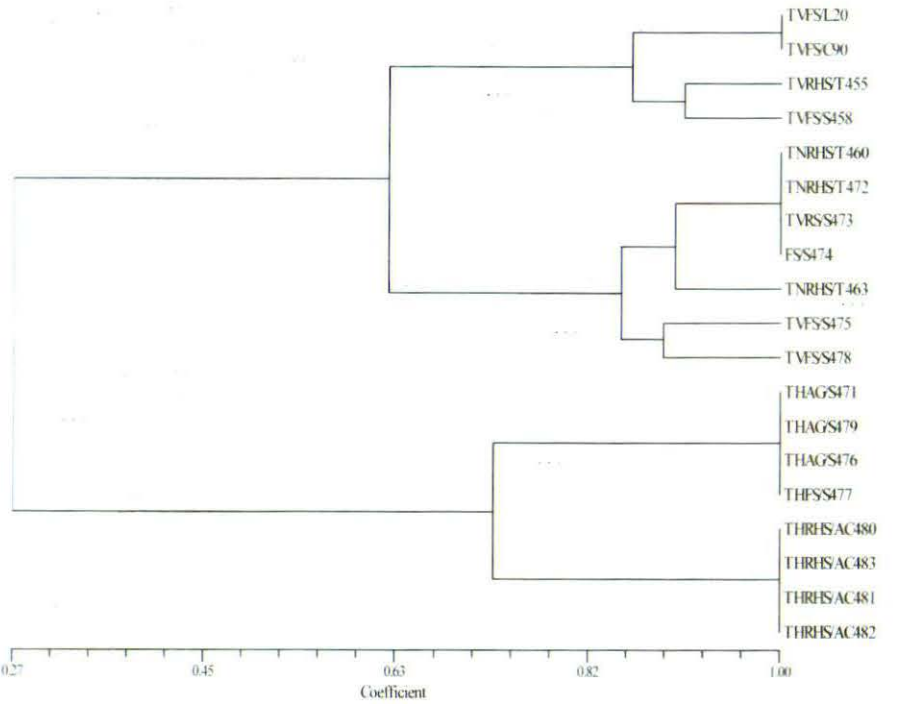

Fig. 4. Dendrogram showing the genetic relationships among *Trichoderma* fungal isolates based on RAPD analysis using NTSYSPc software.

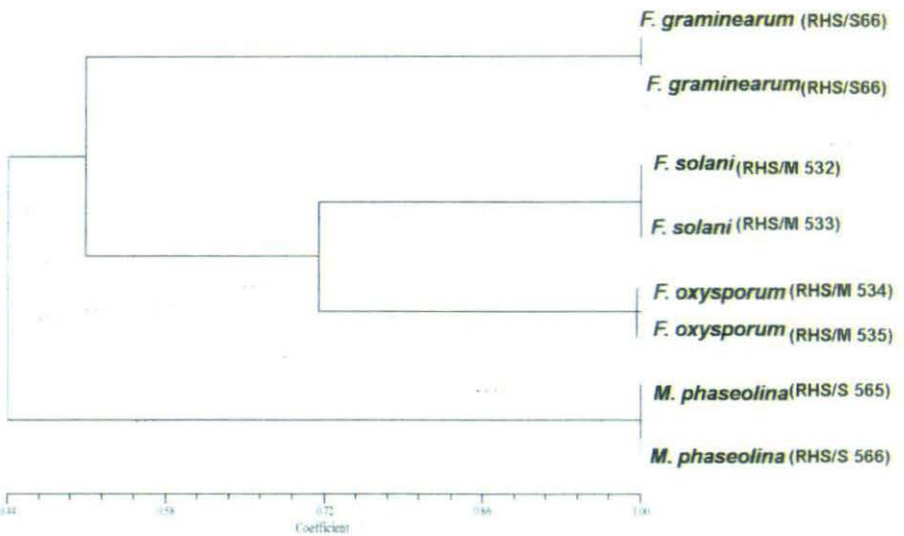

Fig. 5. Dendrogram showing the genetic relationships among *Fusarium graminearum*, *F. solani*, *F. oxysporum*, and *Macrophomina phaseolina* based on RAPD analysis using NTSYSPc software.

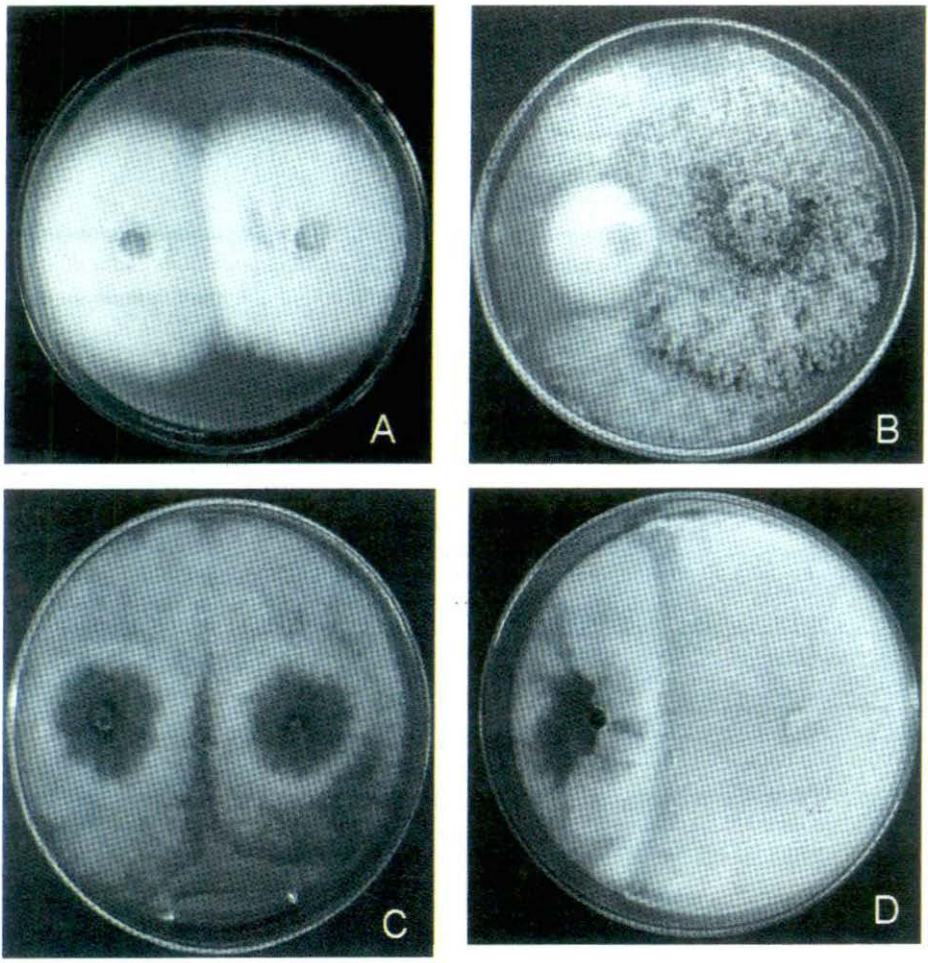

Fig. 6. In vitro test of *Trichoderma harzianum* and *T. viride* against *Fusarium solani* and *Macrophomina phaseolina*. Homologous pairing of *F. solani* (A) and *M. phaseolina* (C). Pairing of *F. solani* with *T. harzianum* (B) and *M. phaseolina* with *T. viride* (D).