Screening of Arbuscular Mycorrhizal Fungi and Plant Growth Promoting Fungi from rhizosphere of *Citrus reticulata* Blanco and their assessment for management of root rot disease

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DECLARATION

I declare that the thesis entitled "**Screening of arbuscular mycorrhizal fungi and plant growth promoting fungi from rhizosphere of *Citrus reticulata* Blanco and their assessment for management of root rot disease"** has been prepared by me under the guidance of Professor B.N. Chakraborty, Immuno Phytopathology Laboratory, Department of Botany, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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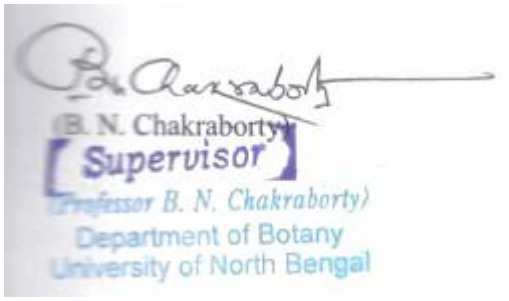
CERTIFICATE

I certify that Ms. Sanjita Allay has prepared the thesis entitled "**Screening of Arbuscular Mycorrhizal Fungi and Plant Growth Promoting Fungi from rhizosphere of *Citrus reticulata* Blanco and their assessment for management of root rot disease"** for the award of Ph.D degree of the University of North Bengal, under my guidance. She has carried out the work at the Department of Botany, University of North Bengal.



ABSTRACT

It is well known that beneficial plant-microbe interaction in the rhizosphere are primary determinants of plant health and soil fertility. Arbuscular mycorrhizal fungi (AMF) are the most important microbial symbiosis for the majority of plants in improving not only their plant health and above ground productivity but also act as bioprotectants against pathogens and toxic stresses. Application of AMF alongwith PGPF has gained increased attention in recent years in improving horticultural benefits. The demand for biological control of plant disease is increasing as it is environment and health friendly.

The present study examined the diversity of Arbuscular Mycorrhizal Fungi (AMF), colonisation nature and histopathological study in *Citrus reticulata*, *C. limoni* and *C. medica* along with isolation of Plant Growth Promoting Fungi (PGPF) and Plant Growth Promoting Rhizobacteria (PGPR) from the rhizosphere and application of AM fungi singly and in combination with potent PGPF to enhance the defence enzymes responsible for disease resistance against root rot caused by *Fusarium* sp.

Mandarin [*Citrus reticulata* Blanco], Rangpur lime [*C. limonia* Osbeck] and Citron [*C. medica* (L.)], all belonging to the family Rutaceae were selected for AMF isolation. Among all the genera, the genus *Glomus* was predominant during summer while *Gigaspora* dominated the population in winter followed by *Acaulospora*, *Scutellospora* and *Sclerocystis*. *Glomus mosseae*, *G. fasciculatum* and *G. badium* are most abundant species of the genus *Glomus* in all the plants; while *Gigaspora gigantea* and *Gi. margarita* are the abundant spores of the genus *Gigaspora*. Root samples taken from each of the five crop plants were examined under microscope and mycorrhization was documented. The physical nature of arbuscules; vesicles, intraradical hyphae etc were studied extensively to determine the colonization impact in these citrus species. Scanning electron microscopic observation was made of three most abundant genera, i.e. *Glomus*, *Gigaspora* and *Acaulospora*.

On the other hand a total of twelve (12) fungal isolates and 14 bacteria have been isolated out of which 6 bacterial isolates were obtained from hills and 8 bacterial isolates were obtained from plains from the rhizosphere soil of mandarin. Isolated fungi and bacteria were studied under microscope after suitable staining and characterized based on morphological and biochemical

studies. Among the fungi isolated three PGPF, two isolates of *T. harzianum* and one isolate of *T. asperellum* and one PGPR *Pseudomonas poae* were isolated from mandarin rhizosphere.

Dot immunobinding assays confirm the effectiveness of raising antibodies against *F. solani* and *F. oxysporum*. Western blot analyses using polyclonal antibody of *F. solani* and *F. oxysporum* revealed that the PAb could show different levels of homologous reactions with the antigens of *F. solani* and *F. oxysporum* respectively.

The main objectives of the present study was to determine the efficacy of these bioformulations using AMF, PGPF and PGPR singly and in combinations on plant growth promotion and biocontrol of root rot diseases of mandarin caused by *Fusarium* sp. along with determination of cell defense responses in mandarin plants associated with induction of resistance toward *Fusarium solani* and *F. oxysporum* by microbial formulation.

A review of literature pertaining to this investigation has been presented which deals mainly with the growth promotion, biological disease control and bioformulations.

Standard methods were used for experimental purpose in order to achieve the above objectives. Mandarin plants which were brought from different hill areas of Darjeeling region were maintained in the Glass house and field of Immunophytopathology Laboratory at Department of Botany, North Bengal University, were used for the experimental purposes.

The fungal isolates RHS/M/511 and RHS/M/512 obtained from mandarin rhizosphere were designated as potential PGPF and both the isolates were confirmed with the help of 18S rDNA sequences. The BLAST query of the 18S rDNA sequence of the isolates against GeneBank database confirmed the identity of the isolate RHS/M/511 as *T. harzianum* and RHS/M/512 and as *T. asperellum*. The sequences have been deposited in NCBI, GeneBank database under the accession no. GQ995194 for *T. harzianum* and HQ265418 for *T. asperellum*. The pathogens *F. solani* and *F. oxysporum* also obtained from mandarin rhizosphere was confirmed with the help of 18S rDNA sequences. The BLAST query of the 18S rDNA sequence of the isolates against GeneBank database confirmed the identity of the isolate RHS/M534 as *F. oxysporum* and RHS/M532 and as *F. solani*. The sequences have been deposited in NCBI, GeneBank database under the accession no. KF952606 for *F. oxysporum* and KF952603 for *F. solani*.

ITS region of rDNA was amplified using genus specific T/ITS1 and T/ITS4 (for *Trichoderma*) and Fcg17F and Fcg17R (for *Fusarium*) primers.

The antagonistic effect of the both the isolates *T. harzianum* and *T. asperellum* showed positive results against mandarin root rot pathogens *viz, Fusarium solani* and *F. oxysporum*

Gigaspora gigantea, *G. margarita*, *Glomus mosseae* and *G. fasciculatum* were mass multiplied in turf grass, sorghum and maize plants and was used singly and in combination with PGPF isolates. Growth enhancement was evaluated in terms of percent increase in height and leaf number over similar increase in control plants after one month and two months of treatment. Results revealed that enhancement of plant growth by single as well as joint application of PGPF and AMF however the results reveals that the growth of mandarin seedlings grown under same environmental and physical conditions were enhanced to a greater extent when PGPF and AMF were applied jointly.

Phosphorus content were significantly enhanced when both PGPR and AMF were applied jointly. Experiments were conducted to assess the effect of single as well as combined application on biochemical components of mandarin root and leaves. Effects of AMF, PGPF and PGPR in management of Fusarium root rot disease of mandarin were tested in pot as well as nursery conditions. Defense enzymes -i.e., peroxidase, phenylalanine ammonia lyase, chitinase, β-1,3 glucanase and phenol metabolism were also determined. Enhanced increase in activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in roots and leaves was observed on application of AMF and PGPR and in another instance application of AMF and PGPF to soil followed by inoculation with *Fusarium*. Induction of flower and fruit was enhanced on application of bioinoculants *Trichoderma* and AMF. Immunological tests confirmed the induction of defense enzymes after application of PGPR and PGPF.

Abiotic stress induced by water logging and drought conditions were determined by biochemical and morphological changes. Marked changes in antioxidative enzymes such as peroxidase, catalase and ascorbate peroxidase was observed during stress in comparison to control plants. Antioxidative activity was seen to be more in the leaves than in the roots.

Analysis of 16s rDNA sequences of a bacterium associated with spores of *Gigaspora* sp (AMF) was done. The identity was confirmed identified as *Bacillus mycoides* and deposited in NCBI with accession no. KJ917554.

Fluorescence studies (FITC and RITC) of AMF spores was also done to locate the presence of AMF in mandarin roots. Strong apple green and red fluorescence were evident in treated spores and roots.

Apart from enzymatic assessment of defense enzymes, an attempt was also made to conduct fluorescent immunological studies to locate the sites of chitinase enzyme expression within the leaf and root tissues. Immunolocalization of chitinase in *T. asperellum* treated and *Fusarium* inoculated as well as control mandarin leaves and roots were observed by immunofluorescence study.

Immunogold localization of defense enzyme (chitinase) in mandarin roots following colonization with AMF, treated with bioinoculant (*T. asperellum*) singly and joint inoculation and subsequently challenge inoculation with *F. solani* through Transmission Electron Microscopy. Heavy deposition of gold particles was observed near the cell wall of inoculated roots.

Mandarin roots following colonization with AMF alone or in combination with PGPF or PGPR could induced resistance against the pathogen. The present investigation was designed to suppress the pathogen in the rhizosphere with the help of AMF and PGPF and AMF and PGPR.

PREFACE

At the very outset, I express my profound sense of gratitude and indebtedness to my supervisor Professor B.N. Chakraborty, immuno-Phytopahtology Laboratory, Dept. of Botany, North Bengal University for his constant guidance throughout my research work. Without his encouragement and tireless mentorship, I would not have completed my thesis. Professor Usha Chakraborty of the Department of Botany needs special mention for her supportive attitude whenever I was in need of her help. Thank you, Madam, for your extra care.

Thanks to the Heads (Past and Present) of this department for all administrative support during the whole time of the course. Without mentioning my humble acknowledgments to all teachers of the department of botany, NBU, my thankfulness is incomplete. I trust all my teachers would accept my respectful offer of gratitude. I also wish to extend my thanks to all the non-teaching staffs of the Department.

I am also indebted to the Honourable Vice Chancellors, both past and present of the University of North Bengal for letting me to carry out my work in this University. My sincere thanks are also due to The Registrar, NBU for his positive response during my registration and submission of Ph.D. Thesis.

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Chapter 1
Introduction

Horticulture indicates importance of plants, their cultivation and their uses for sustainable human existence. Growing of perennial plants and trees for commercial purposes is referred to as orchard. Horticultural cultivation generally includes a wide variety of crops including fruit trees with ground crops and is grown in diverse agro-ecological situations. Horticulture includes food crops such as fruits, vegetables, mushrooms, culinary herbs and non-food crops such as flowers, trees and shrubs, turf grass and medicinal herbs. It also includes plant conservation, landscape restoration, landscape and garden design/construction, maintenance, arboriculture, horticulture therapy and much more. Horticulture development is in demand due to the growing population and rapid urbanisation. New tools and techniques have to be developed to produce more using less land and water. Indian horticulture is the core sector of agriculture, representing a broad spectrum of crops and production of a wide range of horticultural commodities. The issue of climate change and climate variability has thrown up greater uncertainties and risks further imposing constraints on production systems. The challenges ahead are to have sustainability and competitiveness to achieve the targeted production to meet the growing demands in the environment of declining land, water and threat of climate change which needs innovation and its adoption for improving production in challenged environment (Singh and Singh, 2014).

Production and productivity of any cash crop is directly dependent on its health status which is correspondingly related to pest and disease management along with its nutritional input.*Citrus reticulata* (Blanco) is themost extensively grown citrus species in India. It is commonly known as mandarin and Darjeeling mandarin is one of the ancient commercial crop that is cultivated in Darjeeling and Sikkim hills. Besides three more different strains of mandarin cultivated in India viz. Khasi mandarin grown in north-eastern states, Nagpur mandarin grown in Maharashtra, Coorg mandarin grown in south India. In addition, Kinnow mandarin is also grown in north and north-western states of India (Allay and Chakraborty, 2010)

Fig. 1: Mandarin Orchards in Darjeeling hill. (A) Darjeeling mandarin in full bloom at Kalimpong; (B & C) Harvested mandaring for marketing

The Eastern Himalaya and North-Eastern states are considered as the original homeland of citrus in India. Darjeeling mandarin (Fig.1A) have a distinct flavour and quality and hence they have a distinct appreciation in the market (Fig. 1 B&C). Cultivation practices in these areas are very traditional and production is also low. The current annual production of citrus in India is 8.2 million tonnes and that of Darjeeling hills is 67030 fruits/ha (Paulo, 2014). The mandarin cultivation in Darjeeling has shown a massive decline due to various pathological, entomological and nutritional stresses (Fig.2 A-H). The global scenario reflects that citrus production is mostly affected by fungal, bacterial and virus diseases along with pests. In India, more than 250 insect species have been reported which attack citrus but *inter alia* 15-20 major insect pests are of significance. Some local edaphic and climatic factors influence intensely to rank their importance as major pests like trunk borer, fruitfly and citrus psulla are predominant insect pests for Khashi and Darjeeling mandarin whereas blackfly, psylla and bark eating caterpillar are major pests of Nagpur mandarin.In addition to direct damage some of the insect pests act actively as vectors of some deadly diseases like Citrus Tristeza Virus (Fig.2A), psylla with greening and leaf-miner with canker. Insect pest management needs to be dealt effectively within the space and time. Now a days, the emphasis is given to integrated pests management to acquire eco-friendly and eco-feasible production. It has beenobserved that citrus whitefly (*Dialeurodes citri*) cause havoc in Punjab and Vidarbha and loss reached up to 30 per cent. Citrus psylla (*Diaphorina citri*) is a serious insect pest in Maharashtra, Punjab and Himachal Pradesh. In Coorg also, it is a serious problem of citrus growers. Even in the orchards low elevation of Darjeeling hills and Sikkim, citrus psylla has been reported to cause greening disease. They are important mostly as a vector of greening disease. Citrus leaf miner (*Phyllocnistis citrella*) is a serious pest of nurseries, young plantations and tender flushes of citrus groves. Average damage is considered to be 30 per cent but in Nagpur mandarin nurseries total damage goes up to 80 per cent. (Shivankar *et al,* 2006). Lemon butterfly (*Papilio* spp.), is very attractive butterfly of the orchard but its larve cause a considerable damage by feeding leaves causing defoliation (Fig. 2 B). About 25 aphids have been reported to as Citrus aphids around the world. Indian species include black citrus aphid, *Toxoptera aurantii*, brown citrus aphid, *T. citricidus*, green citrus aphid *Aphis citricola* and melon aphid, *Aphis gossypii*. Adult

and nymphs suck the sap from tender leaves and shoots resulting in devitalisation of the plants. Tristeza is considered as the most destructive disease of citrus. It can be transmitted by grating, budding, and dodder and by aphid vectors. The disease symptom includes quick decline, dwarfing, stem pitting to yellowing of seedlings (Gurung, 1989). Citrus Psorosis Virus (CPV) is the oldest documented viral disease. Citrus Ring spot Virus (CRSV) infects several species of citrus, particularly sweet orange, mandarin, lemon and lime.CTV, a phloem limited virus, is transmitted semipersistently by *T.citricidus*. Genetic diversity studies involving indian isolates have confirmed that Indian isolates form distinguishable phylogenetic groups, and majority of them are in phylogenetic association with VT genotype (Tarafdar *et al*., 2014). A high titre of CTV was recorded in the tender shoot bark, leaf petiole and mid rib of seven citrus spp. but pumelo, trifoliate and kumquat were found to be free of CTV infection when tested in DAS-ELISA and reverse transcription polymerase chain reaction (RT-PCR). A higher concentration of CTV was recorded in the older mandarin plants, which might be due to the increase of CTV concentration with age of the plant and multiple inoculations by aphids in older plants (Ghosh *et al.,* 2014). Fruit drop is a major problem in the citrus orchards (Fig.2 F-H). Some 20 to 30 per cent of loss is caused by the fruit fly. In India, fruit fly (*Dacus* spp.) is a major cause of fruit drop which is even true for Darjeeling mandarin.Citrus trunk borer (*Monohammus versteegi*) is a serious insect pest of Khasi and Darjeeling mandarin. Affected branches gradually dry up and the leaves wither away. Nearly 40 per cent of total damage is caused by the borer in West Bengal. Some of the minor insects are scales (50 species have been recorded feeding on citrus), citrus thrips, mealy bug (*Planococcus citri*) (Fig. 2E), citrus mite, barklice (*Psocoptera indet*) (Fig. 2C) cause minor and major damage according micro agro-climatic conditions. It has been observed that nematodes also play a great role in the citrus decline or dieback. *Tylenchulussemipenetrans* is an ectoparasite living on the surface or subsurface tissues of citrus roots. The nematodes remove nutrients from the roots of citrus plants and impair their normal growth and function. The problems and managements of citrus nematode in orchard and nurseries of Darjeeling mandarin have been documented (Thapa, 1991).

Fig 2: Pests and diseases associated with mandarin. (A) Virus infected plant (B) *Papilio demoleus* (C) *Psocoptera indet* (D) *Lepidoptera* sp.(Adult larvae of *P. demoleus*) (E) *Planococcus citri* (F-H) Symptoms of fruit drop

A serious fungal disease of citrus in the subtropical hot and humid climate condition is Gummosis. It appears in the trunk base, where barks cracks and abundantly exudes gum. It is a complex disease caused by various fungi, e.g. *Phytophthora, Diplodia* and *Ceratocystis.* Sometimes affected portion of the bark remains covered with soil so an outstanding hyperplasia of the canker border is present both on the base of the trunk and root whose complexity is referred as root rot. In both cases trees appear yellowish with pale green leaves with yellowish veins, smaller limbs and short flushes branches defoliate and start wilting and in turn during severe attack trees die. The agro climatic conditions of Darjeeling hills favours spread of gummosis.Hot and humid weather followed by a dry hot season is conducive for another important fungal disease called powdery mildew which is caused by *Odium tingitanium.* White powdery growth which later look like ashes, appears on young twigs and especially on the upper surfaces of leaves. Infected portion turn yellow and gradually die. Sometimes, the symptom appears on the skin of the fruit causing either premature fruit drop or degradation of quality. It is also a very common fungal disease of Darjeeling mandarin.

Citrus blight is a disease having great economic significance. A fungal species *Fusarium solani* occurring in the rhizosphere soil of citrus causes fibrous and scaffold root rot on healthy and blight-infected trees but noticeably fewer symptoms occur on healthy-appearing trees (Nemec, 1984). *F. solani* is one the most notorious pathogen causing root rot in Darjeeling mandarin. It is prevalent in almost all orchards of Darjeeling hills and is one of the major reasons of heavy fruit loss due to decline of health of nursery grown plants (Allay and Chakraborty, 2013). Fusarium root rot and dry rot are destructive diseases of citrus trees that gradually affect plants under biotic or abiotic stress. Many *Fusarium* spp. are supposed to be the casual agents of these diseases and their identification could be of an extreme difficulty. *Fusarium* spp. were collected from commercial citrus orchards and nurseries in Italy, Tunisia, Greece and Egypt. Three *Fusarium* species (*F. solani, F. oxysporum* and *F. proliferatum*) were identified according to morphological and molecular characterization, thus *F. solani* and *F. oxysporum* were the most frequent isolated species and, accordingly, were grouped upon the sequence of β-tubulin and α-Elongation Factor loci into nine clusters (Yaseen and

D'Onghia, 2012). Other fungal disease found in citrus growing areas are scab, anthracnose, melanose etc. Though bacterial diseases infecting citrus are limited but two internationally threatening diseases called Greening and Canker are identified as bacterial disease, have been serious problems citrus growers. Greening is systemic and graft transmissible whereas Canker is non-systemic and the symptoms remain restricted to the infected plant parts. Greening implies the greening of fruits as the principal malady. In general, considerable stunting, leaf and fruit drop, and twig dieback occur. It is a serious limiting factor for citrus production in Asia and Africa (Aubert, 1992). In china it was known as "Huang lung bin" which has now been globally known with its acronym HLB. In India it is also referred as "Dieback" or "decline". In Darjeeling mandarin, greening is observed in the orchards of lower elevation (Gurung, 1989). Citrus bacterial Canker is threatening mostly in limes and lemons production. Canker symptoms vary differently according to hosts starting from conspicuous lesion on leaves, twigs and fruits.

Arbuscular mycorrhizal fungi (AMF) the most widespread symbionts on earth receiving attention because of the increasing range of their application in sustainable agriculture and ecosystem management (Gerdemann, 1975; Adholeya, 2012). Root colonization with AMF is a dynamic process which is influenced by several edaphic factors. Mycorrhiza form critical link between the plant and soil structure and make a direct contribution to soil fertility and quality through increased uptake of immobile nutrients especialy 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 parasite water stress tolerance alleviation of salt stress and in sustainable maintenance of plant heath and soil fertility. The extrametrical fungal hyphae can extend several centimeters into the soil and absorb large amounts of nutrients for the host root (Khan *et al.,* 2000).These extraradical hyphal networks and their hyphae help in improving the texture of the soil as they contain and release glomalin, which is a putative glycoprotein, assayed from soil. Glomalin is a Glomalin-related soil protein (GRSP) that is correlated with aggregate water stability (Wright and Upadhyaya, 1998; Rillig, 2004; Rillig and Mummey, 2006). Improved soil structure increases water infiltration and can reduce soil erosion (Tisdall and Oades, 1982). Molecular analysis has provided the first

opportunity to consistently identify the AMF taxa present in the plant roots. Molecular methods allow the identification of AMF taxa and independently of morphological criteria and potentially at low levels of colonization. During colonization AMF can also prevent root infections by reducing the access sites and stimulating host defense (Bhutia *et al.*, 2012). Species richnesss and Shannon-Wiener index of AMF were higher in sod culture treated orchard. The redundancy analysis also showed that community of AMF was greatly influenced by pH, soil management, soil organic matter and phosphorus content (Wang *et al.,* 2014). In order to efficiently use mycorrhizal fungi in agriculture it is necessasry to understand their ecophysiology and metabolic functioning. Concerted efforts shoud be made to develop bioformulations which can be used as biofertilizers and bioprotectors for improvement of plant health status. Howerver stringent quality control measures must be adopted so that farmers get quality products (Chakraborty *et al.*, 2014a).

Plant growth-promoting rhizobacteria (PGPR) colonize the roots of plants following inoculation onto seed before planting and enhance plant growth and/or reduce disease, nematode or insect damage. The work to date is very promising and may offer organic growers with some of their first effective control of serious plant diseases (Chakraborty *et al.*, 2014b). Some PGPR use scarce resources, and thereby prevent or limit the growth of pathogenic microorganisms. Even if nutrients are not limiting, the establishment of beneficial organisms on the roots limits the chance that a pathogenic organism that arrives later will find space to become established. Numerous rhizosphere organisms are capable of producing compounds that are toxic to pathogens like HCN. Arbuscular mycorrhizal fungi (AMF) and bacteria and hormonal elicitors can interact synergistically to stimulate plant growth through a range of mechanisms that include improved nutrient acquisition and inhibition of fungal plant pathogens. These interactions may be of crucial importance within sustainable, low-input agricultural cropping systems that rely on biological processes rather than agrochemicals to maintain soil fertility and plant health (Alizadeh *et al.,* 2013). The symbiotic associations of AMF with most terrestrial plans are well documented but there are only few reports of symbiotic association between mandarin plant and AMF.

Considering the importance of association of AMF with mandarin orange as well as the involvement of biocontrol agents (BCA) for the management of root diseases the present investigation was undertaken with the following objectives.

(A) Isolation and identification of arbuscular mycorrhizal fungi (AMF), plant growth promoting fungi (PGPF) and plant growth promoting rhizobacteria (PGPR) from the rhizosphere of mandarin orchards located in Darjeeling hills.

(B) Selection of microorganisms showing phosphate solubilizing and antifungal activities.

(C) Mass multiplication of AMF in selected host and bioformulation of PGPF and PGPR.

(D) Raising polyclonal antibody against AMF, PGPF and *Fusarium* sp., their immunological detection and identification using immunoassays.

(E) *In vitro* interaction study among bioinoculants (PGPF and PGPR) and root rot pathogen(s).

(F) To assess the efficacy of AMF, PGPF and PGPR singly and in combinations on plant growth promotion and biocontrol of root rot disease of mandarin caused by *Fusarium solani* and *Fusarium oxysporum.*

(G) To evaluate biochemical changes with special reference to defense enzymes [Phenylalanine ammonia lyase (PAL), Chitinase (CHT), β-1,3 Glucanase (β GLU) and Peroxidase (POX)]

(H) To determine cell defense responses associated with localized and induced systemic resistance in mandarin plants using bioinoculants.

Chapter 2

Literature Review

The roots support a unique modified microbial community in an environment termed as rhizosphere. It is the region influenced by the root and its activities. The microbes present in the rhizosphere provide a link between the plants and the environment. Among the important microbes present in the rhizosphere, the mycorrhizae occur in a wide variety of plants. Arbuscular mycorrhizas are mutualistic symbiotic associations between the roots of most vascular plants and a small group of fungi in the new phylum Glomeromycota (Schüßler *et al.*. 2001). It provides phosphorus and nutrition to the plants and itself obtains carbon from the plant to support itself. The association between arbuscular mycorrhizal fungi and plants improves the fitness of both plant and AMF symbionts constituting a mutualism.

Soil pH plays a crucial role in the development of mycorrhizal fungi by affecting the solubility of several compounds. Coughan *et al* (2000) observed in declining maple forest that the site with more acidic soil had larger spore population but lower taxonomic diversity than the healthy site. The quantity of colonization generally increased with pH for both sites. *Gigaspora* appears to be more common in acid soils than *Glomus* sp. and root colonization is generally less in low than in high pH soils (Clark, 1997). Some mycorrhizal fungi grow only in low pH soils whereas others grow after modifying the soil pH with a certain amount of lime (Giri *et al.*, 2003). A few mycorrhizae have a tendency to grow at the pH and soil source from which they have been isolated (Giri and Mukherjee, 2003; Porter *et al.*, 1987).

Although some structural variation exists in this category, most arbuscular mycorrhizas are characterized by the presence of intraradical hyphae (intercellular or intracellular in location), arbuscules (finely branched hyphae involved in nutrient exchange), extraradical mycelium (hyphae that connect the root to the soil), and spores formed in the extraradical mycelium. Two major types of arbuscular mycorrhizas have been described: the *Arum*-type and the *Paris*-type named after the plant genera in the families Araceae and Liliaceae, respectively, in which they were first described (Smith

and Smith, 1997). Since some of the fungal structures of both types are formed within root cells, arbuscular mycorrhizas are classified under the broader term, endomycorrhizas. Unlike ectomycorrhizas, arbuscular mycorrhizas do not produce an obvious change in the root system and the distinguishing characteristics can only be observed with the help of various microscopical methods.

It has been estimated that over 80% of all vascular plants form arbuscular mycorrhizas. A detailed list for the United Kingdom has been published (Harley and Harley, 1987) but such lists generally do not exist for other regions of the world and, in fact, many species of vascular plants have not been assessed for the presence of mycorrhizas. Arbuscular mycorrhizas have been identified in a broad spectrum of plants, including some non-vascular plants, ferns and other seedless vascular plants groups within the gymnosperms including conifers (e.g., *Thuja, Sequoia, Metasequoia*), *Ginkgo biloba*, the cycads, and the majority of angiosperm families. The few angiosperm families that do not have arbuscular mycorrhizas either form other categories of mycorrhizas or lack mycorrhizas. Among the latter families are the Brassicaceae (this family includes canola, mustards, cabbages, etc.) and the Chenopodiaceae (this family includes garden and sugar beets, spinach and the large genus *Chenopodium*), although even here arbuscular mycorrhizal associations have been reported for a few species. A few aquatic plant families and the sedges may have low levels of colonization by arbuscular mycorrhizal fungi, however, these can be overlooked if roots are collected at the wrong time of the year or if the sample size is too small. The relative abundance and the seasonality of arbuscular mycorrhizas in many plant species remain undetermined.

Plant microbe interactions in the rhizosphere are responsible for a number of soil processes that include carbon sequestration, ecosystem services and nutrient recycling. The composition and quantity of microbes in the soil influence the ability of plants to obtain nitrogen and other nutrients. Plant can influence these ecosystem changes through the deposition of root exudates or carbon rich substances into the soil to attract or inhibit the growth of specific organisms. These carbon rich substances can range from less than 10% to as much as 44% of a plant's total carbon production. Soil microbes utilize this abundant carbon source, implying that the plant's selective secretion of specific compounds between plants and microbes may encourage beneficial symbiotic and

protective relationships against pathogenic microbes. Plants basically feed, raise and encourage certain microbes just like farmers raise and feed plants and livestock for food and fiber.

Nutrient uptake and Plant growth promotion

Mycorrhiza plays a very important role on enhancing the plant growth and yield due to an increase supply of phosphorus to the host plant. Mycorrhizal plants can absorb and accumulate several times more phosphate from the soil or solution than non–mycorrhizal plants. Plants inoculated with AMF have been shown to be more resistant to some root diseases also.

Percent Root Length Colonization (PRLC) is the most common metric of arbuscular mycorrhizal fungi by mycorrhizal structures (Treseder, 2013). Frequently, plants with greater PRLC are assumed to receive more nutrients (such as phosphorus) from their mycorrhizal symbionts, leading to greater plant growth.

Benefits of mycorrhizal biofertilizer are as follows:

(a) Allow plants to take up nutrients in unavailable forms or nutrients that are fixed to the soil. In the extreme acidic or basic soil, phosphorus is usually bound to iron, aluminum, calcium, or magnesium, leading to water insolubility, which is not useful for plants. Mycorrhiza plays an important role in phosphorus absorption for plant via cell wall of mycorrhiza to the cell wall of plant root. In addition, mycorrhizal help to absorb other organic substances that are not fully soluble for plants to use, and also help to absorb and dissolve other nutrients for plants by storage in the root it is associated with.

(b) Enhance plant growth, improve crop yield, and increase income for the farmers. As it helps in absorption of water and essential nutrients for plant growth, it leads to improvement in plant photosynthesis, nutrients translocation, and plant metabolism processes. Therefore, the plant has better growth and yield and thus reduces the use of chemical fertilizer. As in the trial involving mycorrhizal biofertilizer on asparagus it was observed that, when the farmers used suggested amount of chemical fertilizer together with mycorrhizal biofertilizer, it was found that the crop yield improved by more than

50%, and the farmers' income increased 61% higher than when chemical fertilizer alone was used.

(c) Improve plant resistance to root rot and collar rot diseases. Mycorrhizal association in plant roots helps plant to resist root rot and collar rot diseases caused by other fungi.

(d) It can be used together with other agricultural chemicals. Mycorrhiza are endurable to several chemical substances; for example; pesticide such as endrin, chlordane, methyl parathion, methomyl carbofuran; herbicide such as glyphosate, fuazifopbutyl; chemical agents for plant disease elimination such as captan, benomyl, maneb triforine, mancozed and zineb.

(e) Increase tolerance to water stress.

(f) Induce greater resistance to pathogens and reduce sensitivity to toxic substances present in the soil.

According to fossil records, plants colonizing terrestrial ecosystems four hundred million years ago were mycorrhizal (Remy *et al.*, 1994). Hence mycorrhizal fungi have been involved in the adaptation of plants to unfavorable conditions from the very beginning (Penninsi, 2004). Root rot of mandarin (*Citrus reticulata*) is a soil borne disease that is incited by many fungal pathogens including *Fusarium* sp. and *Phytopthora* sp. Arbuscular Mycorrhizal Fungi (AMF) are the major components of the rhizosphere of most plants which play an important role in decreasing plant disease incidence (Akthar and Siddique, 2008). Available evidence suggests that plants and rhizosphere organisms function in an interdependent fashion. Rhizosphere organisms depend on plants for continuous supply of reduced carbon and are recognized as playing a significant role in nutrient cycling, thus exerting an influence on plant growth. The increase in the soluble carbon in soil sections close to the root surface is related to the rhizo-deposition of root exudates that include low molecular weight organic acids, carbohydrates, nucleic acids derivatives and amino acids. Microorganisms in turn contribute to the availability and mobilization of nutrients, production of growth regulators, phototoxic substances or by suppression of pathogens and pollutants added to soil. The role of rhizospheric organisms

in mineral phosphate solubilization was known as early as 1903 and the ability of rhizospheric microorganisms to promote growth by phosphate solubilization is also one of the most studied mechanisms involved in plant growth promotion (Misra *et al.*, 2012).

Biological control

Biocontrol may be defined as any condition or practice where by survival or activity of pathogen is reduced through the agency of any other living organism with the result that there is reduction in the incidence of disease caused by the pathogen.

Plant growth promoting fungi (PGPF)

The rationale behind the disease control is to check pathogen's growth in the host and improve the health status of the plant. Disease resistance and susceptibility in plants do not represent any absolute values. Even susceptible variety shows resistance to its pathogen under certain cropping conditions or by treating stress situation. This would suggest that even a susceptible variety has a potentially effective defense mechanism and that by manipulating cropping conditions or by treating stress, it may be possible to elicit the expression of such latent defense potential during host parasite interaction. This constitutes the very basis of induced resistance in plants as a possible disease control measure. Since the first report of induction of resistance in plants against their fungal pathogens by prior inoculation with their less virulent form, the emphasis had been mostly on biological induction of resistance. Considerable evidence has now accumulated to that prior inoculation of susceptible plant host with an avirulent form of pathogen. Cultivars of non pathogenic races of pathogens of both homologous and heterologous nature or non pathogen can provide it significant levels of protection from the subsequent attack of the virulent forms of pathogen (Purkayastha, 1994). Plants so protected develop less disease symptoms. In some cases such induced or acquired resistance is systemic in nature and persists effectively over a fairly long periods. Management of root diseases through the application of beneficial soil microorganisms has been considered as a highly valuable tool to improve productivity without damaging the soil environment. The rhizosphere population may have either a favorable or a detrimental influence upon plant development, because the micro flora is so intimately related with the root system,

partially covering its surface any beneficial or toxic substance produced can cause an immediate and profound response (Singh *et al.,* 2011).

Trichoderma is a filamentous fungus that is widely distributed in the soil, plant material, decaying vegetation and wood (Chet *et al*., 2006). Their defense mechanisms comprise both enzymatic and chemical weapons, which make *Trichoderma* spp. efficient mycoparasites, antagonists, and biocontrol agents—characteristics that can be exploited by using *Trichoderma* spp. or the metabolites secreted by these fungi as biological fungicides to fight plant diseases caused by pathogenic fungi (Vinale *et al*., 2006; Navazio *et al*., 2007; Vinale *et al*., 2009). Hence *Trichoderma* spp. play an important role in the three-way interaction with the plant and the pathogen (Lu *et al*., 2004; Woo *et al*., 2006).

Aspergillus, Tricothecium and Epicoccum are the other fungi used in the biocontrol of soil-borne plant pathogens. Other fungi frequently used in the composition of biofertilizers are Arbuscular Mycorrhizae Fungi (AMF). AMF have developed a symbiotic (mutually beneficial) relationship with the root systems of living plants, from garden vegetables all the way up to old trees. By attaching to the feeder roots, mycorrhizae greatly extend the effective absorbing area available to plants. Mihaela and Mioara (2008) showed that a mixed fertilizer containing both *Trichoderma* and mycorrhizal fungi improved plant development and maybe the defense response, thus improving the defense reaction to pathogens.

Anand and Jayaram (2009) observed the inhibition pattern of 42 *Trichoderma* strains against *Fusarium ciceri* on the third and fifth day after inoculation .The percentage reduction of growth over control for the pathogen *F. ciceri,* was calculated and the isolate T40 (153.892%) was found to be the most effective on the third day after inoculation. On the fifth day of inoculation, T40 (186.173%) followed by T27 (152.099%) were the best performers. The lowest percentage reduction of *F. ciceri* over control was recorded by T38 on the third day (9.98%) and on the fifth day (12.346). Combined inoculation of *T. harzianum* with mycorrhizal fungi (*Glomus constrictum, G. mosseae*, *G. claroideum* and *G. intraradices*) resulted in general synergistic effect on disease control caused by *F. oxysporum* in melon plants, but *T. harzianum* itself was

more effective in suppressing disease development (Mart´ınez-Medina *et al.*, 2009). It was demonstrated that eight years after planting, the container-grown pine seedlings mycorrhized with the fungi *H. crustuliniforme* were affected by the Armillaria root rot disease in relatively lesser degree in comparison with the seedlings subjected to such treatment (Kowalski and Wojnowski, 2009).

Arbuscular Mycorrhizal Fungi and their associated interactions with plants reduce the damage caused by plant pathogens (Allay and Chakraborty, 2010; Chakraborty *et al.*, 2004; Chakraborty *et al.*, 2011; Bhutia *et al.*, 2012; Chakraborty *et al.*, 2012; Chakraborty *et al.*, 2013; Singh *et al.*, 2013, Chakraborty *et al.*, 2014). With the increasing cost of pesticides and the environmental and public health hazards associated with pesticides and pathogens resistant to chemical pesticides, AM fungi may provide a more suitable and environmentally acceptable alternative for sustainable agriculture and forestry (Narayanasamy, 2013). The interactions between different AM fungi and plant pathogens vary with the host plant and the cultural system. Moreover, the protective effect of AM inoculation may be both systemic and localized. Diseases caused by fungal pathogens persist in the soil matrix and in residues on the soil surface. Damage to root and crown tissue is often concealed in the soil; thus, diseases may not be noticed until the above-ground parts of the plant are severely affected. Colonization of the root by AM fungi generally reduces the severity of diseases caused by plant pathogens (Sonia *et al.*, 2013). Reduced damage in mycorrhizal plants may be due to changes in root growth and morphology, histopathological changes in the host root, physiological and biochemical changes within the plant, changes in host nutrition, mycorrhizosphere effects which modify microbial populations, competition for colonization sites and photosynthates, activation of defense mechanisms etc. (Siddiqui and Mahmood, 1996).

Effect of AM fungi on plant pathogens

AM FUNGI	PATHOGEN	DISEASE	CROP	REFERENCE
Glomus. fasciculatum, G. mosseae, Acaulospora laevis	*Cephalosporium acremonium*	Black bundle	Maize	Veerabhadraswamy Garampall, 2011
G. intraradices	*Verticillium dahlia* Kleb.	Cotton wilt	Cotton	Orak and Demir, 2011
G. mosseae, G, intraradices, G. clarum, Gigaspora gigantea, Gi. margarita	*Fusarium solani*	Fusarium root rot	Bean	Al-Askar and Rashad, 2010
G. hoi, G. fasciculatum, Rhizobium leguminosorum	*F. oxysporum* f. sp. *ciceris*	Fusarium wilt	Chickpea	Singh *et al.*, 2010
G. intraradices (BB-E), *G. mosseae* (BEG12)	*Candidatus* Phytoplasma asteris	Chrysanthemum yellows (CY) Phytoplasma infection	Chrysanthemum	Sampo *et al.*, 2012
G. mosseae, T. harzianum, Pseudomonas fluorescens	*Phytopthora parasitica* var. *nicotianae*	Root rot	Papaya	Sukhada *et al.*, 2011
G. mosseae	*Verticillium dahlia* Kleb.	Verticillium wilt	Eggplant and tomato seedling	Karagiannidis *et al.*, 2002
G. mosseae, G. versiforme and Sclerocystis sinuosa	*Verticillium dahlia* Kleb.	Verticillium wilt	Cotton	Liu, 1995
AMF	*F. oxysporum* f. sp. *lini*	Wilt	Flaxseed	Dugassa *et al.*, 1996
Glomus sp., *G. fasciculatum, G. mosseae*	*F. oxysporum* f. sp. *medicaginis*	Verticillium wilt and Fusarium wilt	Alfalfa	Hwang *et al.*, 1992
G. etunicatum	*Verticillium dahlia* Kleb.	Verticillium wilt	Cotton	Kobra *et al.*, 2009

G. mosseae, Scutellospora erythropa, Pseudomonas fluorescens	*Rhizoctonia solani*	Root rot	French bean	Neeraj and Singh, 2009
G. aggregatum	*Fusarium udum*	Wilt	Pigeon pea	Deene *et al.*, 2004
G. mosseae, G, intraradices,	*Phytopthora parasitica*	Phytopthora root rot	Tomato	Pozo *et al.*, 2001
G. mosseae, B. pumilus	*Fusasrium oxysporum*	Fusarium root rot	Mandarin	Chakraborty *et al.*, 2011
G. mosseae, T. hamatum	*Fusarium solani*	Fusarium root rot	Mandarin	Allay and Chakraborty, 2010
G. mosseae, B. pumilus	*Ustulina zonata*	Charcoal stump rot	Tea	Bhutia *et al..*, 2012
G. mosseae, B. pumilus	*Sclerotium rolfsii*	Sclerotial bight	Tea	Chakraborty *et al..*,2012

Plant growth promoting rhizobacteria (PGPR)

On the other hand plant growth promoting rhizobacteria (PGPR) described by Kloepper and Schroth (1978) and their use for sustainable agriculture has increased tremendously in various parts of the world. Studies have also shown that the growth-promoting ability of some bacteria may be highly specific to certain plant species, cultivar and genotype (Lucy *et al.,* 2004). PGPR can affect plant growth by different direct and indirect mechanisms (Gupta *et al.,* 2000). Some examples of these mechanisms includes increased mineral nutrient solubilization and nitrogen fixation, repression of soilborne pathogens (by the production of hydrogen cyanide, siderophores, antibiotics, and/or competition for nutrients), improving plant stress tolerance to drought, salinity, and metal toxicity, production of phytohormones such as Indole-3-acetic acid (Mansoori *et al*., 2013) etc. Moreover, some PGPR have the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which hydrolyses ACC, the immediate precursor of ethylene in plants (Glick *et al.,* 1995). By lowering ethylene concentration in seedlings and thus its inhibitory effect, these PGPR stimulate seedlings root length (Glick *et al.,* 1999). Significant increases in growth and yield of agriculturally important crops in response to inoculation with PGPR have been repeatedly reported (Asghar *et al*., 2002;

Vessey 2003; Gray and Smith, 2005; Peng *et al.,* 2013). Some antagonistic bacteria like *Bacillus subtilis*, *Enterobacter aerogenes*, *Pseudomonas fluorescence*, *Streptomyces spp.* and *Actinomycetes* are also usedin disease control. Diseases caused by soil borne plant pathogen *Rhizoctonia solani* can be controlled by the antifungal activity of *Trichoderma* spp. and *P. fluorescens*. These two antifungal agents produces wide variety of enzymes such as β-1,4 glucanase, β-1,3 glucanase, chitanases etc (Dev and Dawande, 2010).

According to their relationship with the plants, PGPR can be divided into two groups: symbiotic bacteria and free-living rhizobacteria (Khan, 2005). PGPR can also be divided into two groups according to their residing sites: iPGPR (i.e., symbiotic bacteria), which live inside the plant cells, produce nodules, and are localized inside the specialized structures; and ePGPR (i.e., free-living rhizobacteria), which live outside the plant cells and do not produce nodules, but still promote plant growth (Gray and Smith, 2005). The best-known iPGPR are Rhizobia, which produce nodules in leguminous plants (Hayat *et al*., 2010). They facilitate plant growth and development both directly and indirectly (Glick, 1995). PGPR are also termed as plant health promoting rhizobacteria (PHPR) or nodule promoting rhizobacteria (NPR) and are associated with the rhizosphere which is an important soil ecological environment for plant–microbe interactions (Burr and Caesar, 1984). Currently several genera are designated as PGPR and are known to be associated with several crop plants, viz. *Azotobacter*, *Azoarcus*, *Bacillus*, *Burkholderia, Enterobacter, Erwinia, Gluconacetobacter, Klebsiella, Pseudomonas, Serratia, Rhizobium* etc. The mechanisms by which PGPR can influence plant growth may differ from species to species as well as from strain to strain. Several determinants for mechanisms of growth promotion include bacterial synthesis of plant hormones like Indole-3 acetic acid (IAA), cytokinin, gibberellins, breakdown of plant induced ethylene by bacterial production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase and increase mineral and N- availability in the soil (Kloepper, 1992; Glick, 1995). Growth promotion mechanism may be direct i.e. production of growth hormones, phosphate solubilization, nitrogen fixation or indirect viz, suppression of deleterious microorganisms by siderophore production or secretion of antifungal metabolites (Kloepper, 1993). Co-inoculation with two or more microorganisms have also shown to yield similar or better results in field studies Chatterjee *et al.,* (2012) reported the effect

of co-inoculation of three bacterial isolates- *Bacillus firmus* KUCr1, *Cellulosimicrobium cellulans* KUCr3 and *Pseudomonas aeruginosa* KUCd1 on selected growth parameters of amaranth plants. KUCr1 and KUCr3 were reported to be P-solubilizers and indole acetic acid (IAA) producers, and KUCd1 was a siderophore producer. Co-inoculation of the three isolates gave the best results for overall growth of amaranth plants followed by co-inoculation with KUCr1 and KUCd1, then KUCr1 alone. Among the test isolates, KUCr1 and KUCd1 were found to be better rhizosphere colonizers when co-inoculated. KUCr1 and KUCr3 when co-inoculated produced more IAA in liquid medium. Co-inoculation gave insignificant variation in P-solubilization, but siderophore production by KUCd1 was greatly enhanced when inoculated with other isolates in culture conditions. Co-inoculation of microbes promoted plant growth better than individual isolates.

Turan *et al.,* (2012) conducted a green house experiments to determine the effects of plant growth-promoting rhizobacteria (PGPR) and boron (B) treatments, applied either alone or in combination, on yield, plant growth, leaf total chlorophyll content, stomatal conductance, membrane leakage, and leaf relative water content of wheat (*Triticum aestivum* L. cv. *Bezostiya*) and barley (*Hordeum vulgare* L. cv. *Tokak*) plants. Results showed that alone or combined B (0, 1, 3, 6, 9 kg ha^{-1}) and PGPR (*Bacillus megaterium* M3, *Bacillus subtilis* OSU142, *Azospirillum brasilense* Sp245, and *Raoultella terrigena*) treatments positively affected dry weight and physiological parameters in both species. Dubey *et al.,* (2013) isolated a total of eight motile, aerobic, Gram-positive and straight rod-shaped, endospore forming *Bacillus* spp. from the rhizosphere of chickpea plants collected from different agricultural fields. Phylogeny of the isolates was studied by partial sequencing of 16S rDNA and comparative analysis of the sequence data confirmed that the isolates belong to distinct phylogenetic lineage corresponding to *Bacillus*. Phenotyping clusters correlate with ARDRA pattern and showed resemblance to partial 16S rDNA sequencing. *Bacillus* spp. BSK5 and *Bacillus subtilis* BSK17 were the most potent strains for having plant-growth-promoting attributes. These two strains solubilised inorganic phosphate, produced Indole acetic acid, siderophore, Hydrocyanic acid and secreted extracellular chitinase and β-1,3-glucanase which antagonised and caused mycelial deformities in two phytopathogens- *Macrophomina phaseolina* and *Fusarium oxysporum* in dual culture and by culture filtrate. Interest in biological control has

increased recently by public concerns. PGPR have been used as good biocontrol agents against soil borne pathogens. Disease suppression by antagonistic bacteria depends on their ability to colonize roots and to produce substances inhibitory to pathogens. Potential biocontrol agents produce antibiotics, siderophores that cause disease suppression and increase yield of plants. Well characterized antibiotics with biocontrol properties include 2,4-DAPG, phenazine, pyrrolnitrin, HCN and lipopeptides. Some biocontrol PGPB strains protect plants by activating gene encoding defense enzymes- chitinase, β-1,3 glucanase, peroxidase, phenylalanine- ammonia-lyase and other enzymes, involved in synthesis of phytoalexin (Piga *et al.,* 1997).

According to Van Loon *et al.* (1998) non-pathogenic rhizobacteria can induce a systemic resistance (ISR) in plants that is phenotypically similar to pathogen-induced systemic acquired resistance (SAR). SAR develops when plants successfully trigger their defense mechanism in response to primary infection by a pathogen, notably when the latter induces a hypersensitive reaction through which it becomes limited in a local necrotic lesion of brown, desiccated tissue. Bacterial determinants of ISR include lipopolysaccharides, siderophores, and salicylic acid (SA). Whereas some of the rhizobacteria induce resistance through the SA-dependent SAR pathway, others do not and require jasmonic acid and ethylene perception by the plant for ISR to develop. ISR is effective under field conditions and offers a natural mechanism for biological control of plant disease. PGPR mediated induced systemic resistance (ISR) results in alteration of physiological and biological reactions of plant cells and production of pathogenesis related proteins (PR) and phytoalexins. It has been hypothesised that the inducing rhizobacteria in the plant roots produce signal, which spreads systemically within the plant and increases the defensive competence of the distant tissues from the subsequent infection by the pathogens. The efficacy of the biological control agent would largely depend on the type of formulation and delivery technology (Lumsden *et al.,* 1995). Experimental formulations of *Bacillus* sppthat effectively reduced plant disease included peat and chitin (Sid Ahmed *et al.,* 2003). The advantages of using *Bacillus* as a biological control agent is its property to form spores resistant to unfavorable natural conditions and its tolerance to antimicrobial substances released by other microbes in the soil.

Treatment with *Ochrobactrum anthropi* decreased brown root rot of tea, caused by *Phellinus noxius* (Chakraborty *et al*., 2009). Multifold increase in activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in tea plants was observed on application of *O. anthropi* to soil followed by inoculation with *Phellinus noxius*.Kim *et al*., (2009) and Fatima *et al*., (2009) both mentioned the role of *Paenibacillus polymyxa* GBR-462 and *Azotobacter* sp. WPR-51 as biocontrol agents in chili pepper and wheat in controlling diseases caused by *Phytopthora capsici* and *Rhizoctonia solani* respectively. Further, Chakraborty *et al*., (2010) also reported that *Serratia marcescens* (TRS 1) showed antagonism to a number of fungal pathogens *in vitro*. It reduced brown root rot of tea caused by *Fomes lamaoensis*. Urrea *et al.,* (2011) isolated 120 strains of antagonistic bacteria from the rhizosphere of *Physalis peruviana* and five were selected for their high inhibition of *F. oxysporum* growth, conidia production under *in vitro* conditions. These strains inhibited growth by 41–58% and reduced three- five fold conidia production. In the *in vivo* assays, all the tested isolates significantly reduced fungal pathogenicity in terms of virulence. All isolates were identified as belonging to the genus *Pseudomonas* except for A-19 (*Bacillus* sp.). Simonetti *et al.,* (2012) isolated strains of *Pseudomonas fluorescens* and *Bacillus amyloliquefaciens* from soybean rhizosphere, inhibited mycelial growth of *Botrytis cinerea* and *Sclerotinia sclerotiorumin vitro*. Nithya *et al.,* (2013) reported that nine bacterial strains (*Bacillus pumilus* SB 21, *Bacillus megaterium* HiB 9, *Bacillus subtilis* BCB 19, *Pseudomonas plecoglossicida* SRI 156, *Brevibacterium antiquum* SRI 158, *B. pumilus* INR 7, *P. fluorescens* UOM SAR 80, *P. fluorescens* UOM SAR 14 and *B. pumilus* SE 34) were tested to induce systemic resistance in sorghum cultivars 296B and Bulk Y against the highly pathogenic grain mould pathogens-*Curvularia lunata* and *Fusarium proliferatum*, respectively.

Parveen *et al.,*(2004) discussed the mode of antagonisms of *Trichoderma viride* against *Alterneria triticina* causing leaf blight of wheat. Dual culture interaction *in vitro* revealed that mycelial strand of *T. viride* coiled around the hyphae of the test pathogen forming a rope like structure and finally inhibited the growth of *A. triticina in vitro*.

Oyeyiola (2009) isolated and identified fungi present in the rhizosphere and rhizoplane of Okra (*Hibiscus esculentus*). The fungi were *Penicillium frequetans, P.*

oxalicum, P. palitans, Rhizopus stolonifer, R. oligosporus, R. oryzae, Aspergillus niger, A. fumigatus, A. japonicus, A. clavatus, Mucor hiemalis, M. racemosus, Alternaria herbarum and *A. triticina. R. stolonifer* were Predominant mycoflora in both the rhizosphere soil and the rhizoplane were, *A. niger and A. clavatus* while *P. oxalicum* and *A. herbarum* were predominant in the rhizosphere soil only. *Mucor hiemalis, Penicillium frequetans P. palitans, P. oxalicum, A. clavatus, and A. triticina* were present in the rhizosphere soil and/or 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.

Species of *Penicillium* (*P. erythromellis, P. janthinellum, P. raistricki*) and *Trichoderma* (*T. pseudokoningii* and *T. koningii*) were found to be dominant in the rhizosphere of established tea. While seasonal fluctuation 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 temperatures. In general, fungi associated with the tea rhizosphere were found to prefer a mesophillic temperature range (15° C to 35°C). The dominant species of *Penicillium* and *Trichoderma* also exhibited tolerance to lower temperatures, i.e., 5-10°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 *et al.*, 2000)

Rhizosphere of healthy pigeonpea plant was heavily colonized by *Aspergillus niger,Penicillum* sp.*, Trichodermaviride* and *Gliocladiumvirens.* Resident *Trichoderma* and *Gliocladium* was highly antagositic to the pathogen (*Fusarium udum*). *T. viride* formed loops, coiling 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* causes shrinkage and coagulation of cytoplasm of pathogen hyphae (Pandey and Upadhyay, 2000).

Two species of *Aspergilli* and ten other fungi were isolated from rhizosphere mycoflora of onion (*Allium cepa*). *Aspergilli* in general were dominant contributing 38.59% to the total mycoflora by Kallurmath and Rajasab (2000). *A. niger* and *A. flavus* were dominant on onion bulbs with the progress of their maturity.

Dominant fungi in the rhizosphere of established tea bushes and their interaction with the dominant bacteria under *in situ* conditions have been discussed by Pandey *et al.,* (2001). The populations of *Penicillium* and *Trichoderma* species were inversely correlated with the populations 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* conditions.

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 bioremediators. *Pseudomonas*, one of the best root colonizers is 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.

Mulaw *et al.,* (2010) reported the southwestern highlands forests of Ethiopia are the origin of the coffee plant *Coffea arabica*. The production of coffee in this area 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. We 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. Among others, the putative new species were particularly efficient to inhibit growth *of G. xylarioides* polymorphism and clone library sequencing of 16S ribosomal RNA (rRNA) gene fragments.

Mycorrhiza helper bacteria

Mycorrhiza helper bacteria (MHB) are organisms that specifically promote mycorrhiza formation especially ectomycorrhizal fungi by producing growth metabolites that encourages easy proliferation of the fungal hyphae thereby increasing the chances of the fungal hyphae to colonise plant roots with a large surface area. When plant growth promoting rhizobateria (PGPR) are found to stimulate mycorrhizal formation they can be regarded as MHB43 this interchangeable characteristic brings about the overlap that exists between the two groups. Generally the microbes in the mycorrhizosphere affect mycorrhizal functioning and thus some bacteria may interact with the mycorrhizal fungi on more than one metabolic level. It has been reported that some organisms especially those belonging to the genera *Bacillus* can be multifunctional. This means that they are able to perform functional roles such as being N2 fixers P solubilizers or grouped as PGPR or MHB. Few studies have also been carried out on the interaction between AMF and Actinomycetes. Research conducted using both organisms to determine their effect on plant growth showed their individual enhancement whereas dual inoculation of organisms adversely affected plant growth and exhibited antagonistic interaction towards each other. Actinomycetes was said to be responsible for the suppression of AM fungi due to its antagonism and inhibitory effect in the rhizosphere. The production of inhibitory compounds by actinomycetes could be seen as the organism's way of competing with other organisms for nutrients. *Frankia*were able to form a synergistic relationship with AM fungi when inoculated together in actinorrhizal plants such as tibetanseabuckthorn (*Hippophaetibetana*) and *Discariatrinervis* 44. *Streptomyces* is a common soil organism belonging to the actinomycetes. Their effect on AM fungi varies according to species. For example the colonization of finger millet roots by *Glomus fasciculatum* was shown to be inhibited by *Streptomyces cinnamomeous* while *Streptomyces orientalis* produced volatile compounds that stimulated germination of the resting spores of *G. mosseae, Gigaspora margarita* and *Scutellospora heterogama* when cultured auxenically 45. Hence the interactions of AM fungi with soil bacteria can either stimulate or inhibit each other's processes in the rhizosphere.

Mycorrhizal fungi are the best-known examples of fungal and bacterial interactions as the hyphae offer good ecological niches for other microbes. AM fungal spores harbour Bacteria Like Organisms (BLOs) also referred to as endosymbionts in their cytoplasm and these organisms complete their life cycle within the eukaryotic cells giving rise to a further level of symbiosis46. To demonstrate this symbiosis a combination of morphological and molecular techniques were conducted and it was concluded that the AM fungal spores of *Gigaspora margarita* and *Glomus versiforme* spores harboured these BLOs in their cytoplasm. Analysis of the bacterial 16S rRNA gene sequence obtained from the extraction of spore DNA of *G. margarita* inferred that these bacteria are related to the genus *Burkholderia*. Investigation of two geographically separated isolates of *Gigaspora margarita* and four other isolates *G. gigantea, G. rosea* and *Scutellospora persica*showed that four out of the five species had endosymbionts the exception being *G. rosea*. This demonstrates that BLOs are common features in the *Gigaspora* and can possibly be used as a genetic marker for members of this genus. Following further analysis of the morphological and molecular similarities between the endosymbionts found in *G. margarita, S. persica and S. castenea* through the amplification and sequence of partially complete 16S rRNA revealed that all endosymbionts obtained from the three AM fungal species were over 98% similar to each other.

Mycorrhizal symbiosis is generally considered not only plant-fungus interaction but should also be associated with other microorganisms and is termed as ‘mycorrhizosphere’. Although the term ‘mycorrhizosphere’ was coined by Linderman (1988), the importance of mycorrhizas as a fungal derived rhizosphere compartment was first proposed over a decade earlier (Rambelli, 1973). Mycorrhizal fungi are relevant members of the rhizosphere mutualistic microsymbiont populations known to carry out many critical ecosystem functions such as improvement of plant establishment, enhancement of plant nutrient uptake, plant protection against cultural and environmental stresses and improvement of soil structure (Smith & Read, 1997). Bowen and Theodorou (1979) first stated the presence of bacteria that are directly involved in mycorrhiza formation in an experiment where some bacterial isolates promoted and some inhibited

the colonization of *Pinus radiata* roots by *Rhizopogon luteolus*. The presence of bacteria able to promote mycorrhiza formation was confirmed in ectomycorrhiza (Garbaye and Bowen, 1989) and also in orchid mycorrhiza (Wilkinson *et al.*, 1987). Previous studies has shown that the spores of mycorrhizal fungi are colonized by bacteria (Mosse, 1962; Walley and Germida, 1996) that can influence spore germination and growth of arbuscular mycorrhizal fungi (Bianciotto and Bonfante, 2002; Hildebrandt *et al.*, 2002; Xavier and Germida, 2003). Duponnois and Garbaye (1991) proposed the term Mycorrhization Helper Bacteria (MHB), referring only to bacteria that promoted the establishment of the root-fungus symbiosis. Dohroo and Sharma (2012) used mycorrhiza helper bacteria isolated from chlamydospores of AMF to suppress root rot of apple to almost half in a consortium treatment with AMF caused by *Pythium*, *Dematophora*, and *Fusarium*.

The lineages of MHB identified so far belong to many groups and bacterial genera, such as Gram-negative Proteobacteria (*Agrobacterium, Azospirillum, Azotobacter, Burkholderia, Bradyrhizobium, Enterobacter, Pseudomonas, Klebsiella* and *Rhizobium*), Gram-positive Firmicutes (*Bacillus, Brevibacillus,* and *Paenibacillus*) and Gram-positive actinomycetes (*Rhodococcus, Streptomyces* and *Arthrobacter*) (Frey-Klett *et al.*, 2007). Freitas and Vildoso (2004), demonstrated strains of fluorescent *Pseudomonas, Bacillus* and other rhizospheric bacteria may act as growth promoters of citrus plants. Garbaye (1994) suggested that the second hypothesis is supported by the fact that sporocarpof some ectomycorrhizal fungi, as *Laccaria*, *Tuber, Suillus, Hymenogaster* and *Cantharellus* are usually inhabited by large bacterial populations. Furthermore, many isolates of MHB described in the literature have been collected from mycorrhizospheres, fructification bodies of ectomycorrhizal fungi and fungi spores of arbuscular mycorrhizal.

Many MHB are considered now-a-days as Plant Growth Promoting Rhizobacteria (PGPR), such as *Pseudomonas* sp. (Silveira *et al.*, 1995). As reported by Fitter and Garbaye (1994), these classifications may overlap, due to the prominence of *Pseudomonas* and *Bacillus* in both groups. Another factor that complicates the distinction of the two terms (PGPR and MHB) is that studies with PGPR generally exclude the

evaluation of mycorrhization (Probanza *et al.*, 2001). However, it is interesting to note that some fungal signaling pathways are mutually regulated by different rhizobacteria, while others are specific to some MHB (Deveau *et al.*, 2007).

The effect of MHB on ectomycorrhizal associations

Five possible ways of action of MHB on mycorrhiza were proposed by Garbaye (1994), in the receptivity of the root to the mycobiont, in root-fungus recognition, in fungal growth, in the modification of the rhizospheric soil and in germination of fungal propagules. In the ectomycorrhizae studied so far, the stimulus to fungal growth appears to be the primary MHB effect. The germination of spores and the mycelial growth can be stimulated by MHB through the production of growth factors, detoxification of antagonistic substances or inhibition of competitors and antagonists (Frey-Klett *et al.*, 2007). The stimulus to growth represents an adaptive advantage to the fungus, which becomes heavily associated to the host plant and acquires more competitive capacity against other mycobionts in the planting area (Duponnois and Plenchette, 2003). Currently, the contribution of each of these effects has not been fully established, and further studies are needed to elucidate these issues.

One of the features also observed in MHB is the stimulus to the formation of lateral roots in mycorrhizal plants. This fact, associated to the stimulus to fungal growth, could lead to an increase in the number of possible interaction sites between the plant and the fungus (Shilev *et al.*, 2007) and, consequently, promote greater plant mycorrhization by the mycobiont. Furthermore, apparently, different MHB may develop different helper mechanisms, even for the same pair of mycorrhizal symbionts. For example, Poole *et al.* (2001) observed that the MHB *Burkholderia* sp. EJP67 isolated from *Pinussylvestris-Lactariusrufus* ectomycorrhizae stimulated both first- and second-order mycorrhizal roots, while *Paenibacillus* sp. EJP73 isolated from the same ectomycorrhizae only promoted the formation of second-order mycorrhizal roots.

Aspray *et al.*, (2006a) demonstrated that the contact between MHB cells and the symbionts is necessary for the helper effect to be exerted. The MHB can improve the nutrition of the fungus, for example, through the provision of nitrogen in the case of

diazotrophic bacteria, or contribute to the solubilization of minerals by the secretion of protons and complexing agents, such as organic anions of low molecular weight or siderophores. It is possible that the MHB stimulate the production of phenolic compounds by the fungus, such as hypaphorine, and thus enhance the aggressiveness of the mycobiont.

Some strains of MHB are capable of competing with bacteria that inhibit mycorrhization (Garbaye, 1994) and, consequently, reduce the concentration of anti-fungal metabolites in mycorrhizosphere. The fungus favors the MHB by releasing exudates that serve as nutrients for the bacteria. An interesting fact is that the fungus *Amanita muscaria* secret substances (organic acids or protons) that can modulate the spectrum of antibiotics production by MHB (Frey-Klett *et al.*, 2007). Keller *et al.*, (2006) reported that the metabolite auxofuran, produced by *Streptomyces* sp. AcH505, seems to stimulate the pre-symbiotic growth of *A. muscaria* but inhibit the growth of pathogenic fungi.

The researches available so far suggest that MHB may have developed selective mechanisms of interaction with surrounding microorganisms, with neutral or positive effects on mycorrhizal associations, but negative effects on the root pathogens that threaten its habitat (Frey-Klett *et al.*, 2007). However, there are data concerning MHB stimulating phytopathogenous fungi and this should be considered in the biotechnological applications of MHB, for instance, as inoculum for plants. Further researches are necessary to determine whether MHB could promote the colonization of the roots by pathogenic fungi and development of disease.

Specificity of the interaction between MHB and ectomycorrhizal symbiosis

MHB are fungus-specific but not plant-specific (Garbaye, 1994). Many studies have been carried out in order to explore the specificity of the interaction between MHB and the fungi and between MHB and the symbiont plant and diverse results have been obtained (Aspray *et al.*, 2006b, Bending, 2007, Frey-Klett *et al.*, 2005, Garbaye, 1994). Frey-Klett *et al.*, (2007) reported that the MHB *Streptomyces* sp. AcH505 is capable of promoting growth of *A. muscaria* and *Suillusbovinus* and increase the formation of

ectomycorrhizae between *A. muscaria* and *Piceaabies*, but the growth of *Hebeloma cylindrosporum* and pathogenic fungi is inhibited. In general, it is noted that MHB exhibit a degree of specificity with the mycobiont, with some strains apparently specific to certain ectomycorrhizal fungi (Duponnois *et al.*, 1993) and other capable of stimulating the mycorrhization by different ectomycorrhizal fungi (Bending, 2007).

Defense strategy of plants

PR-Proteins

Higher plants protect themselves from various stresses such as pathogen attacks, wounding and application of chemicals including phytohormone and heavy metals, air pollutants like ozone, ultraviolet rays, and harsh growing conditions by changing their physiological conditions. These protective reactions are known as "defense responses" of higher plants, and the proteins actively synthesized in accordance with this reaction are called "defense-related proteins". Protective plant proteins specifically induced in pathological or related situations have been intensively studied from an agricultural perspective and are called "pathogenesis-related proteins" (PR proteins) (Bowles, 1990).

Originally, five main groups of PRs (PR-1 to PR-5) were characterized by both molecular and molecular-genetic techniques in tobacco, numbered in order of decreasing electrophoretic mobility. Each group consists of several members with similar properties (Bol *et al.*, 1990). In 1994 a unifying nomenclature for PRs was proposed based on their grouping into families sharing amino acid sequences, serological relationships, and enzymatic or biological activity. By then eleven families (PR-1 to PR-11) were recognized and classified for tobacco and tomato, with the families PR-8 and PR-10 also being present in cucumber and parsley, respectively (Van Loon *et al.*, 1994). Later three novel families (PR-12, PR-13 and PR-14) were recognized in radish, *Arabidopsis* and barley, respectively (Van Loon and Van Strien, 1999). Germins and germin-like proteins (GLPs) have been classified as PR-15 and PR-16. PR-16 has been isolated from hot pepper during the resistance response to bacterial and viral infection (Park *et al.*, 2004b). Criteria used for the inclusion of new families into PRs are that (a) the protein must be induced by a pathogen in tissues that do not normally express it and (b) induced expression must occur in at least two different plant-pathogen combinations, or

expression in a single plant-pathogen combination must be confirmed independently in different laboratories (Van Loon and Van Strien, 1999).

PRs have dual cellular localisation – vacuolar and apoplastic, the apoplast being the main site of their accumulation. PRs are established in all plant organs i.e. leaves, stems, roots and flowers (Van Loon, 1999), being particularly abundant in the leaves, where they can amount to 5-10% of total leaf proteins. In the leaves PRs are present in mesophyll and epidermal tissues. They are also localized in the abscission zone of leaves and inflorescence, abscission zone at the stem-petiole junction, and vascular tissue of stems and petioles (Del Campillo and Lewis, 1992; Eyal *et al.,* 1993). In inflorescences PRs are detected in sepals, pedicels, anthers, pistils, stigmata and ovaries (Van Loon, 1999; Buchel and Linthorst, 1999). In seeds of maize, sorghum, oat, barley, and wheat a group of PRs is established, commonly named permatins, characterized as PR-5 thaumatin-like proteins (Vigers *et al.,* 1991). Linusitin from flax seeds is referred to the same group (Anzlovar *et al.,* 1998). Noteworthy, specific cell types, such as cultured plant cells, are highly active in PRs expression (Singh *et al.,* 1987). Chemicals, such as salicylic, polyacrylic, and fatty acids, inorganic salts, as well as physical stimuli (wounding, UV-B radiation, osmotic shock, low temperature, water deficit and excess), are involved in PRs induction. A special class of PRs inducers are hormones (ethylene, jasmonates, abscisic acid, kinetin, auxins) (Edreva, 1990, 1991; Tamas *et al.,* 1997; Buchel and Linthorst, 1999; Fujibe *et al.,* 2000). The dissipation of the proton gradient across the plasma membrane, provoked by the fungal toxin fusicoccin, activator of the plasma membrane H+-ATPase, was reported to induce PRs (Schaller *et al.,* 2000). The biochemical and structural properties, as well as the organ-, tissue-, and cell-localization, the induction and regulation of PRs have been discussed by Edreva (2005).

Earlier it was thought that PRs are devoided of enzymatic functions. But Legrand *et al.,* (1987) detected chitinase activity in four members of group 3 tobacco PRs. Later, 1, 3-glucanase activity in four members of group 2 tobacco PRs (Kauffmann *et al.,* 1987) was detected. Later on chitinase activity was detected in PR-4, PR-8 and PR-11, PR-4 being referred to as chitin-binding proteins. Proteinase, peroxidase, ribonuclease and lysozyme activities were established in PR- 7, PR-9, PR-10 and PR-8, respectively. PR-6

was assigned proteinase-inhibitory properties. Membrane-permeabilizing functions are characteristic of defensins, thiols and lipid-transfer proteins (LTPs), referred to as PR-12, PR-13 and PR-14, respectively, and of osmotins and thaumatin-like proteins (PR-5). Multiple enzymatic, structural and receptor functions are detected in "do-all" germins and germin-like proteins referred to as PR-15 and PR-16, respectively (Van Loon and Van Strien, 1999; Van Loon, 2001; Selitrennikoff, 2001; Bernier and Berna, 2001; Park *et al.*, 2004 a,b).

PRs can operate in a distinct pathway involving the hydrolytic release of chitin and glucan fragments from fungal cell walls. These oligosaccharides are endowed with elicitor activity and can induce a chain of defense reactions in the host plant (Ham *et al.*, 1991; Lawrence *et al.*, 2000; Kombrink *et al.*, 2001). The peroxidase activity of PR-9 can contribute to the rigidification and strengthening of plant cell wall in response to pathogen attack (Lagrimini *et al.*, 1987). The cell-, tissue-, organ- and development-specific expression pattern of PRs suggests important functions beyond defense against pathogens. Thus, basic tobacco glucanase PR-2d functions developmentally in seed germination by weakening the endosperm, thus allowing the radicle to protrude (Vögeli-Lange *et al.*, 1994). Chitinases homologous to PR-3 and PR-4 act as morphogenetic factors in carrot embryogenesis (Kragh *et al.*, 1996), and several PRs accumulate upon the transition of plants to flowering and senescence (Fraser, 1981; Hanfrey *et al.*, 1996), also suggestive of a developmental role. Basic PR-5 (osmotins) are abundantly induced in tobacco and tomato cells in response to osmotic stress, thus contributing to osmotic adaptation (Singh *et al.*, 1987).

Systemic Acquired Resistance (SAR) and Induced Systemic Resistance (ISR)

Systemic acquired resistance (SAR) is a mechanism of induced defense which provides long-lasting protection against a broad spectrum of microorganisms. SAR requires the signal molecule salicylic acid (SA) and is associated with accumulation of pathogenesis-related proteins, which are thought to contribute to resistance.Induced resistance is a physiological "state of enhanced defensive capacity" elicited by specific environmental stimuli, whereby the plant's innate defenses are potentiated against subsequent biotic challenges (Van Loon *et al.*, 1998). Systemic acquired resistance

(SAR) and induced systemic resistance (ISR) are two forms of induced resistance; in both SAR and ISR, plant defenses are preconditioned by prior infection or treatment that results in resistance (or tolerance) against subsequent challenge by a pathogen or parasite. This resistance is effective against a broad range of pathogens and parasites, including fungi, bacteria, viruses, nematodes, parasitic plants, and even insect herbivores (Benhamou and Nicole, 1999; Hammerschmidt and Kuc, 1995; Kessler and Baldwin, 2002; McDowell and Dangl, 2000; Sticher *et al.*, 1997; van Loon *et al.*, 1998; Walling, 2000).Vallad and Goodman (2004) worked on the benefits, drawbacks, and future considerations for the improved use of chemical and biological elicitors of induced resistance in conventional agriculture which includes the potential to exploit genetic variability within populations of crop species to improve the utility of SAR and ISR in the field.

Using the model plant *Arabidopsis*, it was discovered that the isochorismate pathway is the major source of SA during SAR. In response to SA, the positive regulator protein NPR1 moves to the nucleus where it interacts with TGA transcription factors to induce defense gene expression, thus activating SAR. It has been suggested that the mobile signal for SAR might be a lipid molecule (Durrant and Dong, 2004). The nature of the mobile signal that travels through the phloem from the site of infection to establish systemic immunity has been sought after for decades. Several candidate signaling molecules have emerged in the past years, including the methylated derivative of a well-known defense hormone (methyl salicylate), the defense hormone jasmonic acid, a yet undefined glycerolipid derived factor, and a group of peptides that is involved in cell-to-cell basal defense signaling. Systemic SAR signal amplification increasingly appears to parallel salicylic acid-dependent defense responses, and is concomitantly fine-tuned by auxin (Vlot *et al.*, 2008).

A mutation affecting the lipid-transfer protein DIR1 (Defective in Induced Resistance 1) renders Arabidopsis incapable of generating/transmitting a functional SAR signal, but does not affect resistance in the inoculated leaf (Maldonado *et al.*, 2002). Another potential lipid-derived SAR signal is the oxylipin- derived defense hormone jasmonic acid (JA), which might be an early signal establishing systemic immunity

(Truman *et al.*, 2007). The apoplastic aspartic protease CDR1 (Constitutive Disease Resistance 1) reportedly generates a small peptidic mobile signal that induces systemic defense gene expression in Arabidopsis (Xia *et al.*, 2004). Other signals that are less well characterized in the context of SAR signaling are generated by MAP kinase signaling cascades. For instance, MAP Kinase Kinase 7 (MKK7), a negative regulator of polar auxin transport, is involved in basal resistance and SAR (Zhang *et al.*, 2007). Non Expressor of PR-1 (NPR1) is one of the main regulators of SA and SAR signaling, and its functions have been extensively reviewed elsewhere (Grant and Lamb, 2006; Dong, 2004).

The diversity of bacteria and fungi present in the soil helps increase its fertility and soil quality by affecting soil agglomeration. They are both important in nutrient cycling and in enhancing plant health through direct or indirect means. Although there have been a number of studies of regarding interactions between AM fungi, plant growth promoting fungi and bacteria, the underlying mechanisms of these associations are not very well understood, and we still need further experimental confirmation. All of them together do help in improving crop yield and reducing many diseases of plants including root rot which is the root cause of decline of many plants.

The use of chemicals to control pests and diseases in plants has proved to be hazardous to human health. So the increase in public health and the environment has increased the need to develop and implement effective biocontrol agents for crop protection. Effective PGPR, PGPF and AMF could be developed for disease control only after understanding its performance in the environment in which it is expected to perform. One needs to understand the mechanisms and performance of the biocontrol agents used to control disease so that one can select the proper biocontrol agent for a particular pathogen. As both pathogen and biocontrol agents do not have similar ecological niche for their growth and survival, the use of mixed inoculums will give better results and consistent performance under diverse environmental conditions.

Chapter 3
Material and methods

3.1. Plant Material

Nursery grown mandarin (*Citrus reticulata*) seedlings, two month old obtained from IARI Kalimpong, Nirmaldass Orchard Gurung Brothers Nursery Baramangwa Busty, Darjeeling, Bijanbari, and Mirik were used for experimental purposes. One year old *C. limonia* seedlings were obtained from CDRS, Kalimpong and *C. medica* from Padmaja park, University of North Bengal. 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 and after two years seedlings were planted in the experimental field. Suitable management practices were adopted in the field throughout the years (Fig 3).

3.2. 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.

Fig 3: Maintenance of mandarin saplings in glass house

3.2.1.Isolation of AMF

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.

3.2.2. Isolation of bacteria and fungi

Isolation of microorganisms from the rhizosphere of *Citrus reticulata* was carried out following, Warcup's soil plate method (1955) with a few modifications. Five grams of soil particles loosely adhering to the roots were collected from from healthy mandarin plants of different geographical locations of hills and plains. 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.

3.2.3. Isolation of Mycorrhiza Helper Bacteria

Mycorrhiza Helper Bacteria (MHB) were isolated as per the protocol of Budi *et al.* (1999) with a few modifications. Spores of *Gi gigantea* (45-50) were sonicated at 25 Hz for two minutes to remove the debris adhered to the spores. They were then washed 3-4 times gently in sterile water to remove loosely adhering particles and transferred to fresh sterile water, vortexed again and rinsed with sterile water until this remained clear. They were then transferred using a sterile Pasteur pipette to a sterile Millipore filtering apparatus (Millipore SA) with a 0.8- mm filter. After a rinse with 50 ml sterile water, the spores where washed successively with three sterilizing solutions: (1) 96% ethanol, (2) a mixture of 2% Chloramine T (w/v), 0.02% streptomycin (w/v), 0.01% gentamycin (w/v) and two drops of Tween 20, (3) 6% calcium (Ca) hypochlorite (w/v), and were plated in NA medium aseptically under laminar air flow. Total number of twelve set ups were prepared for the isolation of MHB.

3.3. Histopathological studies of mandarin roots

Fungal association of AM fungi within the root tissues was observed according to Philips & Hayman (1970). Young roots from mandarin plants were dug out manually. Roots were cut into 1cm or smaller pieces and washed in tap water gently to free them from soil particles. It was boiled in 2% KOH in hot water bath for 1 hour. The KOH was decanted and the roots washed with water for 2-3 times. 1% HCL was added and kept for 30 minutes. After decanting the HCL the sample was washed thrice in tap water and cotton blue, lactic acid and glycerol was added in the ratio 1:1:1 to stain the internal structures of AMF inside the root segments i.e. arbuscules, vesicles, auxiliary cells, and boiled in water bath for 1 hour. The excess stain was decanted and sample placed in 50% glycerol for destaining. The roots were then crushed under pressure in slide and covered with cover slip for microscopic observation. Percent root colonization was determined following the method of Giovanetti and Mosse (1980).

3.4. Morphological characterization of AMF spores

3.4.1. Microscopical

With the help of a simple microscope (20X) parasitized spores, plant debris etc were separated. Spores were sonicated at 30 Hz for two minutes to remove the debris adhered to the spores then 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 -15ºC to -20 ºC 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).

3.4.2. SEM

Dominant mycorrhizal fungal spores were examined under scanning electron microscopy (SEM). Selected AMF spores were sonicated under 35 MHz to followed by washing five times in sterile distilled water, surface disinfected with 4% (wt/vol) chloramine-T and 300 ppm of streptomycin for 1 h, and then rinsed a further five times in sterile distilled water and were stored in eppendorf's tube in room temperature. Each sample was placed within separate aluminium "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-r5 Kev.

3.5 Biochemical tests of microorganisms

3.5.1. Screening for phosphate solubilization

Primary phosphate solubilizing activities of bacterial isolates were carried out by allowing the bacteria to grow in selective medium i.e., Pikovskaya's agar (Himedia-M520; ingredients- yeast extract-0.50 g/l, dextrose- 10.00 g/l, calcium phosphate- 5.00 g/l, ammonium sulphate- 0.50 g/l, potassium chloride- 0.20 g/l, magnesium sulphate-0.10 g/l, manganese sulphate- 0.0001 g/l, ferrous sulphate- 0.0001 g/l and agar- 15.00 g/l) for 7 to 10 days at 37°C (Pikovskaya, 1948). The appearance of transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria.

3.5.2. Siderophore production

The bacterial isolates were characterized for siderophore production following the method of Schwyn and Neiland (1987) using blue indicator dye, chrome azurol S (CAS).

For preparing CAS agar, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM FeCl3.6H2O in 10 mM HCl) and volume made up to 1L. With constant stirring this solution was added to 72.9 mg hexa-decytrimethyl ammonium bromide (HDTMA), dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. The dye solution was mixed into the medium along the glass wall with enough agitation to achieve mixing without the generation of foam, and poured into sterile petriplates (20 ml per plate). The plates were inoculated with the bacteria and incubated for 10-15 days till any change in the color of the medium was observed.

3.5.3. HCN production

Production of hydrocyanic acid was determined using the procedure described by Reddy *et al.*. (1991) with slight modification. The selected bacterial isolates were grown at room temperature (37ºC) on a rotary shaker in nutrient broth(NB) media. Filter paper (Whatman no.1) was cut into uniform strips of 10 cm long and 0.5 cm wide saturated with alkaline picrate solution and placed inside the conical flasks in a hanging position. After incubation at 37ºC for 48 hr, the sodium picrate present in the filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved. The color was eluted by placing the filter paper in a clean test tube containing 10 ml distilled water and the absorbance was measured at 625 nm.

3.5.4. IAA production

For detection and quantification of IAA, the selected bacterial cells were grown for 24 h to 48 h in high C/N ratio medium. Tryptophane (0.1 mM) was added in order to enhance acetic acid (IAA) production by the bacteria (Prinsen *et al.*. 1993). Production of IAA in culture supernatant was assayed by Pillet-Chollet method as described by Dobbelaere *et al.*. (1999). For the reaction, 1 ml of reagent, consisting of 12 g $FeCl_3$ per litre in 7.9 M H_2SO_4 was added to 1 ml of sample supernatant, mixed well, and kept in the dark for 30 min at room temperature. Absorbance was measured at 530 nm.

3.5.5. 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.

3.5.6. Catalase activity

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

3.5.7. Protease production

Protease activity was detected on 3% (wt/vol) powdered milk-agar plates according to Walsh *et al.*. (1995).

3.5.8. H_2S Production

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

3.5.9Urease production

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

3.6. Fungal pathogen

3.6.1. 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.

3.6.2.1. 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 30ºC. 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. Czapek dox agar (CDA):

$NaNo_3$ - 0.20g, $KHPO_4$ - 0.10g, $MgSo_4.7H_20$ - 0.05g, KCl - 0.05g, $FeSo_4.7H_20$ - 0.05g, Sucrose - 3.00g, Agar - 3.00g, Distilled water - 100ml

C. Potato sucrose agar (PSA):

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

3.6.2.2. 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 28ºC. 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) and Richards medium 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 60ºC for 96 h, cooled in desiccators and weighed.

3.7. *In vitro* screening and evaluation of phosphate solubilizing activity of isolated microorganisms

3.7.1. 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±2ºC 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.

3.7.2. 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.

3.8. Antifungal tests against pathogens in Dual Plate Culture

3.8.1. Antifungal test of PGPR

The obtained bacterial isolates were evaluated against root pathogens- *F. solani* and *F. oxysporum* 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 28º±2ºC 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 *F. solani* and *F. oxysporum*, were selected for further evaluation and identification.

3.8.2. Antifungal test of BCA

The efficacy of BCA(*Trichoderma* sp.) isolated from mandarin rhizosphere was tested *in vitro* for inhibiting growth of the pathogen (*F. solani* and *F. oxysporum*) 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 replicate plates were used. The plates were incubated for 7 days (depending upon the growth of the pathogen) at 28ºC 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. Mass multiplication of microorganism and their application

3.9.1. Mass multiplication of AMF

Three hosts were selected for mass multiplication of AMF spores (maize, sorghum and turf grass). Spores of *C. reticulata* were multiplied in all three hosts but spores of *C. medica* and *C. limonia* were multiplied in maize only.AMF spores were isolated from rhizosphere of *C. reticulata*, *C. medica* and *C. limonia*as described.The mass of spores were washed with distilled water several times to remove the adhered debris. Filter paper was cut into small bits about the size of 1 cm. With the help of fine tweezers, 45-50 AMF spores were placed in the filter paper bits. They were then carefully placed onto the roots of the 7-10 days old host seedling in plastic pots (12inch) having autoclaved soil to discard the presence of other fungal propagules. Maize plants were grown both in the field and pots. After 45 days, the presence of spores were verified and inocula were prepared by mixing the chopped roots of maize plants with the potted soil where extra radical spores of AMF were present. Approximately > 175 spores / 100gms could be considered as potent inocula for application.

3.9.1.1. Single cell-line culture

In order to develop pure cell line culture of two specific AM fungi (*Glomus fasciculatum* and *Gigaspora gigantea*) following experimental set up were made using sterilized pre soaked sorghum seedlings. Sorghum seeds were kept in the plate over a wet filter paper and it was covered with black paper to avoid light. Within 96h seeds germinate and shoots came out from the open cut end. Selected AMF spore of *G. fasciculatum* and *Gi. gigantea* were carefully inoculated on the root surface and closed by black paper again.

Then 4-5 days after inoculation the small seedlings were transferred in pot having sterile soil-sand mixture (50: 50). After 90 days the desired spore of a single species were harvested. After harvesting the same process is repeated thrice to get the desired spore and to discard other entities. Once pure line culture is obtained, the same experiment was setup for mandarin seedlings. The inoculated seedlings were then maintained in glass house in sterilized soil (Fig. 4).

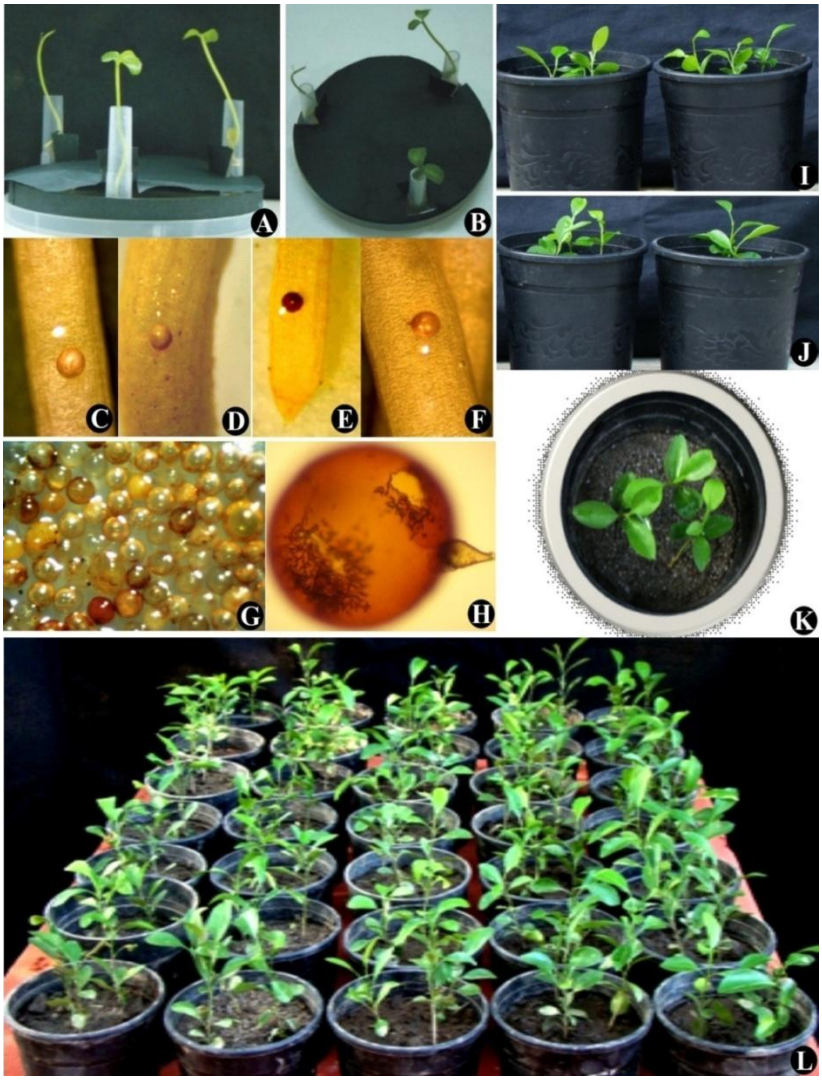


Fig 4: Single spore inoculation of AMF in mandarin roots. Experimental setup (A-F); mass of *Gigaspora* spore (G); Single *Gigaspora* spore (H); Maintenance of inoculated seedlings in sterilized soil (I-L).

3.9.2. Mass multiplication of BCA

3.9.2.1.Wheat bran culture

Inoculum of*Trichoderma asperellum* and *Trichoderma harzianum*were prepared by inoculating wheat bran (sterilized) with 5 mm disc of the fungus and incubating at 28 ºC for 10 days. To each pot of soil (2000 g), 10 g of the wheat bran colonized by *T. asperellum* and *T. harzianum*was mixed to give a concentration of 10^5 cfu / g of soil as described by Chakraborty *et al.*. (2003).

3.9.2.2. Tricho-compost

Six layers of compost materials (each layer about 25 cm thick) was made. 3 parts cellulosic waste (rice straw, grass, corn stalk, spent mushroom substrate) and 1 part mixture of leguminous plant materials (Mungbean, Peanut, Soybean) and animal manure was mixed. Each layer of piled compost materials was sprinkled with 30 litres of Tricho inoculants solution. Additional water is sprinkled to keep the compost heap moist. It was covered with plastic sheet or sack to increase temperature and prevent too much water in case of rainfall. Compost heap was turned from top to bottom after two weeks. The Tricho compost was ready for harvest four weeks after preparation. The compost was stored in sacks or applied directly into the soil (Fig 5).

Fig. 5 Tricho copmpost preparation. Pile of compost materials (A), Turning of compost (B), harvested Tricho-compost in sacks (C)

3.9.3. Mass multiplication of PGPR

3.9.3.1. Soil drench

The bacteria were grown in NB for 48 h at 28ºC 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 seedlings. 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.

3.9.3.2. 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.

3.9.3.3. Talc based formulation

Ten g of carboxy methyl cellulose sodium salt (Himedia) was mixed with one kg of talcum powder and pH was adjusted to 7.0 by adding calcium carbonate. It was then sterilized twice for 30 min each. The bacterium was first grown in nutrient broth and after 48 h the actively growing cells in log phase were harvested by centrifugation at 21 000 g, and aqueous suspension was made to achieve a concentration of 3x 10^9 CFU ml^{-1} which was determined spectrophotometrically. To 1 kg of sterilized talcum powder 400 ml of bacterial inoculum was added and mixed well under sterile condition. The talc mix was dried under shade to bring moisture to less than 20%. The formulation was packed in milky white colour polythene bags to eliminate UV exposure, sealed and stored at room temperature for future use. The talcum based formulation was applied in the field at the rate of 100 g per pot (12 $x10^{10}$ bacterial cells).

3.9.4. Preparation of inoculum of fungal pathogen

Inoculum of*Fusarium solani*and *Fusarium oxysporum* were prepared by inoculating wheat bran (sterilized) with 5 mm disc of the fungus and incubating at 28 ºC for 10 days. To each pot of soil (2000 g), 10 g of the wheat bran colonized by *F.*

*solani*and *F. oxysporum* was mixed to give a concentration of 10^5 cfu / g of soil as described by Chakraborty *et al.*. (2003).

3.10. Growth promotion studies following application of AMF and PGPR

3.10.1. Assessment of plant growth

Plant growth promotion was assessed by examining the plant at intervals of 1 month upto a period of 4 months. The growth parameters such as number of leaves, branches and height were observed.

3.10.2. Assessment of soil phosphate mobilization

3.10.2.1. Extraction of Soil phosphate

Soil sample (1g) was air dried and suspended in 25 ml of the extracting solution (0.025N H_2SO_4, 0.05N HCl) to which activated charcoal (0.01g) was also added, shaken well for 30 min on a rotary shaker and filtered through Whatman No. 2 filter paper (Mehlich, 1984). Quantitative estimation of phosphate was done following ammonium molybdate-ascorbic acid method as described by Knudsen and Beegle (1988).

3.10.2.2. Estimation of soil phosphate

For estimation, 2 ml aliquot of the soil extract was transferred to test tube along with the transfer of 2 ml aliquots of each of the working standards. Then 8 ml of the colorimetric working solution (25 ml conc. ammonium paramolybdate solution, 10 ml ascorbic acid soln., final volume- 1000ml) was added to each test tube and mixed thoroughly. They were allowed to wait for 20 mins for colour development. Finally, % transmittance of all standards and samples on a colorimeter with wavelength set at 882 nm was recorded.

3.11. In vivo assessment of disease

3.11.1. Assessment of root rot index

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).

3.11.2. Assessment of defense enzymes in leaves and roots

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

Estimation of β-1, 3-glucanase activity was done by following the laminarin dinitrosalicylate method described by Pan *et al.*. (1991). The crude enzyme extract of 62.5 µl was added to 62.5 µl of laminarin (4 %) and then incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexed and absorbance was recorded at 500 nm. 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 tissue.

3.11.2.2. Chitinase (E.C. 3.2.1.14)

Chitinase activity was measured according to the method described by Boller and Mauch (1988). Assay mixture consisted of 10µl of 1M Na-acetate buffer (pH4), 0.4 ml enzyme solution and 0.1 ml colloidal chitin. Colloidal chitin was prepared as per the method of Roberts and Selitrennikoff (1988). Incubation was done for 2 hrs at 37°C and centrifuged at 10,000 r.p.m for 3 min. 0.3 ml supernatant, 30µl of 1M K-PO_4 buffer (pH7.1) and 20µl Helicase (3%) were mixed and allowed to incubate for 1 h at 37°C. 70µl of 1M Na-borate buffer (pH9.8) was added to the reaction mixture. The mixture was again incubated in a boiling water bath for 3 min and rapidly cooled in ice water bath. 2 ml DMAB (2% di methyl amino benzaldehyde in 20% HCl) was finally added and incubated for 20 min at 37°C.The amount of GlcNAc released was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as µg GlcNAc released /min/ g fresh wt. tissue.

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

Enzyme was extracted by the method described by Chakraborty *et al.*. (1993). Leaf samples were crushed in liquid nitrogen and extracted using 5 ml of sodium borate buffer (pH8.8) containing 2 mM β mercaptoethanol in ice followed by centrifugation at 15000 rpm for 20 min at 4°C. The supernatant was collected and after recording its volume, used immediately for assay or stored -20°C.

3.11.2.4.Peroxidase (E.C. 1.11.1.7)

For the extraction of peroxidase 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 β mercaptoethanol under ice cold conditions. The homogenate was centrifuged immediately at 15000 rpm for 20 minutes at 4ºC. After centrifugation the supernatant was collected and after recording its volume was immediately used for assay or stored at -20ºC (Chakraborty *et al.*.1993).

3.11.2.5. Extraction and estimation of phenols

3.11.2.5.1. Extraction of phenol

Phenol was extracted from the fresh young leaves and roots following the method of Mahadevan and Sridhar (1982). One g of sample were cut into pieces and immediately immersed in 10 ml of boiling alcohol. After 15 minutes of boiling it was cooled and crushed in mortar using pestle thoroughly at room temperature. The extract was filtered through Whatmann No. 1 filter paper. Final volume was adjusted with 80 % ethanol. The whole extraction of phenol was done in dark to prevent light induced degradation of phenol.

3.11.2.5.2. Estimation

3.11.2.5.2.1. Total phenol

Total phenol content was estimated by Folin Ciocalteau's reagent, following the method of Mahadevan and Sridhar (1982). To 1 ml of the alcoholic extract, 1 ml of 1 N Folin Ciocalteau's reagent followed by 2 ml of 20 % sodium carbonate solution was added in a test tube. The test tube was shaken and heated on a boiling water bath for 1 minute. After cooling, the volume of the reaction mixture was raised to 25 ml. Absorbance of the blue colored solution was measured at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.11.2.5.2.2. O-phenol

O-dihydroxy phenol was also estimated following the method of Mahadevan and Sridhar (1982). 1 ml of alcoholic extract was mixed with 2 ml of 0.05 N HCl, 1 ml of Arnow's reagent ($NaNO_2$ - 10 g, Na_2MoO_4 - 10 g, distilled water - 100 ml) and 2 ml of 1 N NaOH and mixed thoroughly at room temperature following which the volume of the reaction mixture was raised to 10 ml. Absorbance of the colored solution was recorded at

515 nm. Quantity of the O- dihydroxy phenol was estimated using caffeic acid as standard.

3.12. Isozyme analysis of peroxidase

Polyacrylamide gel electrophoresis (PAGE) was performed for isozyme analysis of peroxidase. Extract for isozyme analysis was prepared by crushing 1 g of leaf tissue in a mortar and pestle in 2 M sodium phosphate buffer (pH 7.0) in ice cold condition as described by Davis (1964) and used immediately for the isozyme analysis.

1. Preparation of the stock solution

Solution A: Acrylamide stock solution (Resolving gel)

For the preparation of acrylamide stock solution for resolving gel 28 g of acrylamide and 0.74 g of N' N' methylene bisacrylamide was dissolved in 100 ml of distilled water. The stock solution was filtered with Whatman No. 1 filter paper and stored at 4°C in dark bottle.

Solution B: Acrylamide stock solution (stacking gel)

For the preparation of acrylamide stock solution for stacking gel 10 g of acrylamide and 2.5 g of N' N' bisacrylamide was dissolved in 100 ml of distilled water. The stock solution was filtered and stored at 4°C in dark bottle.

Solution C: Tris- HCl (Resolving gel)

36.6 g of Tris base was mixed with distilled water and 0.25 ml of N, N, N', N'-tetramethyl ethylene diamine (TEMED) was added. The pH was adjusted to 8.9 with concentrated HCL. The volume of the solution was made up to 100 ml with distilled water. The solution was then stored at 4°C for further use.

Solution D: Tris- HCl (Stacking gel)

5.98 g of Tris base was mixed with distilled water and 0.46 ml of TEMED and the pH was adjusted to 6.7 with concentrated HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was stored at 4°C for further use.

Solution E: Ammonium persulphate solution (APS)

Fresh solution of APS was prepared by dissolving 0.15 g of APS in 10 ml of distilled water.

Solution F: Riboflavin solution

Fresh solution of Riboflavin was prepared by dissolving 0.4 mg of riboflavin in 10 ml distilled water. The solution was kept in dark bottle to protect from light.

Solution G: Electrode buffer

Electrode buffer was prepared freshly by dissolving 0.6 g of Tris base and 2.9 g glycine in 1 L of distilled water.

2. Preparation of gel

For the polyacrylamide gel electrophoresis of peroxidase isozymes mini slab gel was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease and then dried. 1.5 mm thick spacers were placed between the glass plates on three sides and these were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. 7.5 % resolving gel was prepared by mixing solution A: C: E: distilled water in the ratio of 1: 1: 4: 1 by pipette leaving sufficient space for (comb + 1 cm) the stacking gel. This resolving gel was immediately over layered with water and kept for polymerization for 2 hours. After polymerization of the resolving gel was complete, over layer was poured off and washed with water to remove any unpolymerized acrylamide. The stacking gel solution was prepared by mixing solutions B: D: F: distilled water in the ratio of 2: 1: 1: 4.

Stacking gel solution was poured over the resolving gel and comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30- 45 minutes in strong sunlight. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was now finally mounted in the electrophoretic apparatus. Tris- Glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

3. Sample Preparation

Sample (32 µl) was prepared by mixing the sample enzyme (20 µl) with gel loading dye (40 % sucrose and 1 % bromophenol blue in distilled water) in cyclomixture in ice cold condition. All the solutions for electrophoresis were cooled. The samples were

immediately loaded in a predetermined order into the bottom of the wells with a microlitre syringe.

4. Electrophoresis

Electrophoresis was performed at constant 15 mA current for a period of 3 - 4 h at 4°C until the dye front reached the bottom of the gel.

5. Fixing and Staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally stained. Staining of the gel was performed following the method of Reddy and Gasber (1973).

The gel was incubated in the aqueous (80 ml) solution of Benzidine (2.08 g), Acetic acid (18 ml), 3 % H_2O_2 (100 ml) for 5 minutes. The reaction was stopped with 7 % Acetic acid. After the appearance of clear blue colored bands, analysis of isozyme was done immediately.

3.13. Extraction and estimation of soluble proteins

3.13.1. 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$\pm$ 1ºC. 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 4ºC and then centrifuged at 10.000rpm for 30 min, at 4ºC to eliminate cell debris. The supernatant was equilibrated to 100% saturated ammonium sulphate under constant stirring in ice bath and kept overnight at 4ºC. After this period, the mixture was centrifuged (10,000rpm) for 30 minute at 4º 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 -20ºC and used as antigen from the preparation of antiserum and other experiment.

3.13.2. Leaf

Soluble protein was extracted from mandarin leaves following the method of Chakraborty *et al..*, (1995). Leaf 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 PVP was added during crushing and centrifuged at 4ºC for 20 min at 12000rpm. The supernatant was used as crude protein extract.

3.13.3. 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 PVP was added during crushing and centrifuged at 4ºC for 20 min at 12000rpm. The supernatant was used as crude protein extract.

3.13.4. 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).

3.14. 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

3.14.1. Preparation of stock solution

Following stock solution were prepared

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

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 4ºC 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 4ºC 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 4ºC 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.

3.14.2. 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.

3.14.3. 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.

3.14.4. 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.

3.14.5. 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 ºC 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 ºC with constant shaking until the background become clear.

3.15. Immunological studies

3.15.1. Preparation of antigen

3.15.1.1. 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 ºC and then centrifuged at 10,000rpm for 30 min at 4 ºC to eliminate cell debris. The supernatant was collected and stored in -20 ºC and used as antigen for the preparation of antiserum.

3.15.1.2. 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 PVP was added during crushing and centrifuged at 4ºC for 20 min at 12000 rpm. The clear supernatant was used as antigen.

3.15.1.3. AMF antigen

Spores of *G. mosseae* and *Gi. gigantea* were isolated from rhizosphere soil of mandarin by wet sieving and decanting method as described before. With the help of a dissecting microscope parasitized spores, plant debris etc were separated and clean spores of *G. mosseae* and *Gi. gigantea* were isolated. Spores were sonicated with 0.1% normal saline under the frequency range of 70-75 mhz as impulse. The supernatant was used as antigen source.

3.16.2. Raising of polyclonal antibodies

3.16.2.1. 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.

3.16.2.2. 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.

3.16.2.3. 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 60º, 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 37ºC 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 -20ºC as crude antisera. The serum was used for double diffusion analysis, dot blots analysis and Enzyme Linked Immunosorbent Assay (ELISA).

3.16.3. Purification of IgG

3.16.3.1 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 20º C in magnetic stirrer. The precipitate thus formed was collected by centrifugation at 12,000 rpm for 1h at 22 º C for 1 h. Supernatant was discarded and pellet was used for further steps.

3.16.3.2 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.

3.16.3.3 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).

3.16.4. Immunological assays

3.16.4.1. Agar gel double diffusion

3.16.4.1.1. 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 º 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.

3.16.4.1.2. 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 25ºC for 72h. Precipitation reaction was observed in the agar gel only in cases where common antigen was present.

3.16.4.1.3. 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 50º C.

3.16.4.2. 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 ºC 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 ºC 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 4ºC 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 ºC 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.

3.16.4.3. 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 ºC 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 37ºC. 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.

3.16.4.4. Western blot analysis

Protein sampleswere 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 (*F. solani* and *F. oxysporum*) 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.

3.16.4.5. 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). Both FITC and RITC were done to locate AMF spores in soil and observe root colonization and cellular location of AMF which was mass multiplied in mandarin roots following colonization with AMF.

3.16.4.5.1. Fungal mycelia

Fungal mycelia were grown in liquid potato dextrose 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.

3.16.4.5.2. Cross section of mandarinroots 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 and leaf sections were observed using Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and photograph was taken.

3.16.4.5.3. AMF in mandarin root

Roots of maize plants (4 months) and mandarin plants (2 years old) in which AMF spores of mandarin were mass multiplied were macerated according to Philips and Hayman method as described by with a few modification. Antigen was given in the dilution 1:50 goat antirabbit IgG after washing with PBS pH 7.2 thrice. The roots were incubated overnight in dark. The next day, the roots were again washed thrice with PBS-Tween and fluorescein isothiocyanate (FITC) and rhodamine isothiocyanate (RITC) was added in the dilution 1:10 and incubated for 45 minutes in dark. The roots were again

washed thrice in PBS and mounted in 10% glycerol in grease free slides. 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.

3.16.4.5.4. AMF spores

Glomus and *Gigaspora* spores along with hyphae were carefully separated from the root and washed. Selected spores were taken in grooved slides and left overnight in antibacterial solution. They were washed thrice with PBS pH 7.2. Antigen was given separately with a dilution of 1:40 and left overnight. The next day the spores were again washed thrice with PBS-Tween. FITC and RITC were given separately at a dilution of 1:10 and again left overnight. Finally, the next day, spores were mounted in grease free slides in 10% glycerol and observed under Leica Leitz Biomed Microscope with fluorescence optics equipped with UV- filter set I-3 and photograph was taken.

3.17. 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.

3.17.1. 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

3.17.2. 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.

3.17.3. 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^oC 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.

3.17.4. 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.

3.17.5. 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.

3.17.5.1. 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.

3.17.5.2. 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.

3.18. RAPD PCR analysis

For RAPD, random primers were selected (Table-1). PCR was programmed with an initial denaturing at 94^{o}C for 4 min. followed by 35cycles of denaturation at 94^{o}C for 1 min, annealing at 36^{o}C for 1 min and extension at 70^{o}C for 90 s and the final extension at 72^{o}C for 7 min. in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µ1) 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.

3.18.1. RAPD primers

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

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC
RAPD primers				
OPA1	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%
AA-04	CAGGCCCTTC	10	38.2	70%

3.18.2. Amplification conditions

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

3.18.3. Analysis of RAPD bands

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

3.18.4. 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.

3.18.5. 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.

3.18.6. 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.

3.19. 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^{o}C for 5 min. followed by 30 cycles of denaturation at 94^{o} C for 30 sec, annealing at 59^{o}C for 30 sec and extension at 70^{o}C for 2 min and the final extension at 72 ^{o}C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8 µ1) 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.

3.19.1. ITS- PCR primers

The following primers were used to amplify ITS regions:

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC	Amplic aon size (bp)	References
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%		

3.19.2. Amplification conditions

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

3.19.3. 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.

3.19.4. Sequence analysis

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

3.19.5. Chomatogram of sequence

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

3.19.6. Editing and alignment of sequence data

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

3.19.7. 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.

3.19.8. 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.

3.20. Extraction and Assay of defense enzyme activity

3.20.1. 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 ºC using mortar and pestle. The extract was then centrifuged at 10000 rpm for 15 min at 4 ºC and the supernatant was used as crude enzyme extract.

Estimation of the ß-1,3-glucanase was done by following theLaminarin 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 40ºC for 10 minutes. The reaction was stopped by adding 375µl dinitrosalicylic reagent and heating for 5 min in 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.

3.20.2. 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 ºC 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 ºC.

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.

3.20.3. 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 ßmercaptoethanol in ice cold temperature. The slurry was centrifuged in 15000 rpm for 20 minutes at 4ºC. Supernatant was collected and after recording its volume, was immediately used for assay or stored at -20ºC.

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 ºC 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.

3.20.4. 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 ºC. After centrifugation the

supernatant was collected and after recording its volume was immediately used for assay or stored at -20 ºC (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_2 O_2 (Chakraborty *et al..*, 1993). Specific activity was expressed as the increase in absorbance at 460 nm g^{-1} tissue/ min $^{-1}$.

3.21. Transmission Electron Microscopy

3.21.1 Specimen preparation

3.21.1.1 Fixation

Control and inoculated root samples (1-2 mm) were excised in 0.1M sodium phosphate buffer pH 7.4. They were immediately transferred to 2.5% Glutaraldehyde in eppendorf tubes for 2-12 hours at room temperature.

3.21.1.2 Dehydration

Dehydration was done in ascending grades of alcohol at intervals of 30 mins in 4° C (30%, 50%, 70%, 80%, 90%) and two changes in absolute alcohol at 1 hr interval each at 4° C in PLT-272(M) Fume Hood (Tanco).

3.21.1.3 Infiltration

Infiltration was done twice in LR White resin (London Redin Co. Ltd) in absolute alcohol (1:1) for 1 hr each at 4° C.

3.21.1.4 Embedding

The samples were dipped in LR White and kept overnight at 4° C. They were kept at room temperature for 3 hrs. A fresh change of LR white was done and kept at 56° C for 36 hrs.

3.21.2 Viewing preparation

3.21.2.1 Trimming

Moulds containing the samples were roughly trimmed with a block trimmer (Reichert TM 60) fitted with a rotating milling cutter.

3.21.2.2 Sectioning

A series of thick sections of the selected blocks were cut with Belgium glass strips in microtome (Leica EM UC7) to observe under an optical microscope. These semithin sections are stained with 1% aqueous toluidine blue solution. These sections can be viewed in light microscope.

3.21.3 Immunogold labeling

Ultrathin sections (60nm) were cut with fresh Belgium glass strips and picked up in nickel grids (100 mesh) for immunogold labeling.

3.21.3.1Primary antibody

The grids containing ultrathin sections were floated in blocking solution containing 2% skimmed milk agar for 30 min. Primary antibody was diluted in 1% fish gelatin in the ratio 1:20. Grids were incubated the PAbs for 24 hrs at 4° C. Grids were washed on drops (100 µl) of fish gelatin pipetted on to parafilm 10X2 min.

3.21.3.2 Secondary antibody

Grids were incubated with anti-rabbit IgG (Whole Molecule) gold antibody produced in goat affinity isolated antibody (Sigma-G7402) diluted in 1:5 in fish gelatin at room temp for 3 hrs.

3.21.3.3 Staining

Sections were stained with 2% uranyl acetate for 15 min. The sections were washed in double distilled water. Post stain was done in 0.2% lead acetate for 5 min. Washed again in double distilled water.

3.21.3.4 Viewing

Ultrastructural analysis of the section was performed with Morgagni 268D with iTEM Imaging System. Specificity of labeling was assessed by the control test by incubating sections with rabbit pre-immune serum instead of the primary antibody.

Chapter 4

Results

4.1. Association of Arbuscular Mycorrhizal fungi in Citrus plants

Arbuscular mycorrhizal fungal spores from the three citrus species *viz.*Darjeeling mandarin (*Citrus reticulata*), Lime (*C. medica*) and Rangpur lime (*C. limoni*) were isolated from different regions of Darjeeling hill areas, Mirik[27° 04'04.74" N 88°11'27.66" E], Kalimpong [27°03'31.34"N 88°28'00.05"E], Bijanbari[26°53'09.47"N 88°10'58.05" E], Kurseong [26°53'54.34"N 88°16'38.59" E], and foothills[26° 42'36.03" N 88°21'05.20" E].Their average spore population and percent root colonization were determined. Spores were identified morphologicallyby examiningtheir variation in size, colour, wall thickness, shape, wall layers specially germinal wall, coriaceous wall, amorphous wall and beaded wall layers, hyphal branching patterns, the diameter, structure and the staining intensity of hyphae.

4.1.1 *Citrus reticulata*

Among the AM fungi, *Gigaspora* sp. especially *Gi. gigantea* and *Gi. margarita* was found to be the dominant genus in mandarin rhizosphere during the winter season; but during the summer season, *Glomus* sp. dominated the spore population from all the soil samples collected from different mandarin orchards. Among the *Glomus* species, *G. mosseae*, *G. fasciculatum* and *G. aggregatum* were dominant. *Scutellospora* and *Acaulospora* were the less common genus found in the soil with a few occurance of *Entrophospora* sp.. *Gi. gigantea*, *G. mosseae*, *G. fasciculatum*, *Scutellospora rubra* and *Acaulospora* sp. were the most common spore found throughout the year in all the soil samples.*G. mosseae*, *G. fasciculatum* and *G. aggregatum* were the most dominant spoes found in the soil from Kurseong whereas *Gi. gigantea*and *G. mosseae* were dominant spores in soil from Kalimpong, Mirik and Bijanbari. Percentage colonization of spores per 100 gm of soil have been represented in Fig (6). According to seasonal variation (summer, monsoon and winter) colonization percentage of spores have been represented respectively in Table 1,2 and 3. Microscopical view of the spores are shown in Fig (7).

Morphological description of spores obtained from the rhizosphere of mandarin plants have been described in Table 4.

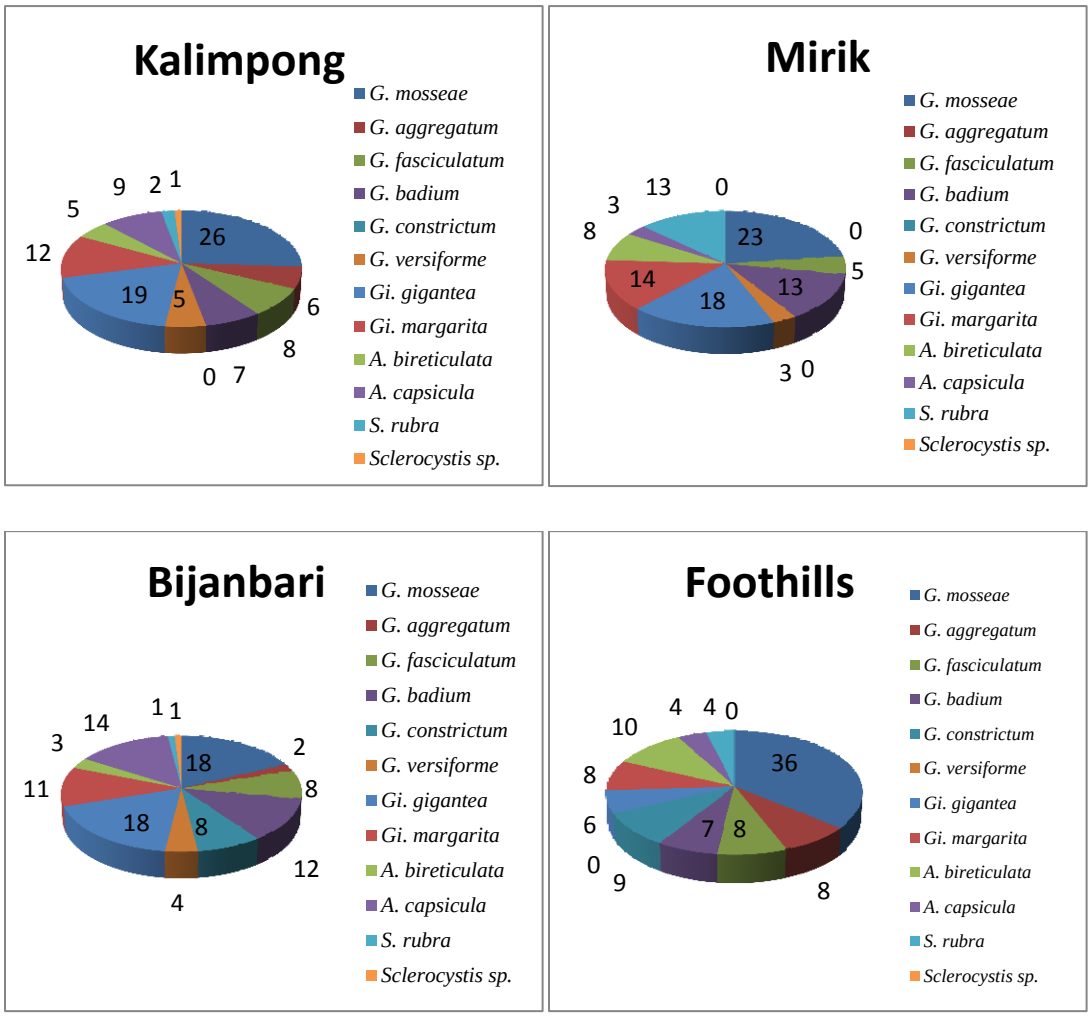


Fig. 6 Percentage population of AMF spores in *Citrus reticulata* of Darjeeling hills per 100 gm of soil

4.1.2 *Citrus medica*

AMF association in *C. medica* from three different sites of Padmaja Park of University of North Bengal was studied.Average spore population and percent root colonization was determined. In *C. medica* the association of *Glomus* sp. was more abundant than when compared to *C. reticulata*. Over all spore count showed four different kinds of *Glomus* species to be dominant in all the soil samples. Among them *G. fasciculatum* and

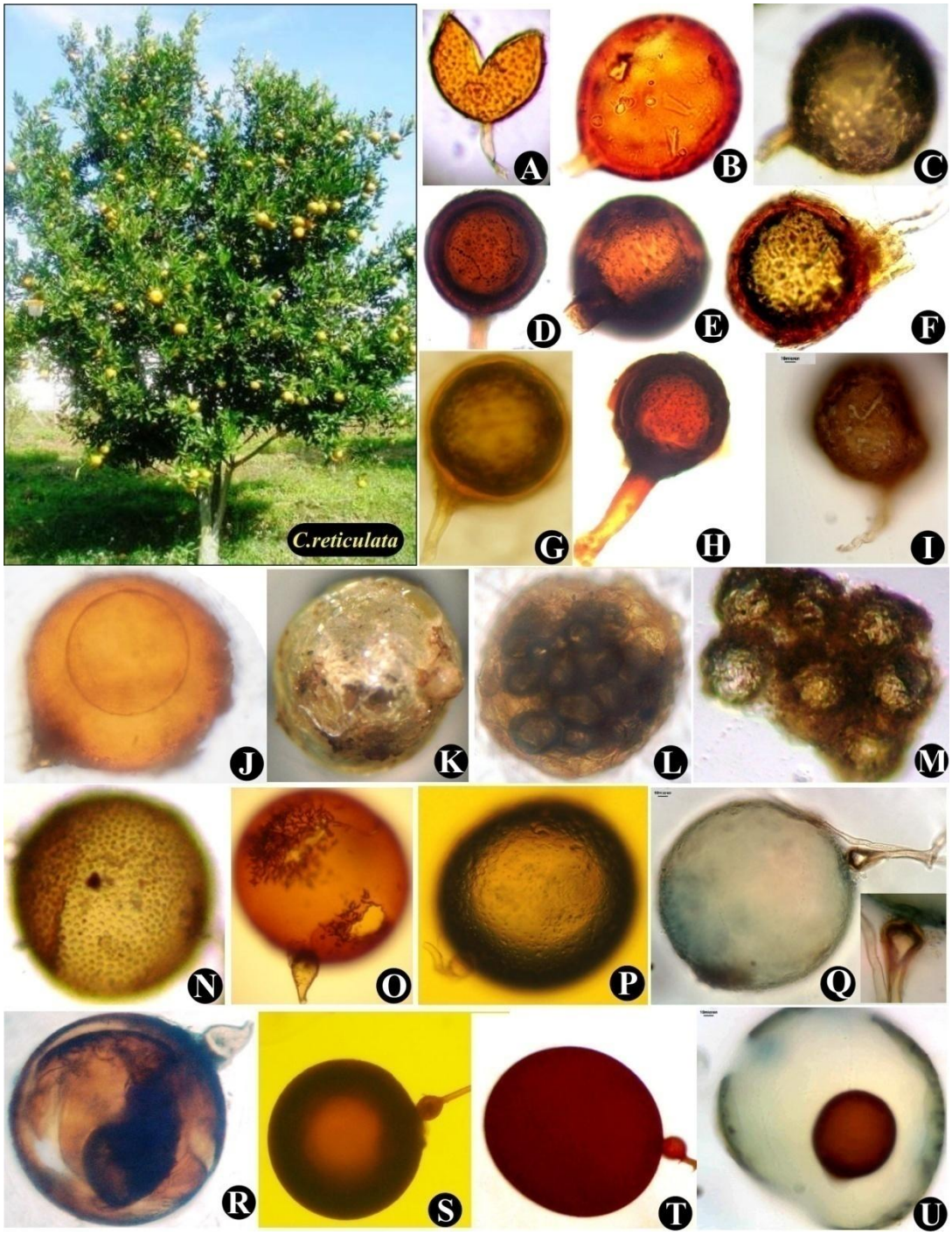


Fig. 7 . Compound microscopic observations of arbuscular mycorrhizal fungal spores obtained from C. *reticulata* plants. (A)broken spore of G*lomus* sp.; (B)G. *ambisporum*;(C)G. *mosseae*; (D)G. *fasciculatum*; (E)*G.constrictum*; (F)G. *ambiosporum*; (G)G*lomus* sp.; (H)G. *badium*; (I)*G. mosseae*; (J)*Glomus* sp; (K)G*igaspora* sp.; (L&M)*Sclerocystis sinuosum*; (N)A*caulospora* sp.; (O)*Gi. margarita*; (P)*Gi.gigantea*; (Q)*Gi. gigantea*; (R)G*igaspora* sp.; (S&T)S. *reticulata*; (U)S. *rubra*

G. mosseae were most abundant. The genus *Acaulospora* comprises of *A.bireticulata, A.capsicula* and *A.delicata*. Among *Gigaspora*, species of *Gi. gigantea* and *Gi. margarita* are common and few unidentified spores of Acaulospora. *Scutellospora* are fewer in comparison to others and presence of Sclerocystis was rare. Microscopical views of the spores are presented in Fig 8.

4.1.3 *Citrus limonia*

AMF association in *C. limonia* from three different sites of Padmaja Park of University of North Bengal was studied.Average spore population and percent root colonization was determined. In *C. limonia* also, the association of *Glomus* sp. was more dominant. *Glomus fasiculatum, Glomus mosseae, G. ambisporum, G. multicaule, Gigaspora gigantea, Gi. margarita, Gi. albida, Acaulospora cavernata, A. bireticulata* and *Scutellospora sp.* are some of the common spores found in *C. limonia*. Microscopical views of the spores are presented in Fig 9. Average population of AMF spores per 100 gm of soil obtained from *Citrus reticulata, C. limonia* and *C. medica* plants found grown in foot hills have been presented in Fig 10.

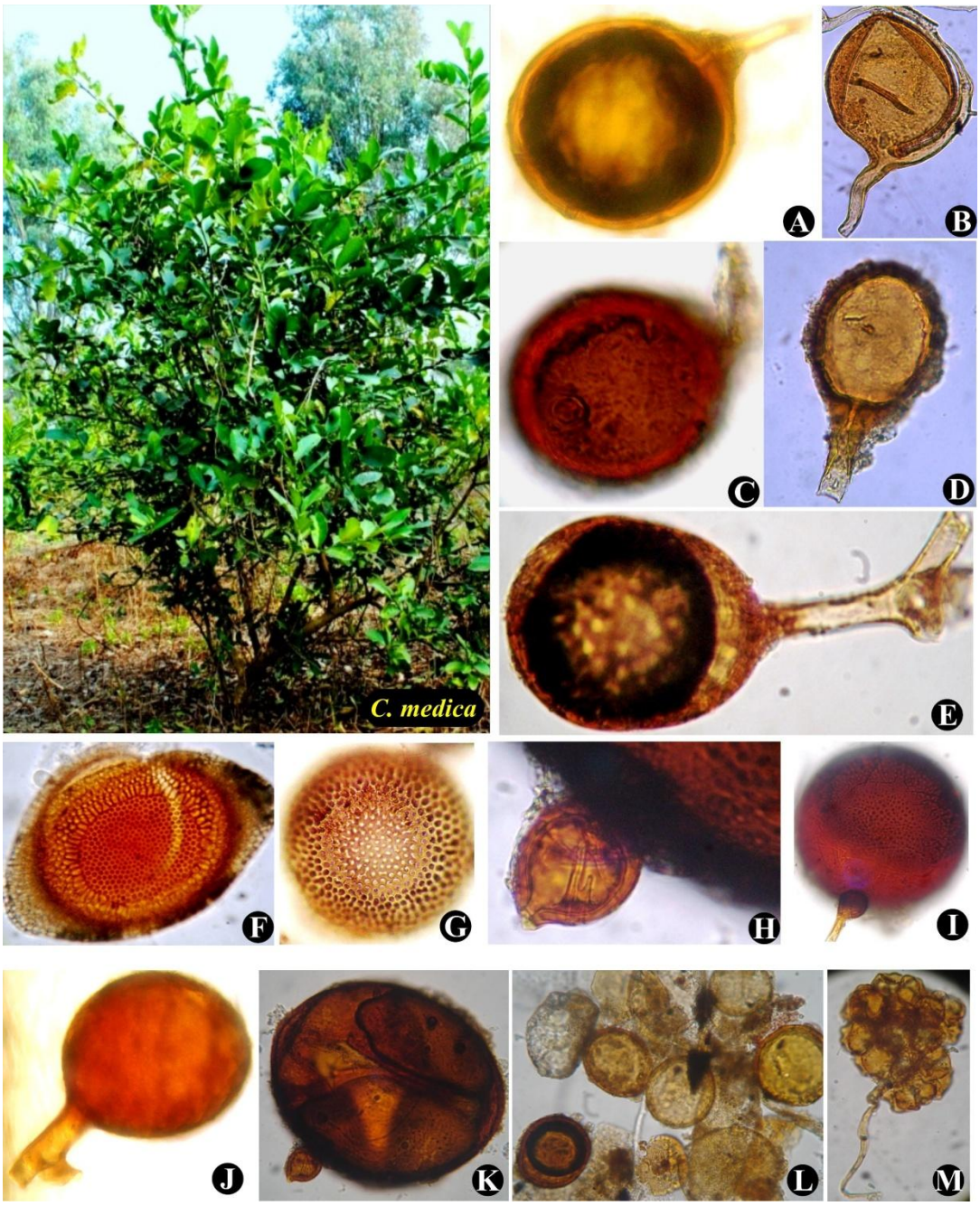


Fig. 8. Compound microscopic observations of Arbuscular Mycorrhizal Fungal spores obtained from *C. medica* plants. (A)*Glomus*; (B)*G. mosseae*; (C)*Glomus* sp.; (D)*G. mosseae*; (E)*G. ambisporum*; (F)*Entrophospora* sp. (G)*A. bireticulata*; (H)Close up of bulbous suspensor of *Gi. albida*; (I)*Gi. albida*; (J)Glomus sp.; (K)*Gi. gigantea*; (L)*Rhizophagus aggregatus*; (M)*Sclerocystis sinuosum*

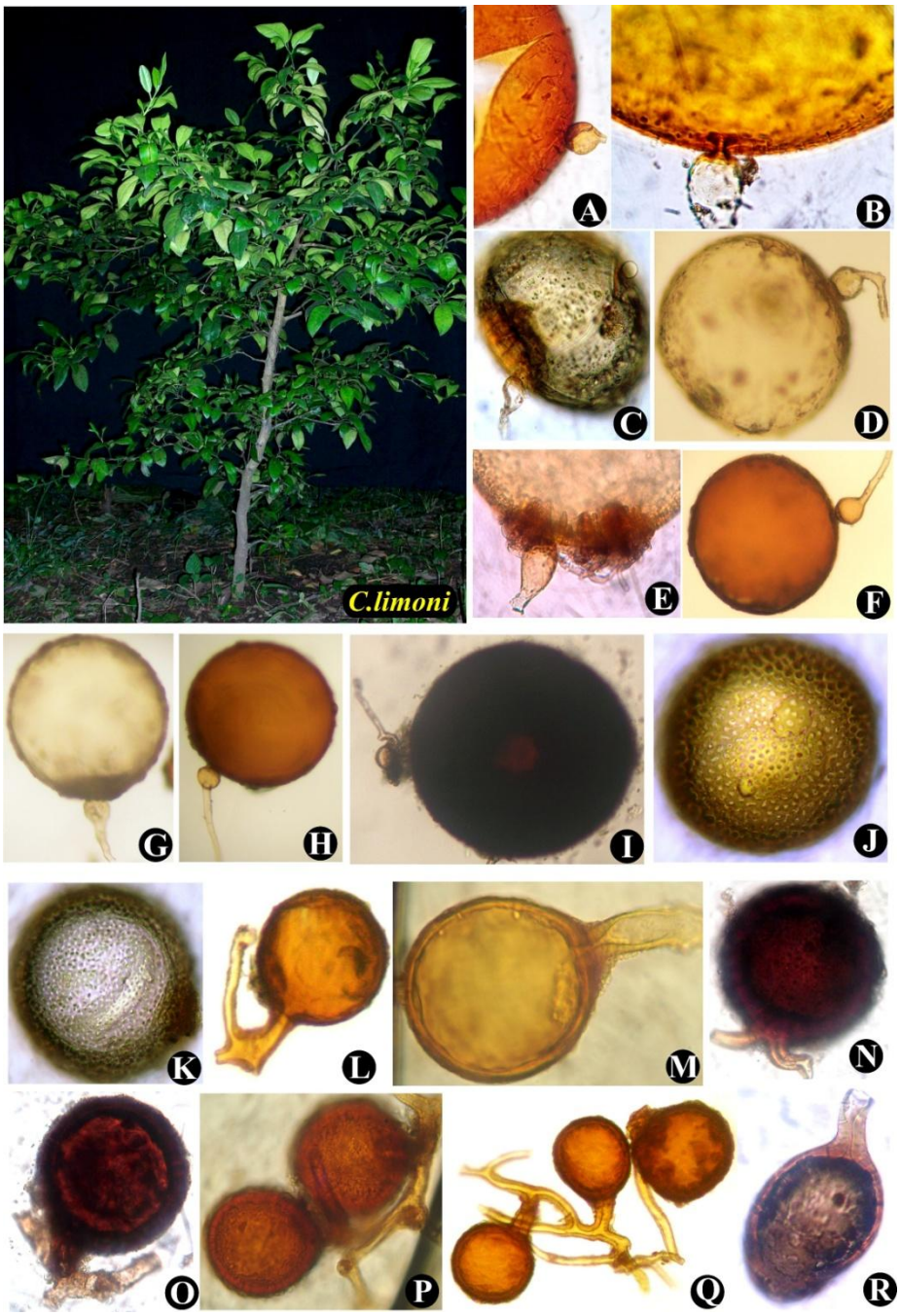


Fig. 9. Compound microscopic observations of Arbuscular Mycorrhizal Fungal spores obtained from *C. limonia* plants. (A)Broken spore of *Gi. decipiens*; (B)Sporogenous cell of *Gi. gigantea*; (C)*G. mosseae*; (C)*Gi. margarita*;(D)*Gi. decipiens*; (E)Bulbous suspensor of *Gi. albida*; (F & H)*Scutellospora reticulata*; (G)*Gi. margarita*; (I)*Gigaspora* sp.; (J)*A. bireticulata*; (K)*A. cavernata*; (L)*Glomus* sp.; (M)*G. mosseae*; (N)*Glomus multicaule*;(O)*G.ambisporum*; (P)*Glomus* sp.; (Q)*G. fasciculatum*; (R)*Glomus* sp.

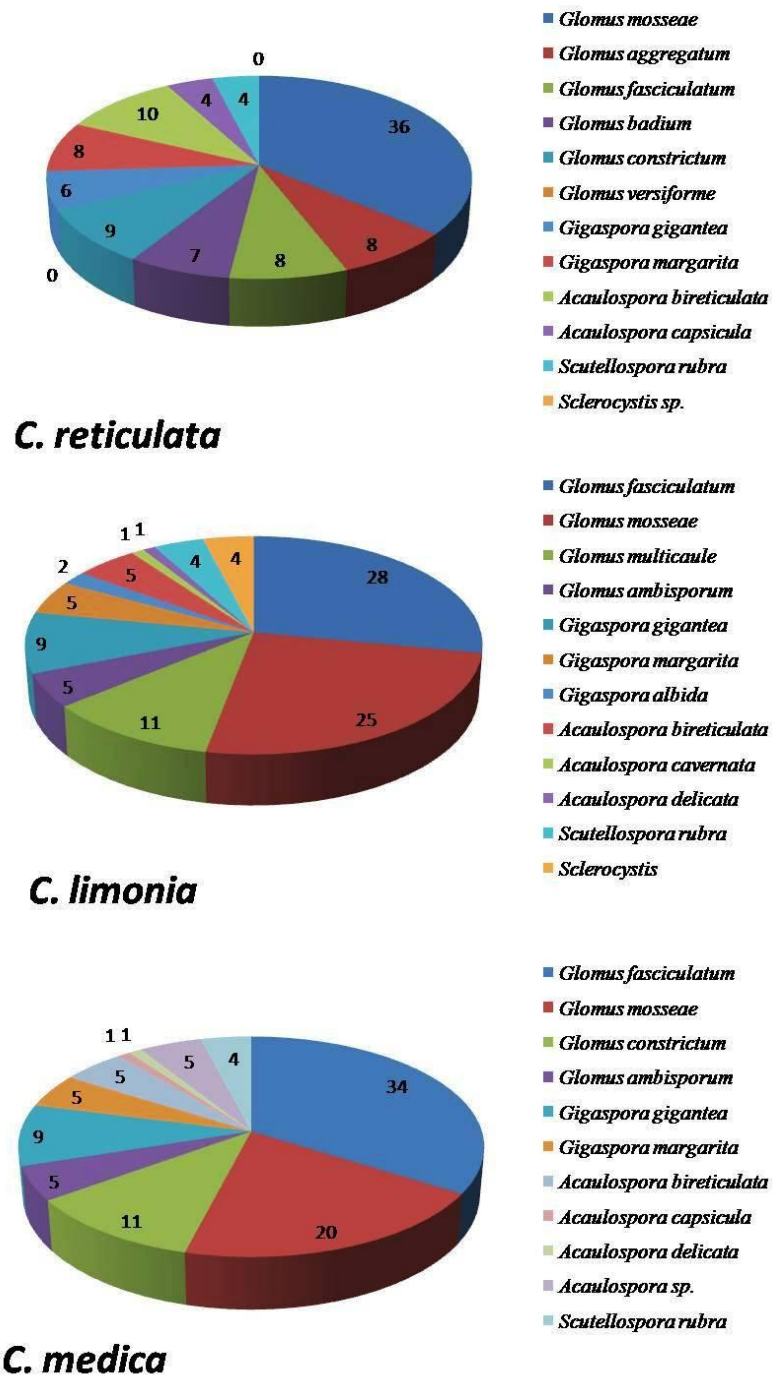


Fig. 10. Percentage population per 100 gm of soil of dominant AMF spores of *Citrus reticulata*; *C. limonia* and *C. medica* plants found grown in foot hills.

Table 1. Percentage of spores in 100gm soil in summer season

Name of AMF	**Location**				
	Kalimpong 27°03'31.34"N 88°28'00.05"E	Mirik 27° 04'04.74" N 88°11'27.66" E	Bijanbari 26°53'09.47"N 88°10'58.05" E	Kurseong 26°53'54.34"N 88°16'38.59" E	Foothills 26° 42'36.03" N 88°21'05.20" E
Glomus mosseae	26±0.471	23±0.471	18±1.41	30±0.942	36±0.471
Glomusaggregatum	06±0.471	00±0.000	02±0.471	23±0.472	08±0.471
Glomus fasciculatum	08±0.942	05±0.942	08±0.471	16±0.472	08±0.470
Glomusbadium	07±0.471	13±0.471	12±0.470	00±0.000	07±0.471
Glomusconstrictum	00±0.000	00±0.000	08±0.471	00±0.000	09±0.942
Glomusversiforme	05±0.471	03±0.942	04±0.470	00±0.000	00±0.000
Gigaspora gigantea	19±0.942	18±0.942	18±0.471	12±0.942	06±0.470
Gigaspora margarita	12±0.471	14±0.471	11±0.470	10±0.471	08±0.471
Acaulosporabireticulata	05±0.471	08±0.471	03±0.471	04±0.471	10±0.470
Acaulosporacapsicula	09±0.471	03±0.470	14±0.471	00±0.000	04±0.471
Scutellosporarubra	02±0.471	13±0.471	01±0.272	05±0.943	04±0.942
Sclerocystis sp.	01±0.272	00±0.000	01±0.272	00±0.000	00±0.000

±=SE

Table 2. Percentage of spores in 100gm soil in monsoon season

Name of AMF	Location				
	Kalimpong 27°03'31.34"N 88°28'00.05"E	Mirik 27° 04'04.74" N 88°11'27.66" E	Bijanbari 26°53'09.47"N 88°10'58.05" E	Kurseong 26°53'54.34"N 88°16'38.59" E	Foothills 26° 42'36.03" N 88°21'05.20" E
Glomus mosseae	10±0.471	18±0.577	12±1.24	14±1.15	23±1.73
Glomus aggregatum	08±0.471	00±0.00	02±1.15	08±1.73	08±0.471
Glomus fasciculatum	15±0.942	09±0.471	14±0.95	18±1.15	10±0.47
Glomus badium	04±0.471	05±0.63	12±0.99	02±0.98	07±0.63
Glomus constrictum	00±0.00	00±0.00	08±0.69	00±0.00	09±0.69
Glomus versiforme	03±0.471	02±0.58	04±0.63	00±0.00	00±0.00
Gigaspora gigantea	29±0.942	23±0.577	18±0.57	30±1.15	13±1.15
Gigaspora margarita	16±0.471	15±0.272	11±0.58	10±1.73	11±0.98
Acaulospora bireticulata	04±0.470	12±0.942	03±0.73	04±0.69	10±0.69
Acaulospora capsicula	07±0.471	03±0.272	02±0.77	00±0.00	04±0.95
Scutellospora rubra	03±0.470	13±0.115	13±1.15	14±0.63	05±0.73
Sclerocystis sp.	01±0.272	00±0.98	01±1.73	00±0.00	00±0.00

±=SE

Table 3. Percentage of spores in 100gm soil in winter season

Name of AMF	Location				
	Kalimpong 27°03'31.34"N 88°28'00.05"E	Mirik 27° 04'04.74" N 88°11'27.66" E	Bijanbari 26°53'09.47"N 88°10'58.05" E	Kurseong 26°53'54.34"N 88°16'38.59" E	Foothills 26° 42'36.03" N 88°21'05.20" E
Glomus mosseae	14±0.471	23±0.471	18±1.41	30±0.942	36±0.471
Glomus aggregatum	06±0.471	00±0.000	02±0.471	23±0.472	08±0.471
Glomus fasciculatum	18±0.942	05±0.942	08±0.471	16±0.472	08±0.470
Glomus badium	06±0.471	13±0.471	12±0.470	00±0.000	07±0.471
Glomus constrictum	00±0.000	00±0.000	08±0.471	00±0.000	09±0.942
Glomus versiforme	03±0.471	03±0.942	04±0.470	00±0.000	00±0.000
Gigaspora gigantea	21±0.942	18±0.942	18±0.471	12±0.942	06±0.470
Gigaspora margarita	12±0.471	14±0.471	11±0.470	10±0.471	08±0.471
Acaulospora bireticulata	05±0.471	08±0.471	03±0.471	04±0.471	10±0.470
Acaulospora capsicula	05±0.471	03±0.470	14±0.471	00±0.000	04±0.471
Scutellospora rubra	09±0.471	13±0.471	01±0.272	05±0.943	04±0.942
Sclerocystis sp.	01±0.272	00±0.000	01±0.272	00±0.000	00±0.000

±=SE

Table 4. Description of AM Fungi found in rhizosphere of citrus plants

AMF spore	Colour	Size (µm)	Shape	No. of wall layers	Other characteristics
Glomus mosseae(Nicol. & Gerd.) Gerd. & Trappe	Brown to orange-brown	200	Globose to sub-globose	3	Hyphae double layered
Glomus badiumOehl, Redecker & Sieverd	Brownish orange to reddish brown	200-500 x 290-260	Mainly ovoid to irregular sometimes globose to sub-globose	3	Spores formed blastically at the tip of hyphae developing from a hyphal plexus positioned in the centre of sporocarp
Glomus fasciculatum(Thaxt.) Gerd. & Trappe emend. C. Walker & Koske	Pale yellow to bright brown	70-120	Globose to sub-globose	3	Spores produced directly with one or more subtending hyphae
Glomus aggregatumN.C. Schenck & G.S. Sm. emend. Kosk**e**	Pale yellow	200-1800 x 200-1400	Globose to oval	1-2	Sporocarps formed in loose clusters
Glomus intraradiscesN.C. Schenck & G.S. Sm.	White pale cream to yellow brown	40-140	Globose to sub-globose	3	Outer two layers only present in young spores, both thin and degrade with spore maturation.
G. versiforme	Orange to re-brown in sporocarp, white to white cream-coloured to pale orange yellow in pot cultures	60-160	Globose to subglobose sometimes ovoid	2	Recurved septum positioned at the innermost sublayer of the laminate layer, giving the appearance of being inserted.
Glomus ambisporum Smith & Schenck, sp.nov.	Dark brown to black	315-690 X 424-776	Subglobose to highly variable	2-3	Outer wall hyaline
Glomus constrictum Trappe	Brownish orange to dark brown	160	Globose to sub-globose	1-2	Usually markedly constricted at the base of spore

Glomus intraradiscesN.C. Schenck & G.S. Sm.	White to pale cream, sometimes yellow brown	40-140	Globose to sub-globose	3	single subtending hyphae attached with the spore
Glomus multicauleGerdemann and Bakshi	Brownish orange to dark brown	149-249 X 124-162	Ellipsoid, broadly ellipsoid, subglobose or triangular	1	Subtending hyphae varies from 1-4, thick ornamented spore
Gigaspora gigantea(Nicol. & Gerd.) Gerd. & Trappe	Greenish yellow	260-318	Globose to subglobose sometimes ovoid	2	Spores formed from bulbous sporogenous cell.
Gigaspora margarita W.N. Becker & I.R. Hall	Yellowish white to sunflower yellow	320-370	Globose to subglobose sometimes ovoid	2	Spores produced singly at the tip of bulbous sporogenous cell
Gigaspora roseaT.H. Nicolson & N.C. Schenck	Pale cream with pale pink tint in new healthy spores	160-280	Globose to subglobose	3	Auxiliary cells in aggregates of 4-20 borne on tightly coiled hyaline hyphae
Gigaspora decipiens (Hall & Abbot)	Bright white to cream to yellow-brown	280-440	Globose to subglobose	3	Multiple germ tubes arise from areas where papillae are mostly concentrated usually near the sporogenous cell
Gigaspora albida (Reference accession BR214)	Cream with pale green tint	200-280	Globose to subglobose	3	Germ tubes form in vicinity of warty protruberances on inner surface of layer 3 of the spore wall
Acaulospora spinosaC. Walker & Trappe	Cream to pale orange brown	140-220	Globose to subglobose	2	Spores formed from sporiferous saccule.
Acaulospora bireticulataF.M. Rothwell & Trappe	Brownish	280-410	Globose	3	Spores borne laterally from neck of sporiferous saccule
Acaulospora capsicula Blaszk.	Bright red or orange red	150-300	Globose to subglobose	3	No presence of subtending hyphae

Acaulospora delicate C. Walker, C.M. Pfeiff. & Bloss	Pale yellow with green tint	80-100	Globose to subglobose	2	Layer 2 thickens by formation of pale yellow sub layers
Acaulospora spinosaC. Walker & Trappe	cream to pale orange brown	140-220	Globose to subglobose	2	Layer 2 thickens by formation of pale yellow sub layers followed by synthesis of closely packed rounded spines.
Scutellospora rubra Stürmer & J.B. Morton	Dark orange brown to red brown	340-640	Globose, subglobose often elliptical	2	Spores develop blastically from a hyphal tip which swells to form the sporogenous cell
Scutellospora pellucida (T.H. Nicolson & N.C. Schenck) C. Walker & F.E. Sanders	Hyaline to yolk yellow	130-155 x 160-235	Globose to subglobose	2	No presence of subtending hyphae
Scutellospora rubra Stürmer & J.B. Morton	Dark orange brown to red brown at maturity	180	Globose to subglobose	2	No presence of subtending hyphae
Scutellospora calospora (Koske & C. Walker) C. Walker & F.E. Sanders	Pale yellow with a greenish tint	120-220	Subglobose to oblong, sometimes irregular	2	No presence of subtending hyphae
Archaeospora trappeiR.N. Ames & Linderman.	Completely hyaline, rarely creamy white	40-80	Globose to subglobose	3	Single subtending hyphae attached with the spore

4.1.4. Scanning Electron Microscopy of dominant AMF spores

Scanning electron microscopic observation was made of three most abundant genera *vizGlomus*, *Gigaspora* and *Acaulospora*. Different species of *Glomus*, *Glomus mosseae*, *Glomus constrictum* and *Glomus intraradisces* obtained from mandarin rhizosphere showed difference in their wall characters and ornamentations. The basal attachments of all the different species showed distinct variation in their shape and size from one another (Fig. 11 &12).Distinct Bulbous suspensor of*Gigaspora gigantea*reveals the outer hyaline layer and the conspicuous curved hyphal attachment(Fig. 11 B) whereas adhered hyphae of *Glomus fasciculatum*(Fig. 12 B) and *Acaulospora* sp.(Fig. 12 E&F) is shownwith its sloughed and eroded outer hyaline layer covering the whole surface area. *Gi. gigantea* shows few pores in the spore surface where MHB (Mycorrhiza

Helper Bacteria) might persist. Image shows attachment of bulbous suspensor of *Gigaspora* sp. (Fig. 11 E & F) attached to the spore wall. *Acaulospora bireticulata* with ornamentation consists of hyaline to round-tipped polygonal structures and the attached sporiferous sacule (Fig.12 E & F).

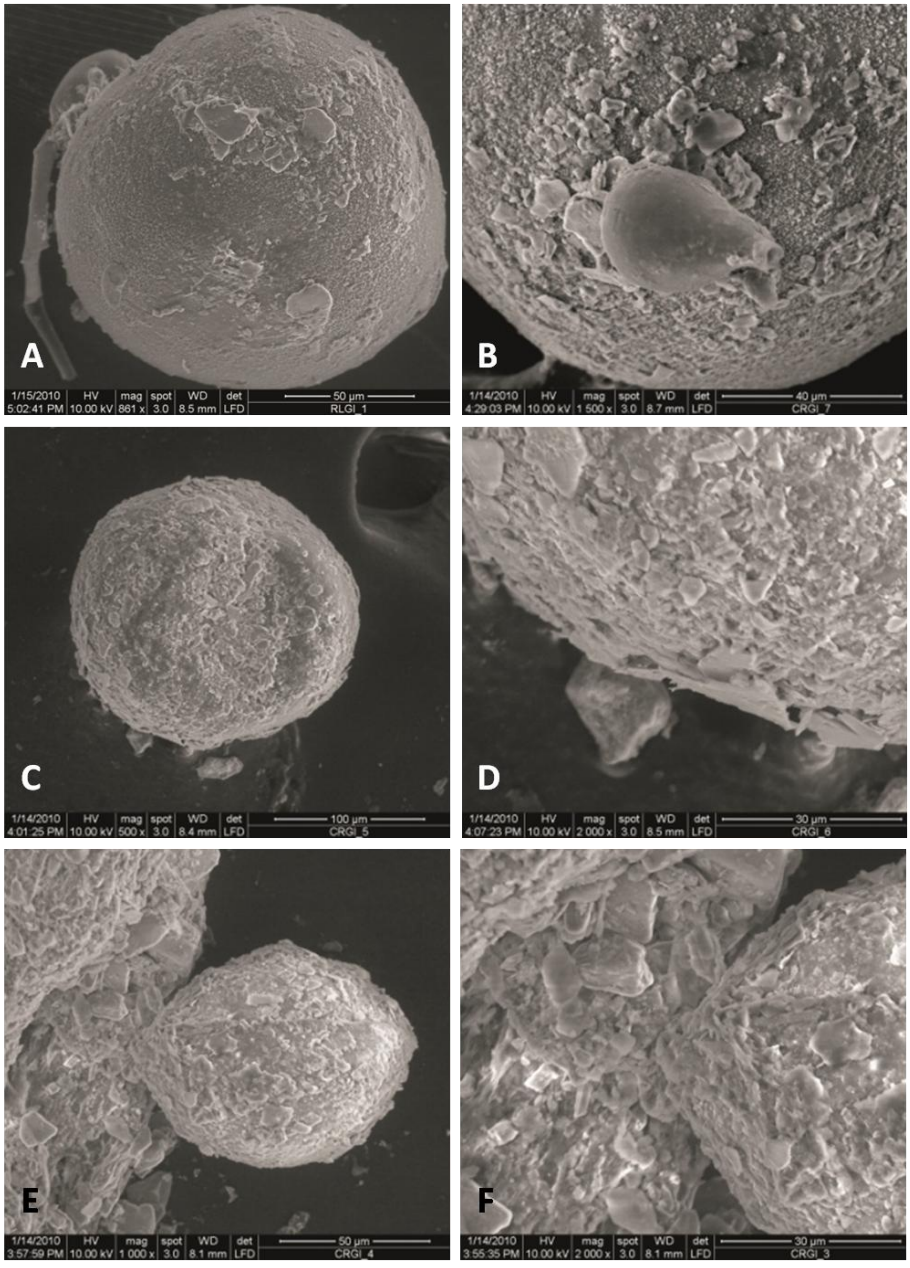


Fig. 11. SEM of *Gigaspora* sp. spores of *C. reticulata*. (A-B) *Gigaspora gigantea*, (C-D) *Gi. margarita*, (E-F) close up of bulbous suspensor of *Gigaspora* sp.

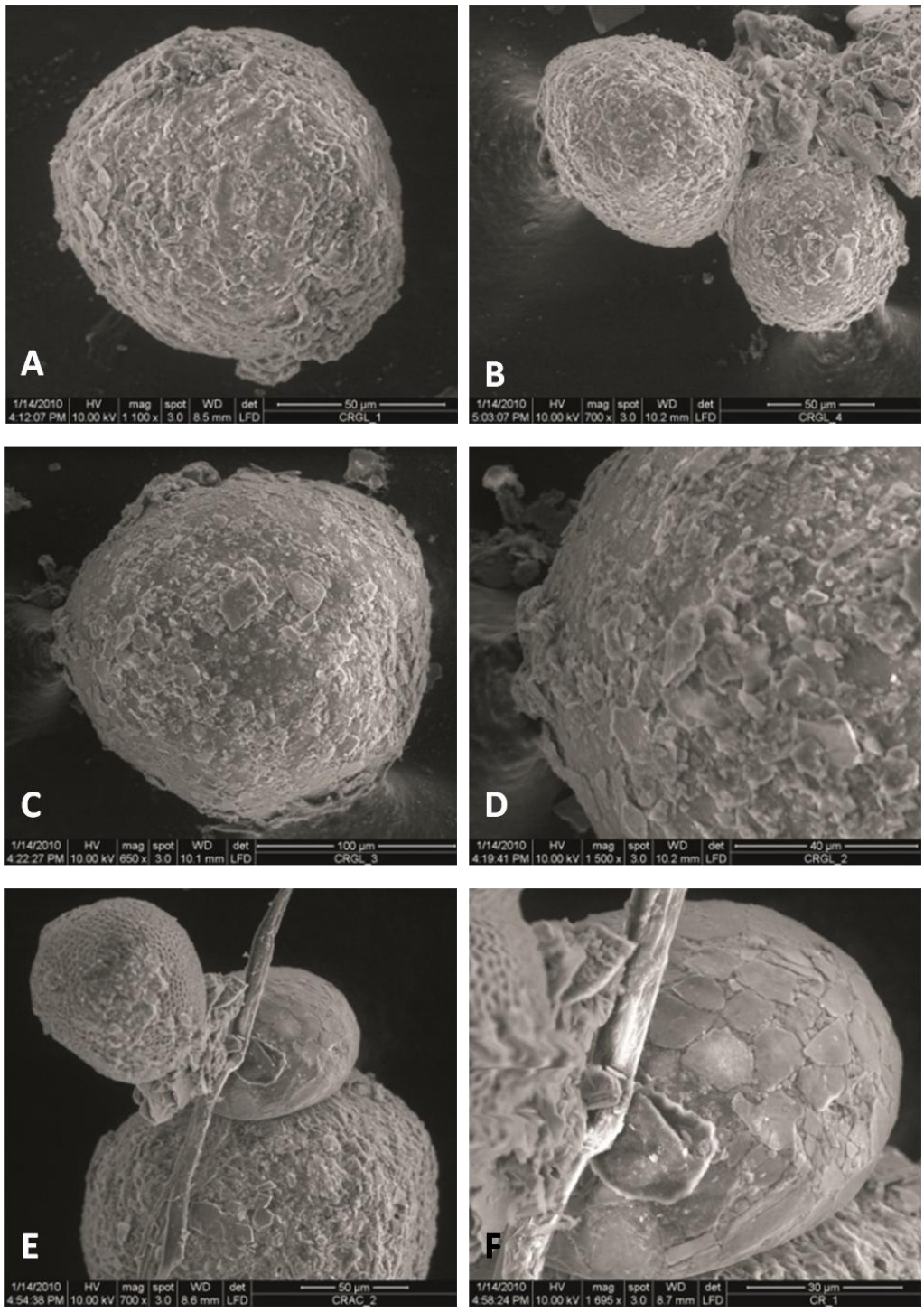


Fig. 12. SEM of dominant AMF spores of *C. reticulata*. (A & B) *G. fasciculatum;* (C & D) *Glomus* sp.; (E & F) *Acaulospora bireticulata*

4.2. Histopathology and root colonization in citrus plants

Mycorrhization pattern of the roots and percent root colonization of all three citrus plants were determined.Plant species differ in the pattern of AMF root colonization. Since variations in vesicles, hyphal branching patterns, structure of hyphae and staining intensity of hyphae are different for each genus, it is possible to identify Glomeromycotan fungi upto genus level but it is difficult to separate species.

4.2.1 *Citrus reticulata*

Root samples of mandarin plants from Kalimpong, Mirik, Bijanbari, Kurseong and foothills were carefully examined to study the histopathological variation. Histopathological colonization patterns are represented in Fig. 13. About 72-75 percent root colonization was found in 2-3 year old seedlings while 85-89 percent colonization was observed in 7-8 years old trees. Abundant filamentous structures known as extraradical hyphae were observed. They comprise the fungal thallus (body) in the soil. These Extraradical hyphae bear profuse spores. Mature spores were found attached to the roots. The hyphae penetrated the root surface at entry points. Extrametrical hyphae with single oil-filled spores were observed. Presence of profuse arbuscules was observed. Oval shaped and flattened vesicles (Fig 13 E & I) present in abundance. Subglobose oil-filled structures (Fig.13 D) have a thicker wall than typical vesicles suggesting they may be intraradical spores. These vesicles took dark stain. Vesicles are formed by hyphal swellingsthat may be both inter or intra cellular. Cells of mandarin root are very small so it is quiet difficult to observe the hyphal pattern, structure of arbuscules and infection peg. But after extensive study, young and mature arbuscules and thick irregularly coiled hyphae were observed. Both arum and paris type of hyphae are present that suggests the presence of both *Glomus* and *Gigaspora* infestation. Arbuscules were visible only under high magnification (100). Dark Septate Endophyte (DSE) were rarely present in root cortex (Table 5). DSE also formed arbuscules which are called sclerotia. These dark septate hyphae and the sclerotia formed do not take the blue stain.

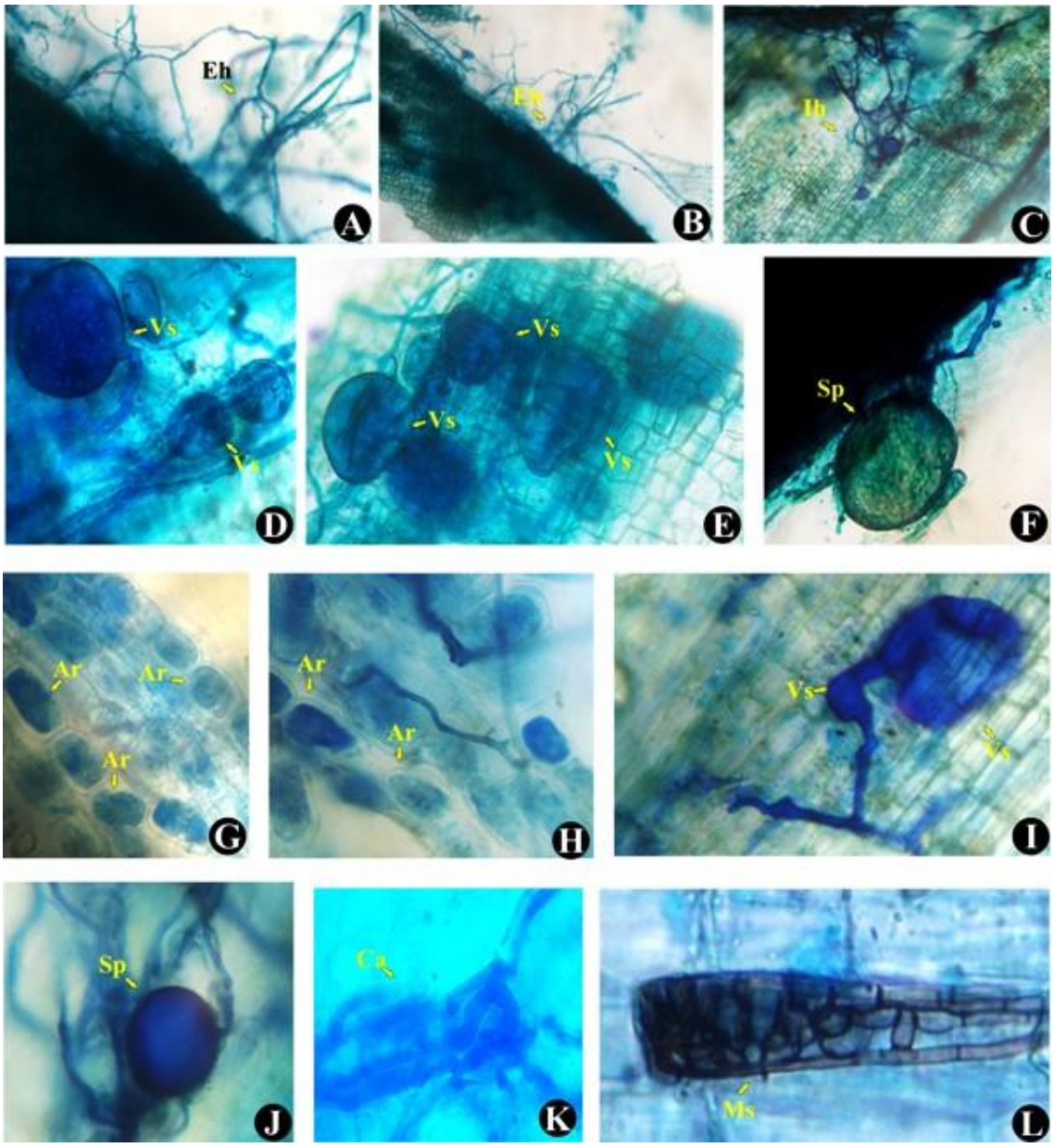


Fig. 13. Histopathological study of *C. reticulata* showing vesicles, hyphae, arbuscules. (A-B) Extraradical hyphae, (C) Intraradical hyphae, (D) Oil-filled structure, (E) Oval vesicles (F) mature spore attached to hyphae, (G-H) Arbuscules, (I) Flattened vesicle, (J) Extraradicle spore, (K) Coiled hyphae, (L) Microsclerotia

4.2.2 *Citrus medica*

Presence of numerous extraradical hyphae. Both intercellular and intracellular hyphae ramify along the whole length of the plant root. Mostly, *Arum*-type arbuscular mycorrhizal association observed. Lemon shaped vesicle (Fig 14 F) with vacuole and oil globules present. Appresoria with infection pegs were clearly seen. The regions along the root at which appresoria form and where hyphae enter the epidermis are referred to as entry points. Often an appressorium forms between epidermal cells (Fig 14 H) and hyphae formed from the appressorium penetrate adjacent epidermal cells. Finely branched spreading arbuscules with thick trunk were present in abundance. Such straight intraradicular hyphae and fine spreading arbuscules are characteristic of Glomus species. Vesicles are globose shaped with presence of oil droplets and is the most characteristic cytological features of mature vesicles. Vesicles frequently enlarge to occupy the entire volume of the cell. The hyphal tip swells to form vesicles. A few DSE and melanized hyphae with haustorium were also observed. These melanized hyphae run in parallel with intraradicular hyphae. Microsclerotia (Fig. 14 N) and sclerotia (Fig 14 M) formed by melanized hyphae. Fine endophyte mycelium traversed the root length. Percent colonization is 76-78% in all the root samples observed.

4.2.3 *Citrus limonia*

The root system showed diverse characters of colonization. Both Arum and Paris type hyphae are abundant in all the varieties. In Arum type association hyphae proliferated in the cortex longitudinally in the root system. Paris type hyphae have spread and formed several coiled structures. Both intra radical and extra radical hyphae are present. Profuse thin extraradical highly branched hyphae with single spores attached to roots (Fig 15 D). These thin hyphae are also known as “absorptive” hyphae. Oval shaped vesicles which are darkly stained and irregular shaped vesicles present in plenty (Fig.15 G, H). Rectangular vesicles (Fig 15 J) are also present in a few number. Presence of such vesicles indicate the occurance of Acaulosporaceae spores. Dark Septate Endophyte colonized roots of *C. limonia*.

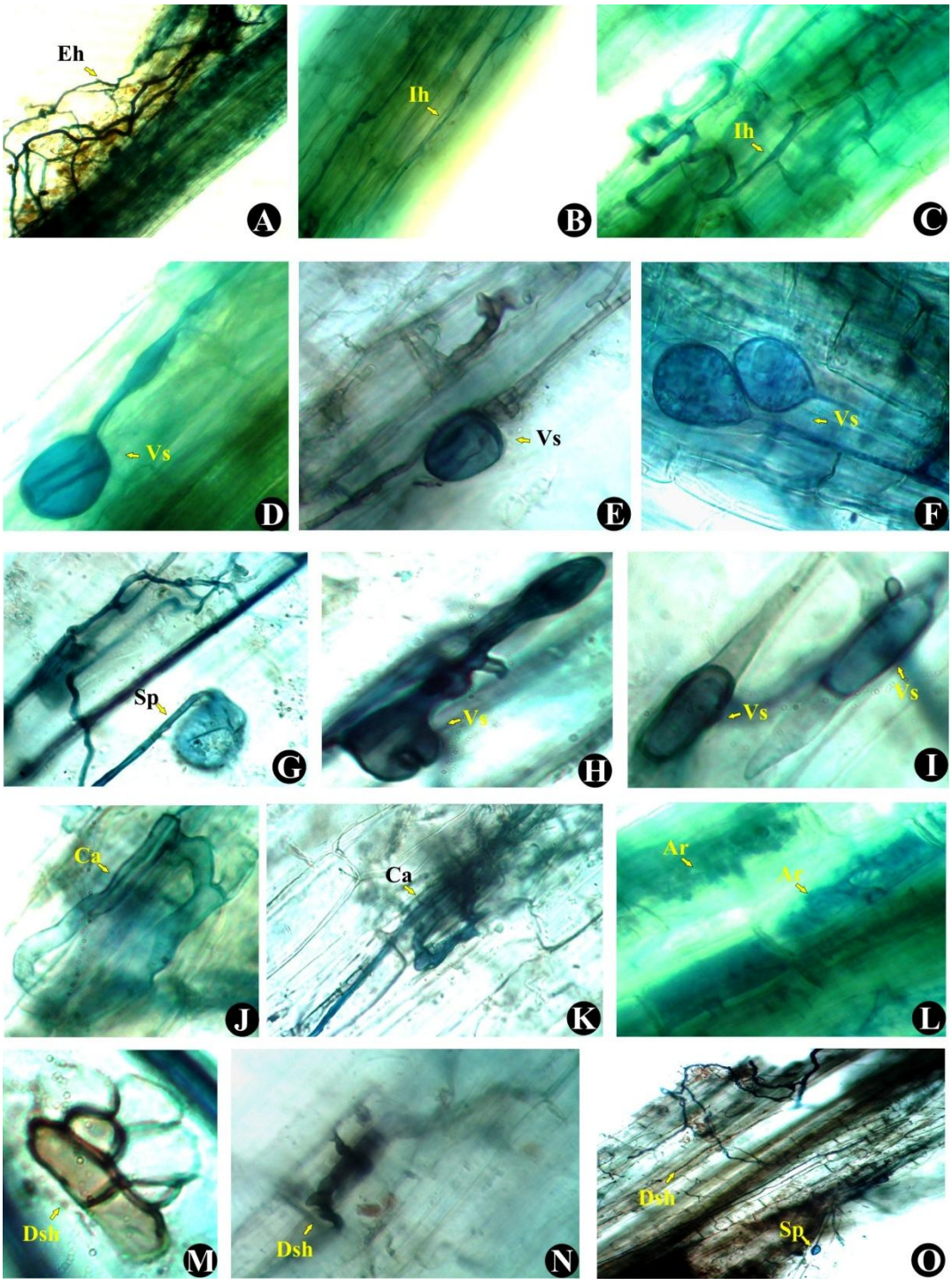


Fig. 14. Histopathological study of *C. medica (*A-C) Extraradical, Intraradical and interradical hyphae, (D-F) vesicles, (G) Intraradical hyphae with spore, (H) mature vesicle with infection nearby, (I) vesicles, (J-K) coiled arbuscule, (L) arbuscules, (M-O) dark septate hyphae

The colonization percent of DSE is 10-15 in all root specimen observed. The DSE wit microsclerotia of different shapes and sizes were highly melanized (Fig. 15 L-N). Microsclerotia formed by DSE are evident which looks like cluster of grapes. Two types of microsclerotia were observed. One was linear with a single row of small grape-like structure (Fig 15 M) while the other was a cluster which more or less looked like Sclerocystis (Fig. 15 N). Mature microsclerotia did not show well developed zone of tissue. The central part was made up of pseudoparenchymatous tissue but he hyphal nature exists. Towards the outside of the microsclerotia the hyphae were more loosely arranged (Fig 15 L). Morphology of DSE of *C. limonia* differed from those found in *C. medica* roots. Percent colonization is 75-80 % in all the studied root samples. Few arbuscules (Fig 15 K) composed of highly coiled hyphae also present.

4.3. Mass multiplication of AMF in different host plants

AMF were isolated from rhizosphere of *C. reticulata, C. medica* and *C. limonia*. Spores were observed under dissecting microscope and dead sporeswere discarded. Fresh clean spores were selected and mass multiplied in maize (*Zea mays*), sorghum (*Sorghum bicolor*) and turf grass (*Cynodon dactylon*) respectively. These three different plants were identified to be host plants for mass multiplication as AMF more efficiently colonize with the roots of these plants. Root maceration technique was again followed to observe the colonization in roots. Maize plants were grown in field (Fig. 16 A & B) and also maintained in pots in sterilized soil (Fig 16 C & D) to discard other fungal propagules but sorghum (Fig. 17 A) and turf grass (Fig. 17 B) were maintained only in pots in sterilized soil.

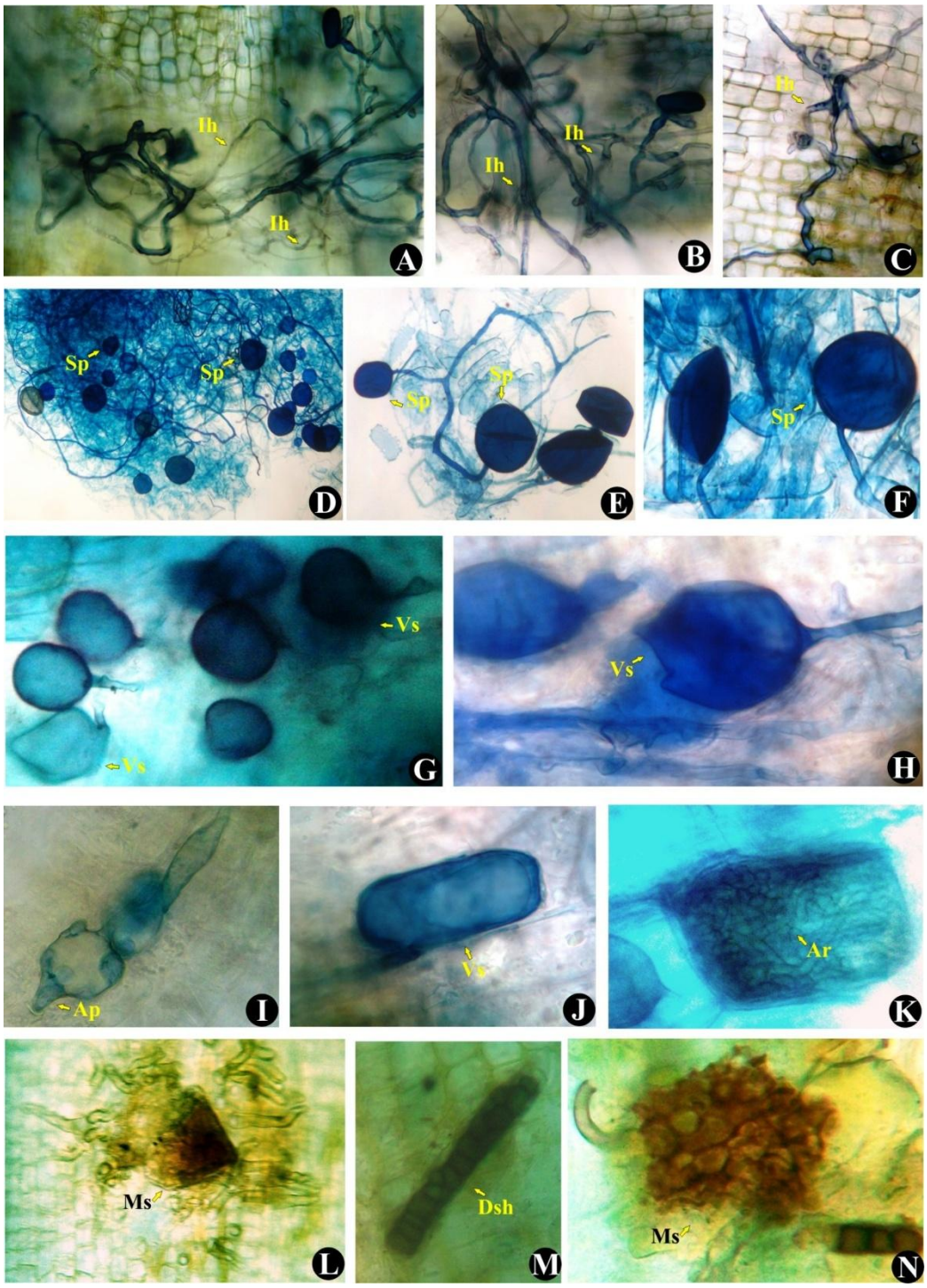


Fig. 15. Histopathological study of *C. limonia* showing different shapes of vesicles, hyphae, arbuscules and dark septate hyphae. (A-C) Extraradical hyphae (D-F) Extraradical thin hyphae with single spores, (G-H) Oval shaped vesicles, (I) Appresorium, (J) Rectangular shaped vesicle, (K) Arbuscles, (L&N) Microsclerotia (M) Dark septate hyphae

Table 5. AMF and DSE associations of citrus plants

Citrus plants	No. of spores / 100gm soil	Percent colonization	No. of Vesicles/root	Vesicle / cm root	DSE
C. reticulata Kalimpong	136	76	78	04	+++
C. reticulata Mirik	142	84	82	06	++
C. reticulata Bijanbari	117	69	54	01	++
C. reticulata Kurseong	103	52	36	01	+
C. medica site 1	139	87	48	04	+++
C. medica site 2	95	86	66	06	+++
C. medica site 3	108	59	46	04	++
C. limonia site 1	115	88	72	05	+++
C. limonia site 2	89	63	57	03	++
C. limonia site 3	93	75	63	02	+++

4.3.1. Maize

Spores isolated from *C. reticulata* were mass multiplied in maize in both pots and field conditions, sorghum and turf grass. Whereas AMF spores isolated from *C. medica* and *C. limonia* were mass multiplied in maize plant only (Fig 16,17).

The plants were regularly watered for 60 days after inoculation. The root colonization behavior of the AMF spores within the root tissues of the inoculated maize plants were studied. Presence of many vesicles, arbuscules and extraradical hyphae with spores were observed. Arum type of hyphae was mostly observed. Diversity of vesicles were present in maize root. Oval vesicles usually produced by *Glomus* sp. and lobed vesicles produced by *Acaulospora* sp. were found in plenty. Numerous single spores around root amongst masses of extrametrical hyphae were found abundantly. Subglobose oil filled structures having a thicker wall than typical vesicles were present in abundance suggesting they may be intraradical spores. Appresorium, infection peg and few dark septate hyphae was observed in maize roots colonized with AMF spores of *C. limonia* (Fig. 18 A-I).

4.3.2. Sorghum

Root colonization was observed after 45 days of inoculation. Two types of hyphae was observed in sorghum roots. One type is straight type of hyphae ramifying along the root cortex and the other is looping hyphae which are irregularly branched, thin walled and stain weakly stained. Arbuscule formation with the hyphal growth progressing outwards from the entry points were observed. Arbuscules are short-lived and begin to collapse after a few days. Root colonization patterns are represented in (Fig 18 J-L).

4.3.3. Turf grass

Turf grass is a perennial herb. As a result, it also serves the purpose of storage of the AMF spores year after year alongwith its maintenance and mass multiplication in the rhizosphere. Vesicles, arbuscules and hyphae with infection pegs were observed. DSE hyphae were also present throughout the length of the root. Paris type of hyphae was usually present (Fig 18 M-O).

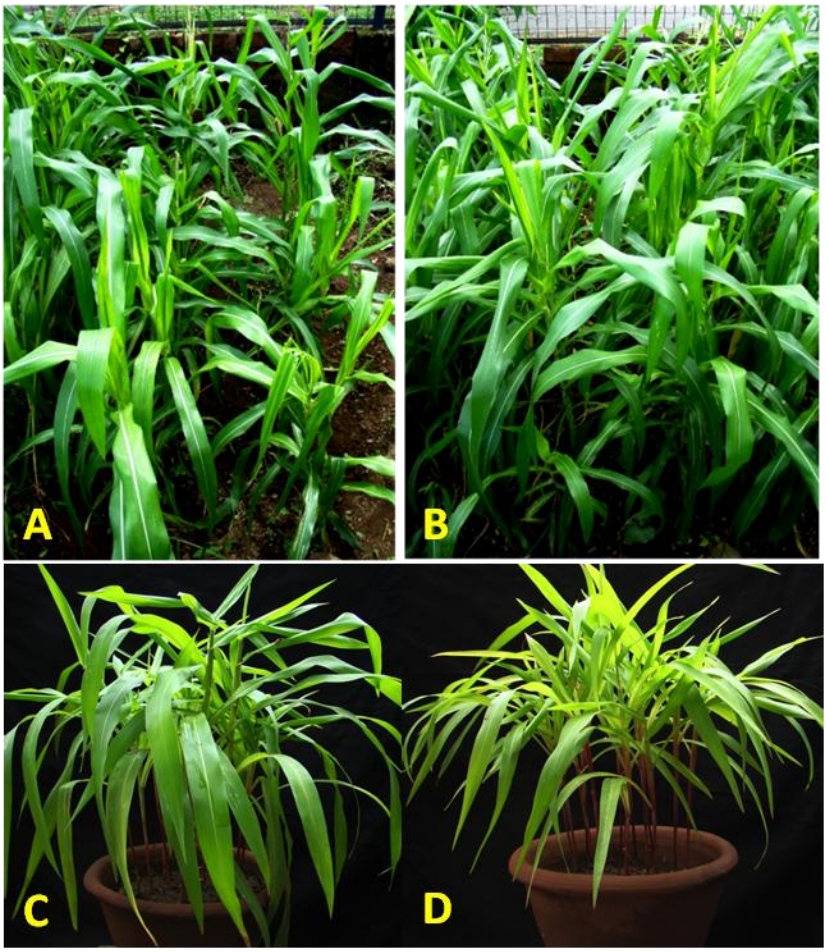


Fig.16. Mass multiplication of AMF spores in maize plant (A & B) grown in field; (C & D) earthen pots

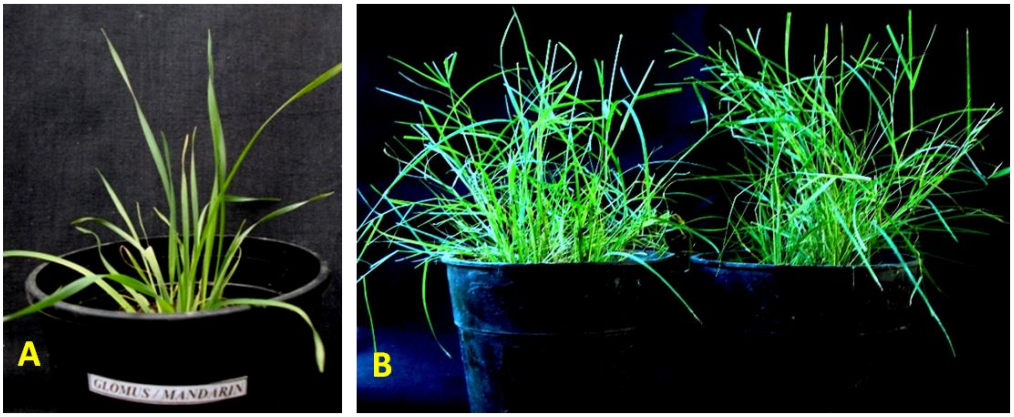


Fig 17. Mass multiplication of AMF spores in (A) sorghum and (B) turf grass

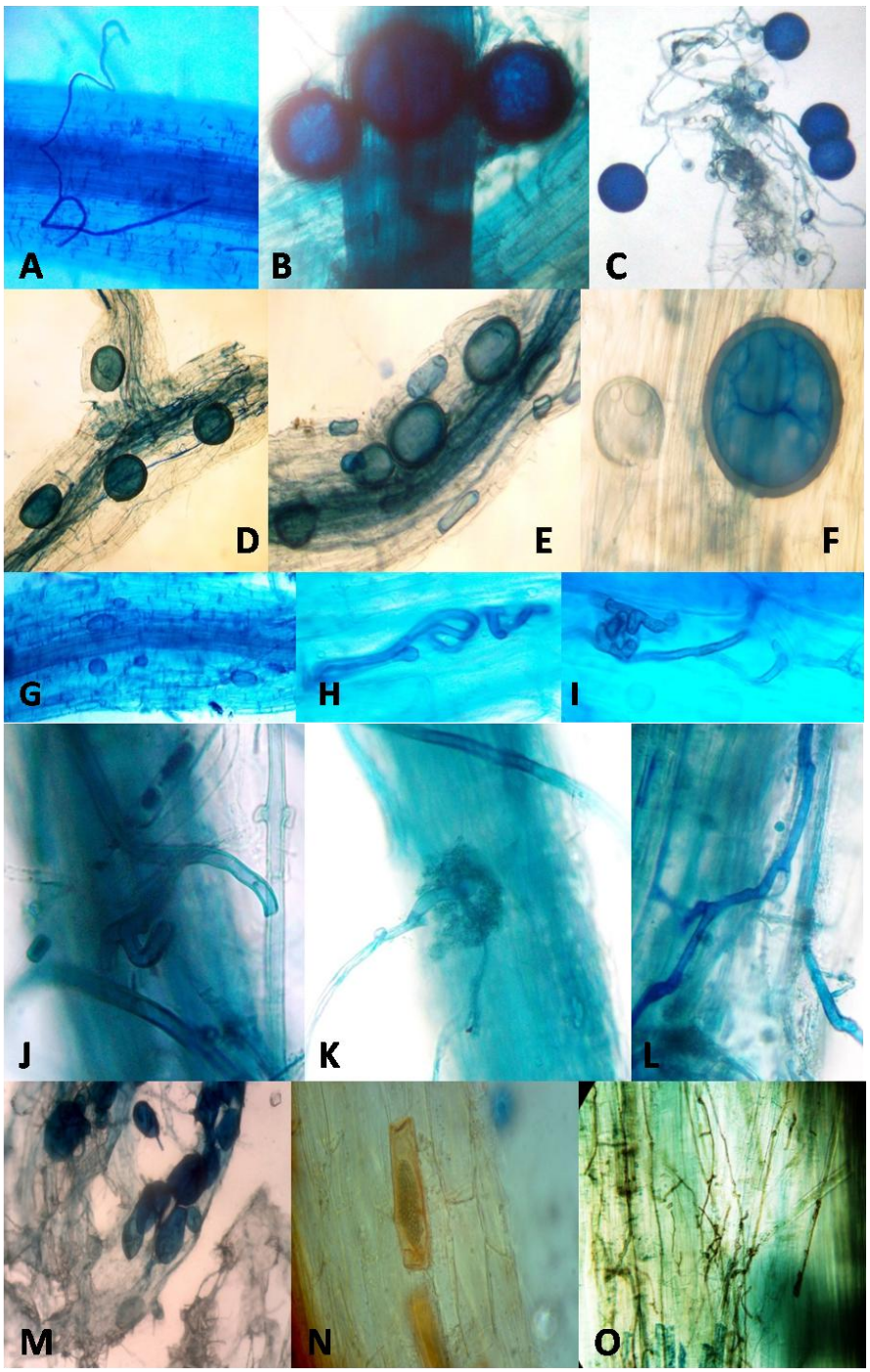


Fig. 18. Histopathological study of AMF colonized roots. (A-I) Maize- (A) Extraradical hyphae, (B-C) Extraradical spores, (D-G) Vesicles of different size and shape, (H-I) Coiled hyphae; (J-L) Sorghum- (J,L) Intraradical hyphae, (K) Arbuscule; (M-O) Turf grass- (M) Vesicles,(N) Arbuscule, (O) Thin intraradical hyphae

4.4 Screening of resistance in mandarin plants against *Fusarium* sp.

Mandarin (*Citrus reticulata*) seedlings were collected from eight different locations of Darjeeling hill areas, viz. Bijanbari, Kurseong, Mirik, Kalimpong Block I and Block II, Sukhia pokhari, Rangli Rangliot and Gorubathan, maintained in glass house and inoculated separately with *F. solani* and *F. oxysporum.* Healthy mandarin seedlings were dipped in culture filtrates of *F. solani* and *F. oxysporum* separately in culture tubes. Wilting symptoms were observed after 7 days of inoculation (Fig 19). Mandarin seedlings were also grown in earthernware pots inoculated separately with the above organisms and incubated for a period of 45 days (Fig 20). Pathogenecity of *F. solani* and *F. oxysporum* was tested on ten mandarin plants each of the eight different locations. The inoculated plants were examined after 15, 30 and 45 days of inoculation. Colour of root, root rot index and percentage loss in dry weight of roots were noted (Table 24). Young seedlings showed light brown discoloration of the root which gradually turned to dark brown and finally to black. Symptoms were also observed in leaves which shrivelled up showing wilting symptoms. In advanced stages, defoliation of the leaves was evident where only the dark brown hard branches were left. The roots had become soft and emitted a foul smell.

Table 6. Screening for resistance of mandarin (*Citrus reticulata)* against *Fusarium solani* and *F. oxysporum*

Root samples (Location)	* Loss in dry mass of roots %		Color intensity of infected roots	
	F. solani	*F. oxysporum*	*F. solani*	*F. oxysporum*
Bijanbari	42.5	40.1	Blackish brown	Blackish brown
Kurseong	39.7	46.0	Deep brown	Blackish brown
Mirik	82.0	79.8	Black	Black
Kalimpong Block I	87.5	56.4	Black	Deep brown
Block II	79.6	86.5	Black	Black
Sukhia pokhari	73.4	68.0	Black	Black
Rangli Rangliot	73.5	56.5	Black	Black
Gorubathan	48.7	55.5	Blackish brown	Blackish brown

* In relation to control on the basis of 3 separate trials of 10 plants each

Fig. 19: Screening for resistance of *C. reticulata* against *F. solani* and *F. oxysporum*

The loss in dry mass of root was very low at the initial stage of infection which increased as the intensity of the disease increased. Mandarin seedlings of Mirik and Kalimpong Block II were found to be highly susceptible to both the pathogen.

Fig. 20. Mandarin plants artificially inoculated with *F. solani* showing wilt symptoms. (A) Healthy plant, (B-C) Plants inoculated with *F. solani* (D) conidia of *F. solani*

4.5. Isolation of microorganism from mandarin rhizosphere and their identification

4.5.1. Analyses of soil samples of mandarin orchards

Soil samples from eight different locations of Darjeeling hill areas i.e. Kurseong, Kalimpong Block I, Kalimpong Block II, Mirik, Sukhia Pokhari, Bijanbari, Rangli Rangliot and Gorubathan and Foothills were collected. The soil samples were analysed for moisture content, pH, soil type, soil texture, carbon and nitrogen availability (Table 7) in Soil testing Laboratory, institute of Plantation Science and Management, North Bengal University. Soil type was mostly sandy clay which is ideal for mandarin growth. Citrus usually prefer a soil pH of 5.5–7.0. Citrus does not grow well in very acid soils (pH

below 5.0) nor very alkaline soils (pH above 8.0). Very acid soils are deficient in some essential plant nutrients such as calcium and magnesium, and are oversupplied with others such as aluminium and/or manganese. Calcareous soils that are high in free lime are also unsuitable, as they cause lime-induced chlorosis, an excessive yellowing of the tree and can affect yield and tree health.

Table 7. Analysis of soil samples collected from mandarin orchards

Factors	Kurseong	Kalimpong Block I	Kalimpong Block II	Mirik	Sukhia Pokhari	Bijanbari	Rangli Rangliot	Gorubathan	Foothills
Soil type:	Sandy clay	Clay	Sandy clay	Sandy clay	Sandy clay	Clay	Clay	Sandy clay	Clay
Sand (%)	52	48	58	54	42	42	40	56	46
Silt (%)	14	10	11	04	2	16	02	12	14
Clay (%)	42	42	40	42	49	42	51	39	40
Ph	6.3	6.01	5.93	4.81	4.66	4.31	4.36	5.85	5.07
Moisture (%)	23.26	21.64	19.66	19.27	11.62	11.62	16.66	22.12	20.95
P_2O_5 (ppm)	19.26	23.94	23.04	34.82	20.67	20.67	38.06	46.76	31.35
K_2O (ppm)	54.20	31.34	30.06	197.67	84.89	84.89	120.09	145.23	48.97
Organic carbon (%)	0.83	0.48	0.59	1.11	1.31	1.31	1.35	1.29	0.75
Nitrogen (%)	0.09	0.05	0.06	0.11	0.14	0.14	.11	.12	0.08

4.5.2. Fungal isolates

The fungi isolated from mandarin rhizosphere were grown in Petri dishes containing sterile PDA medium for 4-6 days at 28^0C. Nature of mycelial growth, rate of growth and time of sporulation were noted. Radial growth patterns of different fungal isolates have been presented. Morphological characters and microscopic observations under bright field of the isolated fungi have been presented in detail in Table 8. On the basis of colony character, hyphal structure, mycelia, structure of conidiophore and conidia these were identified. It was found that most of the fungal isolates belonged to the genera *Aspergillus, Trichoderma, Fusarium, Gongronella Sporotrichum* and *Emericella* (Fig. 21).

Table 8. Morphology and microscopical characters of cultures isolated from mandarin rhizosphere

Fungal isolates	**NBAIM (NAIMCC) Acc. No.**	**Suitable temperature**	**Colonies**	**Conidiophores**	**Conidia**
A. fumigatus	NAIMCC-F-02891	24-26ºC	Bluish green	Clavate vesicles arising from differentiated thick-walled foot cells	Globose to subglobose, echinulate
A. niger	NAIMCC-F-02890	24-26ºC	Black powdery	Arising from long broad thick-walled, mostly brownish foot cells	Large radiating heads, mostly globose, irregularly roughened
A. oryzae	NAIMCC-F-02914	24-26ºC	Light brownish green	Warty or rarely smooth	Slightly roughened or finely echinulate
A. versicolor	NAIMCC-F-02911	12-37ºC	Ochre or orange yellow	Smooth-walled	Globose, echinulate
Emericella nidulans	NAIMCC-F-02892	24-26ºC	Dark green to Brownish	Arising from well differentiated foot cells, smooth-walled, pigmented	Globose, verrucose to rugose, somewhat wrinkled
Trichoderma asperellum	NAIMCC-F-01963	20ºC	Dark green	Short branches appearing near the tip	Almost globose, distinctly roughened

Fungal isolates	NBAIM (NAIMCC) Acc. No.	Suitable temperature	Colonies	Conidiophores	Conidia
Trichoderma harzianum (*Hypocrea lixii*) (RHS/M501)	NAIMCC-F-01964	20ºC	Grayish green	Pruinose pustules arise from a cover of mostly curled sterile conidiophore ends	Short-cylindrical, smooth-walled
Trichoderma harzianum (*Hypocrea lixii*) (RHS/M511)	NAIMCC-F-01961	25 ºC	Grayish green, flat	Perithecia embedded in fleshy stromata formed by pseudoparenchymatous tissue or highly compacted hyphae.	Green and smooth, globose
Sporotrichum pruinosum	NAIMCC-F-02893	36-40ºC	Interwoven appressed or erect branched hyphae, mealy floccose. White to cream coloured	Simple or typically branched	Blastoconidia from branched conidiophores hyaline, subglobose to ellipsoidal to ovoid. Blastoconidia from unbranched conidiophores ellipsoidal to ovoid pyriform or nearly cylindrical. All blastoconidia broadly attached and becoming thick walled, multinucleate
Gongronella butlerii	NAIMCC-F-02894	24ºC	White turf	Sporangiophores 2.1-3.1 µm wide, hyaline, smooth to very faintly roughened, always with a septum under the apophysis beneath the sporangium, branching simple or irregular. Rhizoids present.	Sporangia globose, borne on straight to curved sporangiophores. Columella reduced, hemispherical to dome-shaped, smooth, 2.1-4.2 µm in height, with collar around the base.

Fungal isolates	**Suitable temperature**	**Colonies**	**Conidiophores**	**Conidia**
F. solani RHS/M532	25-29 ºC	White to cream	Short multi-branched which may form sporodochia.	Macroconidia are formed after 4-7 days from They are 3- to 5-septate often moderately curved, with an indistinctly pedicellate foot cell and a short blunt apical cell, 28-42 x 4-6 µm. Microconidia are usually abundant, cylindrical to oval, one- to two-celled and formed from long lateral phialides, 8-16 x 2-4.5 µm
F. oxysporum RHS/M534	25-28 ºC	White	Short, single, lateral monophialides in the aerial mycelium, later arranged in densely branched clusters.	Macroconidia are fusiform, slightly curved, pointed at the tip, mostly three septate, basal cells pedicellate, 23-54 x 3-4.5 µm. Microconidia are abundant, never in chains, mostly non-septate, ellipsoidal to cylindrical, straight or often curved, 5-12 x 2.3 - 3.5 µm.

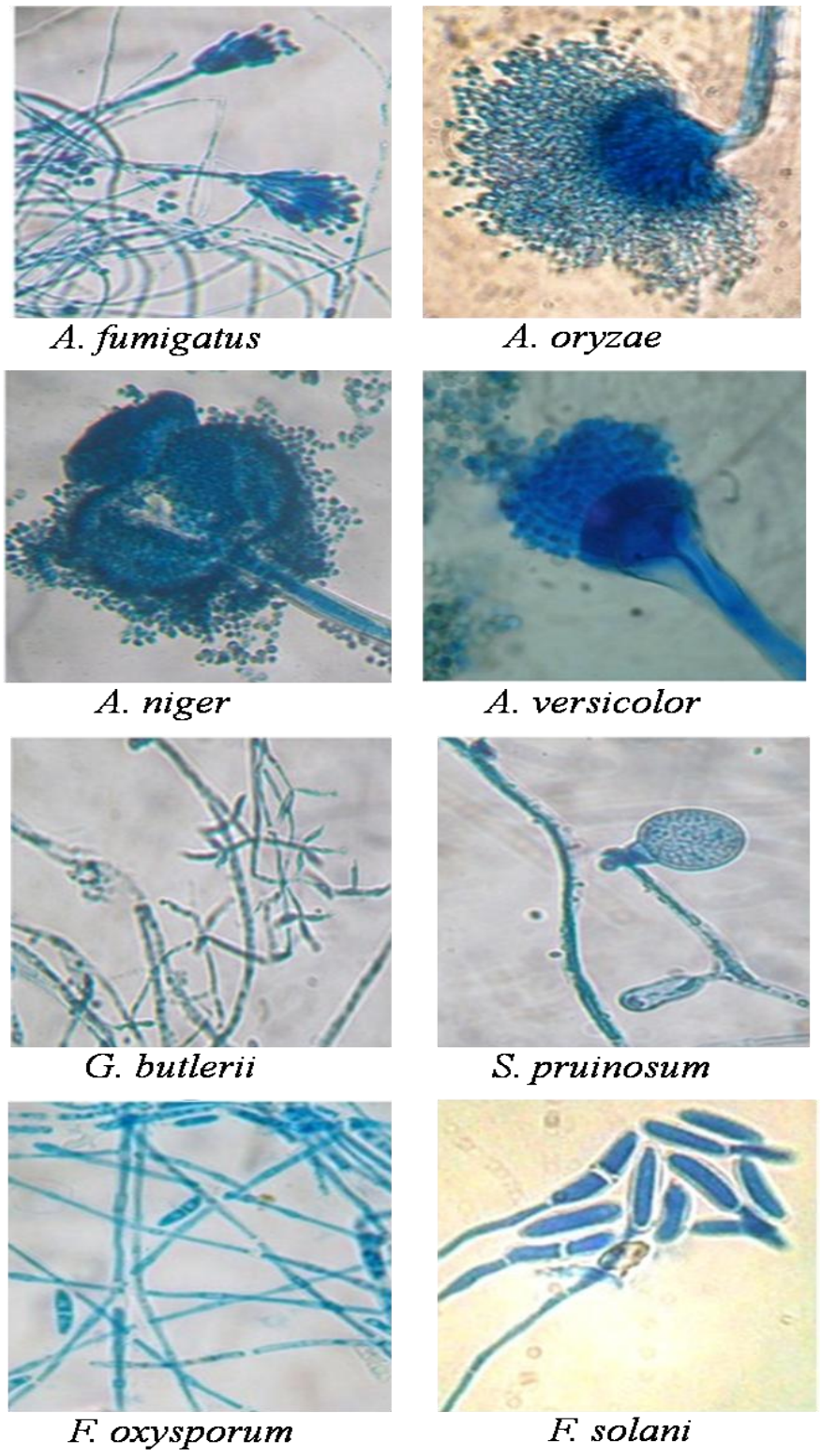


Fig. 21. Microscopic view of fungal isolates of mandarin rhizosphere

4.5.3. Bacterial isolates

Bacteria isolated from mandarin were studied under microscope after suitable staining and characterized based on morphological and biochemical studies following Bergey's Manual of Systemic Bacteriology. A total of 14 bacteria have been isolated out of which 6 bacterial isolates were obtained from hills and 8 bacterial isolates were obtained from plains. Morphological, physiological and biochemical tests were the basis for identification of bacterial tests.

Table 9. Morphology and biochemical tests of bacterial isolates obtained from mandarin plant

Code	Shape	Gram reaction	IAA	Phosphate solubilization	Protease production	Catalase activity	Siderophore production	HCN production	H_2S production	Urease activity
RMK01	Coccus	+	+	-	+	-	-	-	-	-
RMK02	Small rod	+	+	-	+	++	+	-	-	-
RMK03	Small rod	-	+	+	-	+++	+	-	-	-
RMK04	Rod	+	+	-	-	-	-	-	-	-
RMK05	Rod	-	+	-	+	-	-	-	-	-
RMK06	Rod	+	+	-	+	+	-	-	-	-
RMIP01	Small rod	-	+	-	-	+	-	-	-	-
RMIP02	Rod	+	+	-	-	-	-	-	-	-
RMIP03	Rod	-	+	-	-	-	-	-	-	-
RMIP04	Rod	+	+	-	-	-	-	-	-	-
RMIP05	Small rod	-	+	-	-	-	-	-	-	-
RMIP06	Rod	+	+	-	-	-	-	-	-	-
RMIP07	Small rod	+	+	-	-	+	-	-	-	-
RMIP08	Coccus	-	+	-	-	+	+	-	-	-

Isolates were characterized for H2S production, phosphate solubilization, protease production, siderophore production, HCN production, catalase production and indole production. All bacterial isolates have shown positive for indole production butThe overall results for biochemical characterization of all the bacterial isolates have been presented in Table 9.Among all the bacterial isolates one bacterial isolate designated as RMK03 obtained from the rhizosphere of mandarin from Kalimpong hills showed positive tests for phosphate solubilization, catalase activity, IAA and siderophore production. This isolate (RMK03) has been sequenced and has been identified as *Pseudomonas poae*.

4.6. *In vitro* screening of fungal and bacterial isolates of mandarin rhizosphere for plant growth promoting activities

4.6.1. Screening in solid 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 streaks of bacterial isolates was made onto the plates under aseptic conditions. They were incubated at 28±2ºC 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.

Four fungal isolates and one bacterial isolate showed phosphate solubilizing activities. All fungal isolates showed activity up till the third day and then remained constant till the end of the week. In case of *A. niger*(NAIMCC-F-0289) solubilization started after 24 h, reached maximum value on day four and remained constant throughout the week. Highest phosphate solubilizing activity was shown by *A. fumigatus* (NAIMCC-F-02891) where activity grew constantly uoto day four and then became stable. *A. oryzae* (NAIMCC-F-02914) showed a slow and steady growthnin activity. The bacterial isolate *Pseudomonas poae* (RMK03) also showed a slow and steady growth in activity reaching a maximum in day 5 and then remaining constant (Table 10).

Table 10. *In vitro* screening for phosphate solubilizing activities of fungal and bacterial isolates of mandarin rhizosphere

Fungal and bacterial isolates	Diameter of Clear zone (cm)					
	24h	48h	72h	96h	120h	144 h
A. niger (NAIMCC-F-02890)	-	0.3	0.5	0.5	0.6	0.6
A. versicolor (NAIMCC-F-02911)	0.1	0.3	0.6	0.6	0.6	0.6
A. fumigatus (NAIMCC-F-02891)	0.1	0.2	0.5	0.7	0.7	0.7
A. oryzae (NAIMCC-F-02914)	0.2	0.2	0.3	0.3	0.6	0.6
P. poae (RMK/3)	0.2	0.3	0.5	0.6	0.7	0.7

*Average of three replicates

4.6.2. Evaluation in liquid media

Four PSF isolates *A. niger*(NAIMCC-F-0289), *A. versicolor* (NAIMCC-F-02911), *A. fumigatus* (NAIMCC-F-02891) and *A.oryzae* (NAIMCC-F-02914) were further evaluated in PVK liquid media amended with tricalcium phosphate (TCP) and rock phosphate (RP) to assess their phosphorus solubilization capacity.The pH of the cultural broth samplesdropped significantly as compared to the control whereit remained constant around pH 7.0. A decrease in pH from 7 to 3.7 was noticed in *A. niger*(NAIMCC-F-0289) which was attributed to the varying diffusion rates of different organic acids secreted by the tested organisms. *A. versicolor* (NAIMCC-F-02911) and *A. oryzae* (NAIMCC-F-02914) showed better efficiency of TCP solubilization after seven days of incubation (Table 11)

Table 11. 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 (NAIMCC-F-02890)	860	3.1	345	3.5
A. versicolor (NAIMCC-F-02911)	871	3.3	321	3.5
A. fumigatus (NAIMCC-F-02891)	869	4.0	341	3.4
A. oryzae (NAIMCC-F-02914)	895	3.2	366	2.5

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

4.7. Antagonistic activities of bioinoculants against *F. solani* and *F. oxysporum*

4.7.1. Antagonistic effect of *Trichoderma* isolates

Two isolates of *Trichoderma harzianum* and one isolate of *T. asperellum* isolated from mandarin rhizosphere were initially taken up for their antagonistic effects against the fungal pathogens *F. solani* and *F. oxysporum*. For each of the antagonistic tests, 5mm disc of fungal isolates were taken from 5 days old culture and placed at the periphery of the petri plate. Similarly, agar disc of 5 mm from pathogen culture was placed in the same petri plate on the opposite end. The percent inhibition in the radial colony was calculated by the following formula-

Percent inhibition = C-T/CX100

Where C= radial growth in control

T= radial growth in control

Their inhibition percent is placed in the table (Table 12). *T. harzianum* (isolate RHS/M 511) and *T. asperellum* (isolate RHS/M 512) showed maximum inhibitory activities (Fig. 22).

Table 12. *In vitro* antagonistic tests of PGPF isolates against root rot pathogens (*F. solani* and *F. oxysporum*)

Interacting microorganisms	Diameter of fungal colony	% inhibition
F. solani	8.3±0.24	-
F. solani + T. harzianum	1.6±0.08	80.72
F. solani + T. harzianum	1.4±0.09	83.13
F. solani + T. asperellum	1.5±0.07	81.92
F. oxysporum	8.6±0.33	-
F. oxysporum +T. harzianum	2.1±0.23	75.58
F. oxysporum +T. harzianum	2.0±0.21	76.74
F. oxysporum +T. asperellum	1.9±0.20	77.90

Values are average of three replicate experiments. ±= SE

4.7.2. Antagonistic effect of PGPR isolate against mandarin root pathogen

Pseudomonas poae (isolate RMK03), one of the PGPR isolates which showed positive for most of the plant growth promoting characters viz. IAA, phosphate solubilization, catalase and siderophore production was tested against mandarin root pathogens *F. solani* and *F. oxysporum*. The results of the interactions have been presented in Table 13. *P. poae* (isolate RMK03) inhibited the growth of *F. solani* (Fig.23) and *F. oxysporum* markedly.

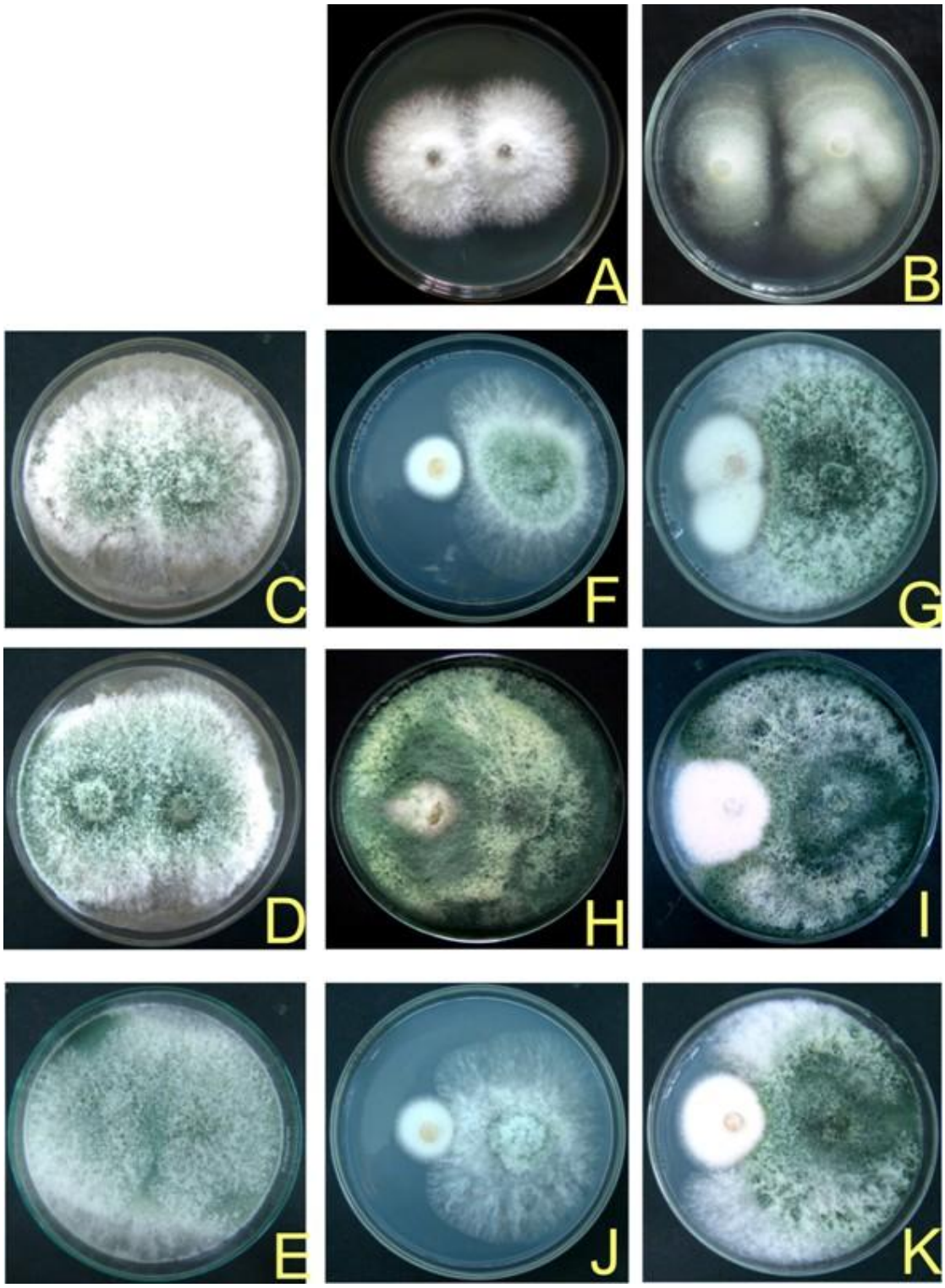


Fig. 22. *In vitro* antagonistic test of *Trichoderma* isolates with *F. oxysporum* and *F. solani*

Table 13. ***In vitro*** **antagonistic tests of PGPR isolates against fugal test pathogens**

Interacting microorganisms	**Diameter of fungal colony**	**% inhibition**
F. solani	8.8±1.3	-
F. solani + *P. poae* (RMK/03)	6.2±0.16	29.54
F. oxysporum	8.6±0.20	-
F. oxysporum + *P. poae* (RMK/03)	3.8±0.09	55.81

Values are average of three replicate experiments. ±= SE

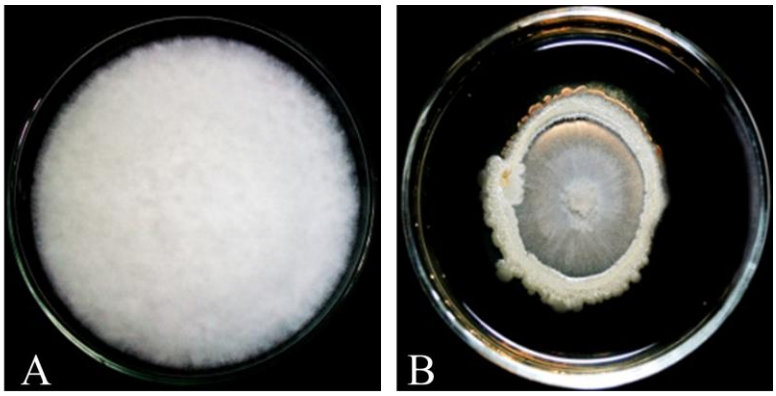


Fig. 23. *In vitro* antagonistic test of *P. poae* with *F. oxysporum*

4.8. Serologicalcharacterization of *F. solani* and *F. oxysporum*

4.8.1. Soluble protein

Mycelia antigen of the two pathogens (*F. oxysporum* and *F. solani*) was initially analysed by SDS-PAGE. The molecular weight of protein bands visualized after staining with coomassie blue were determined from the known molecular weight marker. Mycelia protein of *F. oxysporum* exhibited 12 bands and *F. solani* exhibited 22 bands in SDS-PAGE ranging in molecular weight (Ca. 97.4 kDa to 29 kDa). Bands of varying

intensities and more proteins of lower molecular weight were present especially in *F. oxysporum*.

4.8.2. Immunological assays

Immunological assays were performed using Polyclonal antibodies (PAb) raised against mycelia protein of *F. oxysporum* and *F. solani* in rabbit. Effectiveness of antigen in raising antibodies were checked initially using agar gel double diffusion technique followed by dot immunobinding assay and western blot analysis. 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

Antigens were prepared from root samples collected from eight different locations of Darjeeling hills as well as from fungal pathogens (*F. oxysporum* and *F. solani*). PAbs raised against *F. oxysporum* and *F. solani* were tested with homologous and heterologous antigens of mandarin roots. Among the root antigens of mandarin plants of eight different locations tested against PAbs of *F. solani*, strong and positive reactions were noticed in root antigens of four specific locations (Sukhia pokhari, Mirik, Kalimpong Block I and block II), two showed weak reaction while there was no reaction in root antigens of two locations (Table 14). Similar reactions were also noticed in heterologous reaction of root antigens and PAbs of *F. oxysporum*. Strong precipitin reactions occurred in homologous reactions in immunodiffusion test). Agar gel double diffusion test was performed using the mycelial antigens of *F. oxysporum* and *F. solani* and homologous PAb. Strong precipitin reactions occurred in homologous reactions in immunodiffusion test which was evident by intense precipitation, *F. oxysporum* (Fig 24 C) and *F. solani* (Fig 25 C). Common antigenic relationship shared between host and pathogen was evident in such cases where susceptible reactions were noticed in pathogenicity test.

Table 14. Detection of cross reactive antigens among *C. reticulata* and *F. solani and F. oxysporum* using agar gel double diffusion

Root antigens of	PAb of fungal pathogens	
C. reticulata	*F. solani*	*F. oxysporum*
Rangli Rangliot	Absent	Absent
Bijanbari	Absent	Present
Sukhia Pokhari	Present	Absent
Kurseong	Weak	Weak
Mirik	Present	Present
Kalimpong Block I	Present	Present
Kalimpong BlockII	Present	Weak
Gorubathan	Weak	Present

4.8.2.2. PTA-ELISA

Purified IgGs were tested against homologous and heterologous antigens at 25 µg/l. Doubling dilutions of *F. solani* and *F. oxysporum* ranging from 1:125 to 1;4000 were initially tested. ELISA values decreased with the dilution. IgG dilution of 1:125 were 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 antigen levels. Concentrations as low as 25 µg/l could easily be detected by ELISA at both antisera dilutions. PTA-ELISA could readily detect reaction between root antigen and PAb of pathogens. Antigens extracted from healthy and artificially inoculated with *F. solani* and *F. oxysporum* were tested against PAbs of the pathogens separately. 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 2mg/l. (Table 15).

Table 15 . PTA-ELISA values showing reaction of PAbs of *Fusarium solani* and *F. oxysporum* with antigens of healthy and inoculated mandarin roots

Citrus saplings	Root antigen concentration (40mg/L)		
Locality	Healthy	Inoculated[a]	
		F. solani	*F. oxysporum*
Rangli Rangliot	0.812	1.182	1.173
Bijanbari	0.89	1.139	1.143
Sukhia Pokhari	0.715	1.345	1.289
Kurseong	0.972	1.265	1.191
Mirik	0.664	1.876	1.64
Kalimpong Block I	0.907	1.766	1.91
Kalimpong Block II	0.787	1.98	1.99
Gorubathan	0.938	1.765	1.638

Absorbance at 405 nm

PAb of *F. solani* and*F. oxysporum* (1:125 dilution)

[a] 3 days after inoculation

4.8.2.3. Dot immunobinding assay

Dot immunobinding assay using mycelia antigen and PAb of *F. oxysporum* and *F. solani* was also standardized. Dot immunobinding assays confirm the effectiveness of raising antibodies against *F. oxysporum* and *F. solani*. Soluble protein obtained from seven-day old mycelia of *F. oxysporum* and *F. solani* were reacted on nitrocellulose paper with PAb of *F. oxysporum* and *F. solani.* Result shows development of deep violet colour in homologous reactions (Table 16) indicating a positive reaction suggestive of effectiveness of mycelial antigen in raising PAb against the pathogen. However, faint violet colour was observed in heterologous reactions (Fig 24 C & 25 C).

Table 16. Antigen – antibody Reaction through Dot-blot assay

Antibody Source	Antigen Source	Intensity of dots
Fusarium oxysporum	*F. oxysporum*	++++
	F. solani	++
Fusarium solani	*F. solani*	++++
	F. oxysporum	+++

++++ Very deep pinkish; Deep pinkish; ++ Light pinkish; +Very light pinkish; - Negative

4.8.2.4. Western blot analysis

Western blot analysis using PAb of *F. oxysporum* and *F. solani* were performed to develop strategies for rapid detection of the pathogen. Four antibodies (1st bleed to 4th bleed) were used to confirm the precipitin reaction done with PAb raised against mycelial protein. For this total soluble protein of 7 days old mycelia was used as antigen source. SDS-PAGE was performed as described previously followed by probing of the localized antigen with alkaline phosphatase conjugate. Sharp bands were produced which was stained blue. The bands on nitrocellulose membrane was compared with corresponding protein bands on the SDS-PAGE. Bands of varying intensities was observed ranging from 25 KDa to 69 KDa (Fig 24 D & 25 D). Bands of lower molecular weight were more in number. The intensity of the bands increased with increase in the bleeds. The bands obtained using the fourth bleed was much more intense and dark than the bands obtained using the first bleed which was much lighter and easier to observe in *F. oxysporum* but in the case of *F. solani*, the antibody of the first bleed did not show any reaction. Intense bands were developed from the second bleed.

4.8.2.5. Indirect immunofluorescence

Indirect immunoflouorescence of young hyphae of *F. oxysporum* and *F. solani* was conducted with homologous antibody (PAb of *F. oxysporum* and*F. solani*) and reacted with fluorescein isothiocyanate (FITC) labeled antibodies of goat specific for rabbit globulin.

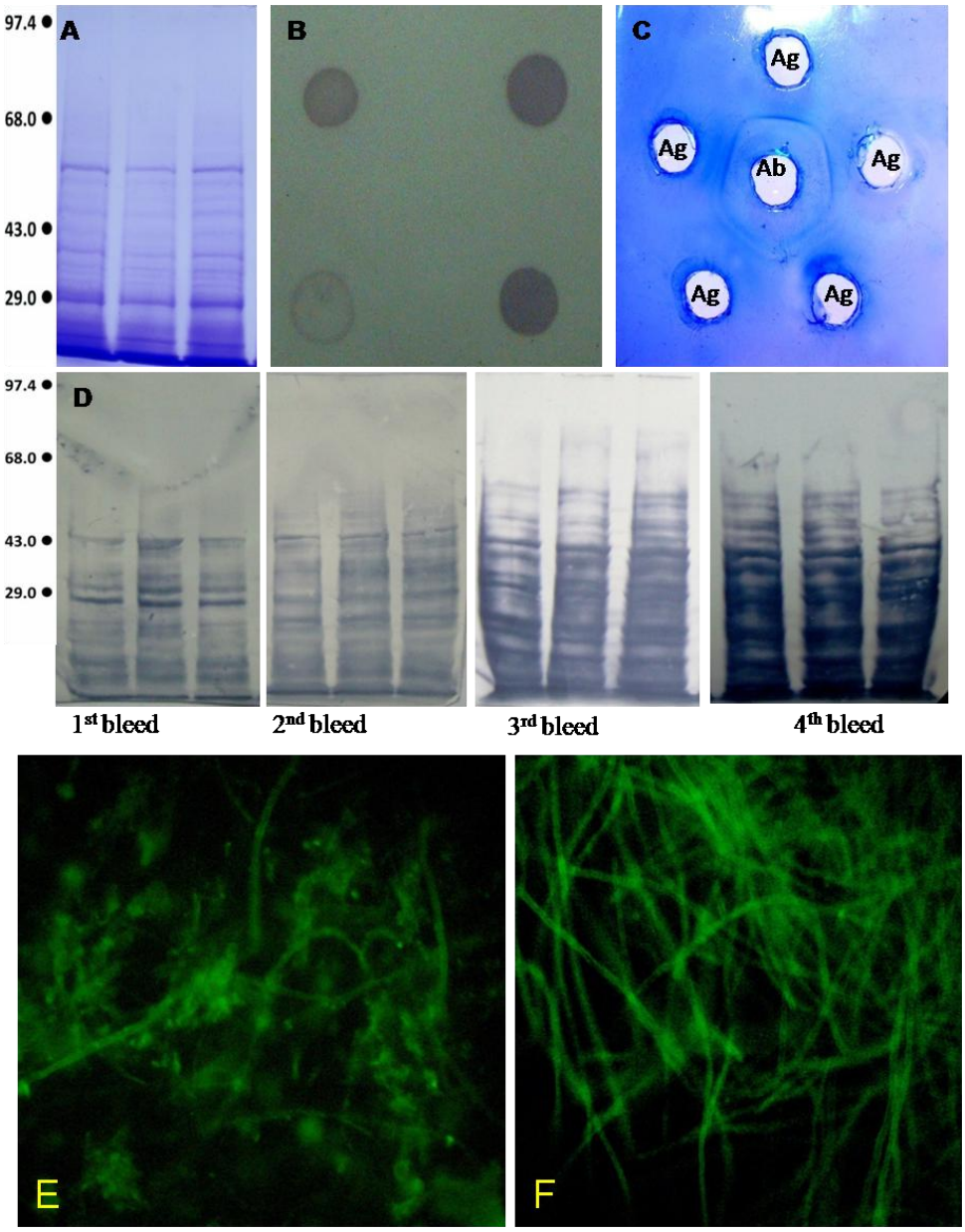


Fig.24. Serological assays of *F. oxysporum*; (A) SDS-PAGE, (B) Dot-blot (C) Immunodiffusion, (D)Western blot of 1st, 2nd, 3rd and 4th bleed, (E-F) Immunofluorescence of young mycelia of *F. oxysporum* using PAb of *F. oxysporum* and labelled with FITC

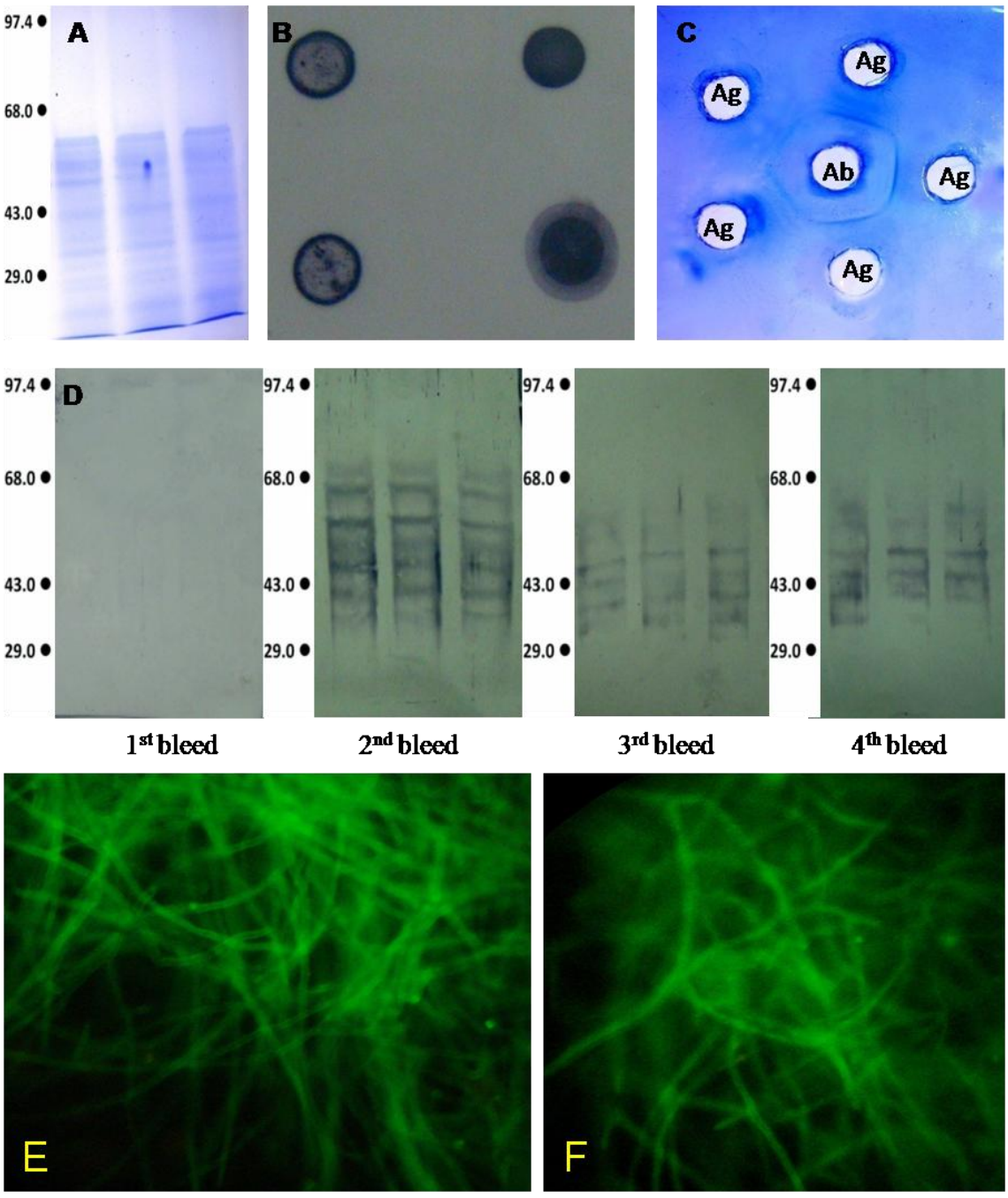


Fig. 25. Serological assays of *F. solani*; (A) SDS-PAGE, (B) Dot-blot (C) Immunodiffusion, (D) Western blot of 1st, 2nd, 3rd and 4th bleed, (E-F) Immunofluorescence of young mycelia of *F. solani* using PAb of *F. solani* and labelled with FITC

Antibody labeling with fluorescein isothiocyanate is known to be one of the powerful techniques to determine the cell or tissue location of major cross reactive antigens shared by host and parasite. Specific detection of cross reactive antigens were confirmed as apple green fluorescence in young mycelia of the pathogen (Fig 24 E & F and 25 E & F).

4.9. Molecular characterization of pathogen

In this present investigation, two important root rot pathogens of mandarin plants (*F. oxysporum* and *F. solani*) were taken for molecular characterization.

4.9.1. RAPD-PCR and phylogenetic analysis of *Fusarium* isolates of mandarin rhizosphere

Genomic DNA of *Fusarium* spp. viz., *F. solani, F. oxysporum*and *F. graminearum* were 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. ITS region of rDNA of *Fusarium*was amplified using genus specific Fcg17F and Fcg17R primers. Amplified products of 550bp size was produced by *Fusarium* (Fig. 26), The primer pairs Fcg17F and Fcg17R were highly specific for *Fusarium* genus.The genetic relatedness among isolates of *F. solani, F. oxysporum,* and *F. graminearum*wereanalyzed by random primers to generate reproducible polymorphisms. All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicated the genetic diversity of all isolates. A total of 54 reproducible and scorable polymorphic bands of 6000bp were generated in *F. solani, F oxysporum* and *F. graminearum* (Table 17, Fig. 26). Relationships among the isolates were evaluated by cluster analysis of the data based on the similarity matrix. The dendogram was generated by un weighted pair-group methods with arithmetic mean (UPGMA) using NTSYSpc software. Based on the results obtained all the four isolates can be grouped into two main clusters. TheRAPD profiles showed that primer OPA-4 showed maximum polymorphism among the isolates of *F. solani, F. oxysporum* and *F. graminearum.*In the case of the RAPD profile of *F. solani,*

*F. graminearum*and *F. oxysporum* grouped into three main clusters. The clusters consist of *F.solani, F. graminearum*and *F. oxysporum* respectively. RAPD banding patterns reveled that the isolates of *F. solani, F. graminearum* and *F. oxysporum* were genetically different and showed polymorphism among each other.

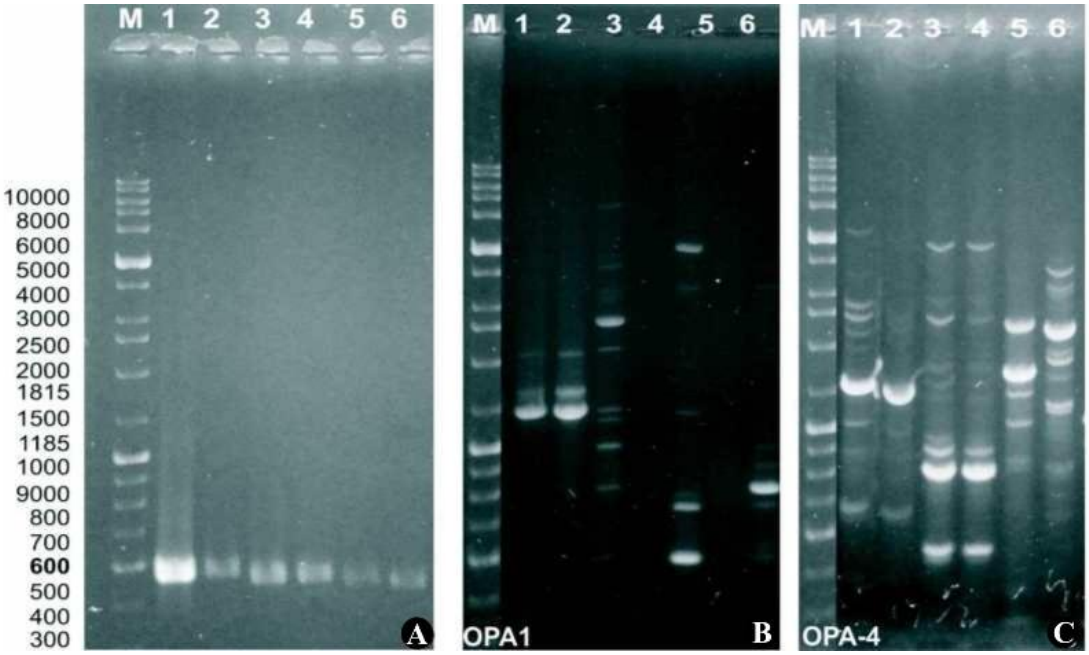


Fig. 26 RAPD-PCR analysis of *Fusarium* isolates of mandarin rhizosphere**;** (A) Agarose gel (2%) electrophoresis of ITS region. Lane M-high range DNA ladder, lane 1-2 for *F. solani*, 3-4 for *F. graminearum*, 5-6 for *F. oxysporum*;. (B) Agarose gel (2%) electrophoresis of RAPD. Lane M-high range DNA ladder, 1-2 for *F. solani*, 3-4 for *F. graminearum*, 5-6 for *F. oxysporum* analysed with primer OPA1; (C) Agarose gel (2%) electrophoresis of RAPD. Lane M-high range DNA ladder, Lane M-high range DNA ladder, 1-2 for *F. solani*, 3-4 for *F. graminearum*, 5-6 for *F. oxysporum* analysed with primer OPA-4;

Table 17 : Analysis of the polymorphism obtained with RAPD markers in *F. solani* , *F. graminearum* and *F. oxysporum*

Seq Name	Total no RAPD bands	Approximate band size (bp)		Monomorphic bands	Polymorphic bands	Polymorphic (%)
		Min	Max.			
OPA1	11	200	6000	0	11	100
OPA-4	17	200	6000	0	17	100
A-5	15	100	6000	0	15	100
A-11	11	100	6000	0	11	100

4.9.2. 18 S rDNA sequence and Phylogenetic analysis for identification of pathogens

Genomic DNA of both the fungal pathogens *F. oxysporum* and *F. solani* were suspended in 100µl 1X TE buffer treated with RNAse (60 µg) until further use. Agarose gel electrophoresis of genomic DNA revealed that they were RNA free. Purity of DNA evaluated in terms of the ratio between absorbance of A_{260} and A_{280} showed that genomic DNA was ~1.8

ITS region of rDNA was amplified using genus specific Fcg17F & Fcg17R *Fusarium* primers where the amplified products of 550 bp size was produced by both the primers.

4.9.2.1. *F. oxysporum*

4.9.2.1.1. Chromatogram

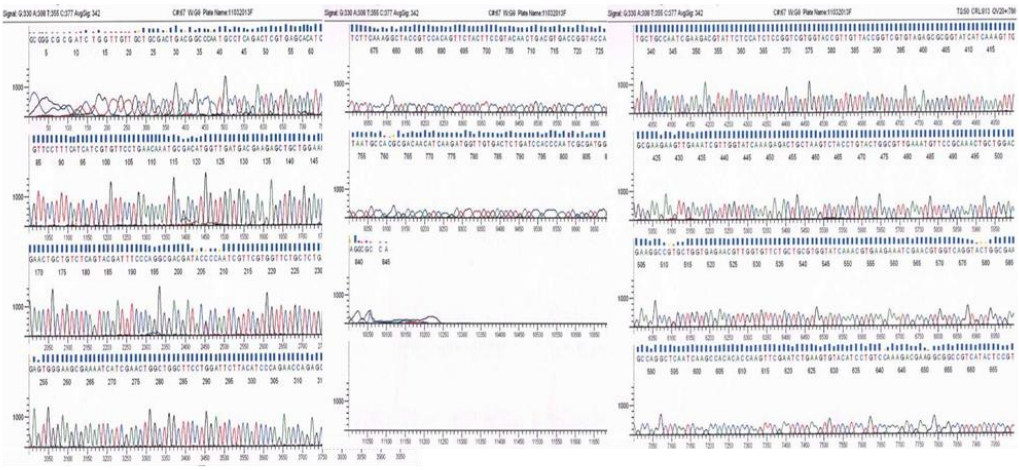
The BLAST query of the 18S rDNA sequence of *F. oxysporum*against GenBank database confirmed its identity**.** The sequences have been deposited in NCBI, GenBank database under the accession no. KF952602.The sequence chromatograms have been represented in Fig 27.

4.9.2.1.2. Multiple sequence alignment

A multiple sequence alignment of ITS gene sequences of *F. oxysporum* was conducted. Sequences of other strains obtained from NCBI Genbank database showing maximum homology with our strain was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. There were quite a number of gaps that were introduced in the multiple

sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Fig.28). Phylogenetic analysis was carried out with Ex-type strain sequences obtained from NCBI Genbank database which showed maximum homology with *F. oxysporum* (KF952602) (Table 18).

Chromatogram



Partial sequence of 18S ribosomal RNA gene

CCCTGTGACATACCACTTGTTGCCTCGGCGGATCAGCCCGCTCCCGGTAAAACGGGA CGGCCCGCCAGAGGACCCCTAAACTCTGTTTCTATATGTAACTTCTGAGTAAAACCAT AAATAAATCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGC AGCAAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAA CGCACATTGCGCCCGCCAGTTTCTGGCGGGATTGCCTTGTTCGAGCGTCATTTCAACC CTCAAGCACAGCTTGGTGTTGGGACTCGCGTTTAATTCGCGTTCCCCAAATTGATTGG CGG

Sequence deposited: NCBI

Title: ***Fusarium oxysporum*** **strain RHS/M534 18S ribosomal RNA gene, partial sequence**

ACCESSION:KF952602
VERSION:KF952602.1
GI:588480870DNA linear:349 bp

ORIGIN

1 ccctgtgaca taccacttgt tgcctcggcg gatcagcccg ctcccggtaa aacgggacgg
61 cccgccagag gacccctaaa ctctgtttct atatgtaact tctgagtaaa accataaata
121 aatcaaaact ttcaacaacg gatctcttgg ttctggcatc gatgaagaac gcagcaaaat
181 gcgataagta atgtgaattg cagaattcag tgaatcatcg aatctttgaa cgcacattgc
241 gcccgccagt ttctggcggg attgccttgt tcgagcgtca tttcaaccct caagcacagc
301 ttggtgttgg gactcgcgtt taattcgcgt tccccaaatt gattggcgg

Fig. 27. Chromatogram and sequence deposition of 18S r DNA region of *F. oxysporum* strain RHS/M534

Fig. 28. 18S r DNA sequence alignments of *F. oxysporum*(RHS/M534) with other extypes isolate. The conserved regions of the gene are demonstrated in different colours

Table 18. Genbank Accession Numbers and Geographic locations of the Ex-Type strains of *F. oxysporum* that showed homology with the isolate

Accession No.	Strain/isolate	rDNA Sequence	Country	Source
AF219123	Ag149	1477 bp	China	Rhizosphere
Z94126	CBS619.87	421 bp	Netherlands	Rhizosphere
Z94127	CWB1	421 bp	Netherlands	Rhizosphere
FN666092	PUS	1453 bp	Lithuania	Soil
FN666090	KAL1	1337 bp	Lithuania	Soil
AF219122	Ag149	1477 bp	China	Rhizosphere
AF219124	Ag149-III	1477 bp	China	Rhizosphere
Z94128	h309	421 bp	Netherlands	Rhizosphere
HF566400	DBT-99	631 bp	India	Rhizosphere
HF566401	DBT-101	531 bp	India	Rhizosphere
FR851229	DBT-20Akola	486 bp	India	Rhizosphere
HG529205	FPV 38	482 bp	Italy	Rhizosphere
FN393417	2476	470 bp	Spain	Rhizosphere
HG529207	FPV 40	482 bp	Italy	Rhizosphere
FR852561	ISPaVe1070	563 bp	Italy	Rhizosphere
KF952602	**RHS/M534**	**349 bp**	**India**	**Rhizosphere**
HF548706	KAUEF20	483 bp	Saudi Arabia	Rhizosphere
AB518683	MAFF 239849	566 bp	Japan	Soil
AB513655	WF-6	205 bp	Japan	Soil
AB470904	DSCF5	568 bp	China	Soil
KF914481	FO40	466	India	Wilted tomato plant
KF914476	FO15	480	India	Wilted tomato plant
KF914449	FO22	478	India	Rhizosphere
KF914447	FO1	478	India	Wilted tomato plant
KF998987	CEF-068	517	China	Rhizosphere
KF986683	CDR1P1F1	517	India	Rhizosphere
KF864555	POS3	486	India	Stem
KC594035	4	505	China	Rhizosphere
JX896995	41	505	USA	Root

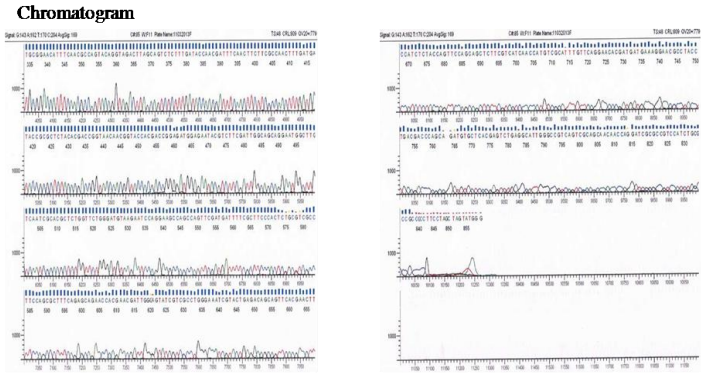
4.9.2.2. *F. solani*

4.9.2.2.1. Chromatogram

The BLAST query of the 18S rDNA sequence of *F. solani*against GenBank database confirmed its identity**.** The sequences have been deposited in NCBI, GenBank database under the accession no. KF952603**.** The sequence chromatograms have been represented in Fig 29.

4.9.2.2.2. Multiple sequence alignment

A multiple sequence alignment of ITS gene sequences of *F. solani* was conducted. Sequences of other strains obtained from NCBI Genbank database showing maximum homology with our strain was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. There were quite a number of gaps that were introduced in the multiple sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Fig.30). Phylogenetic analysis was carried out with Ex-type strain sequences obtained from NCBI Genbank database which showed maximum homology with *F. solani* (KF952603) (Table 19).



Chromatogram

Partial sequence of 18S ribosomal RNA gene

CATACCTAAAACGTTGCCTCGGCGGGAACAGACGGCCCCGTAACACGGGCCGCCCCCGCCAGAGGACCCC
CTAACTCTGTTTCTATAATGTTTCTTCTGAGTAAACAAGCAAATAAATTAAAACTTTCAACAACGGATCT
CTTGGCTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGAATTCAGTGAAT
CATCGAATCTTTGAACGCACATTGCGCCCGCCAGTATTCTGGCGGGCATGCCTGTTCGAGCGTCATTACA
ACCCTCAGGCCCCCGGCCC

Sequence deposited: NCBI Title: ***Fusarium solani*** **strain RHS/M532 18S ribosomal RNA gene, partial sequence.**

ACCESSION:KF952603
VERSION:KF952603.1
GI:588480871
DNA linearL: 299 bp

ORIGIN

1 catacctaaa acgttgcctc ggcgggaaca gacggccccg taacacgggc cgcccccgcc
61 agaggacccc ctaactctgt ttctataatg tttcttctga gtaaacaagc aaataaatta
121 aaactttcaa caacggatct cttggctctg gcatcgatga agaacgcagc gaaatgcgat
181 aagtaatgtg aattgcagaa ttcagtgaat catcgaatct ttgaacgcac attgcgcccg
241 ccagtattct ggcgggcatg cctgttcgag cgtcattaca accctcaggc ccccggccc

Fig. 29. Chromatogram and sequence deposition of 18S r DNA region of *F. solani* strain RHS/M532

Table 19. Genbank Accession Numbers and Geographic locations of the Ex-Type strains of *F. solani* that showed homology with the isolate

Accession No.	Strain/Isolate	rDNA Sequence	Country	Source
KC577182	FsolteaIN1	555 bp	India	Rhizosphere
KC013591	SSLF2	542 bp	Korea	Rhizosphere
KC577185	FsolPlvIN1	592 bp	India	Rhizosphere
KC577184	FsolSIN5	575 bp	India	Soil
KC577186	FsolCpIN1	564 bp	India	Rhizosphere
KC202940	FsD1A4	553 bp	China	Rhizosphere
KC013590	SSLF1	541 bp	South Korea	Rhizosphere
AB498983	MAFF 240345	1138 bp	Japan	Rhizosphere
AB498985	MAFF 240347	1138 bp	Japan	Rhizosphere
AB498984	MAFF 240346	1138 bp	Japan	Rhizosphere
HM064429	F.S.0613	565 bp	Taiwan	Rhizosphere
JF817274	CanR-19	566 bp	China	Rhizosphere
KC202941	FsD7A27	559 bp	China	Rhizosphere
AB513851	MAFF 731042	1135 bp	Japan	Rhizosphere
JX114792	F6RS3	547 bp	Algeria	Rhizosphere
KF952603	**RHS/M532**	**299 bp**	**India**	**Rhizosphere**
JN419024	DMR12	222 bp	India	Rhizosphere
AB518683	MAFF 239849	566 bp	Japan	Soil
AB513655	WF-6	205 bp	Japan	Soil
AB470904	DSCF5	568 bp	China	Soil

Fig. 30 18S r DNA sequence alignments of *F. solani*(RHS/M532) with other extypes isolate. The conserved regions of the gene are demonstrated in different colours

4.9.3. Phylogenetic analysis of *F. oxysporum*

Phylogenetic analysis of F. oxysporum was done (Fig 31). The evolutionary history was inferred using the UPGMA method [Sneath & Sokal, 1973]. The optimal tree with the sum of branch length = 0.55343450 is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method [Tamura *et al.*. 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 154 positions in the final datas*et* . Phylogenetic analyses were conducted in MEGA4 [Tamura *et al.*, 2007].

4.9.4. Phylogenetic analysis of *F. solani*

The evolutionary history was inferred (Fig 32) using the Neighbor-Joining method (Saitou and Nei, 1987). The optimal tree with the sum of branch length = 0.10563138 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 (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 *et al.*, 2004) and are in the units of the number of base substitutions per site. The analysis involved 20 nucleotide sequences. All positions containing gaps and missing data were eliminated. There were a total of 146 positions in the final datas*et* Evolutionary analyses were conducted in MEGA6 (Tamura *et al.*, 2013)

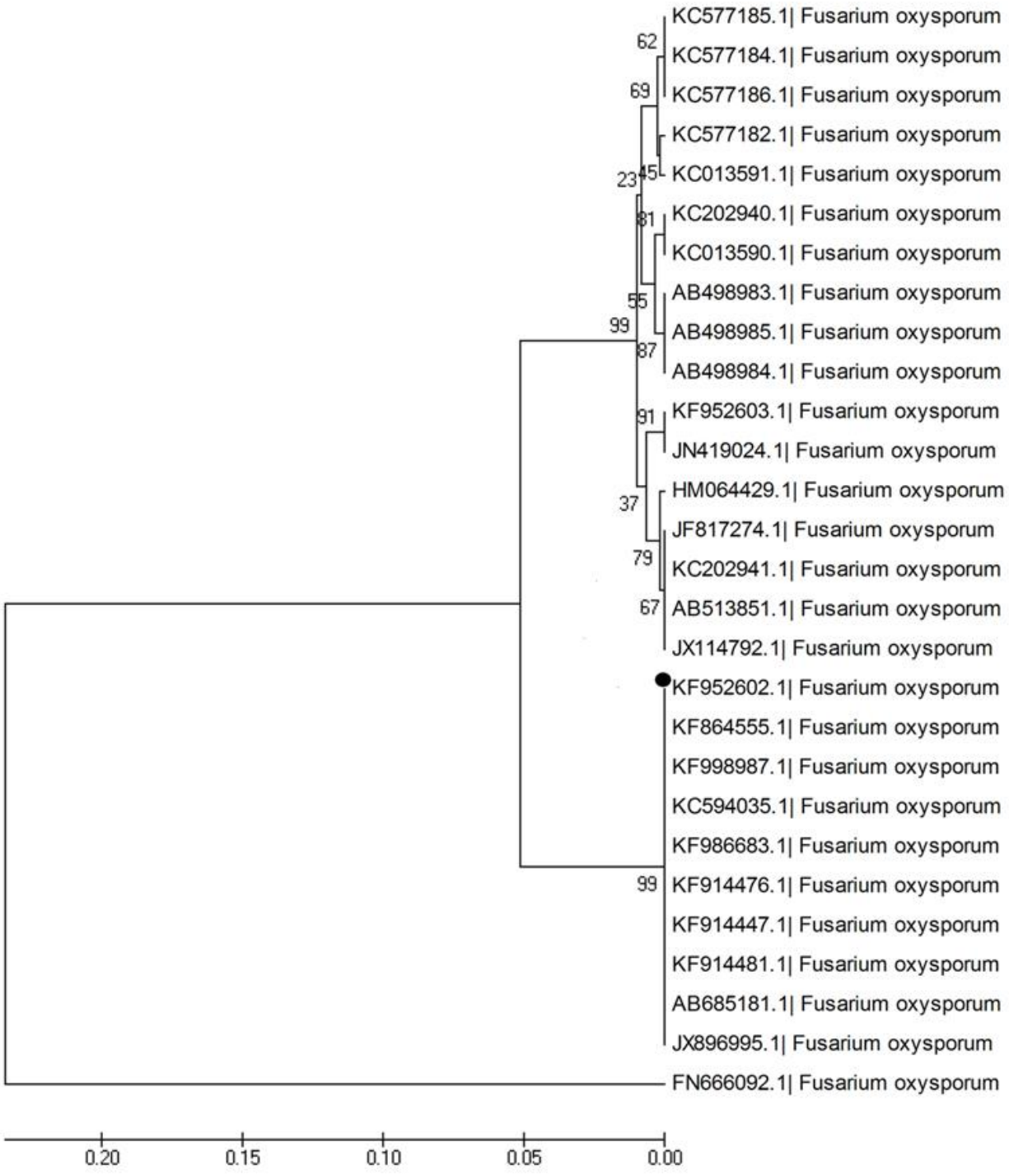


Fig. 31. Phylogenetic placement of *F. oxysporum* (RHS/M534) with other ex-type strain sequences obtained from NCBI GenBank Database

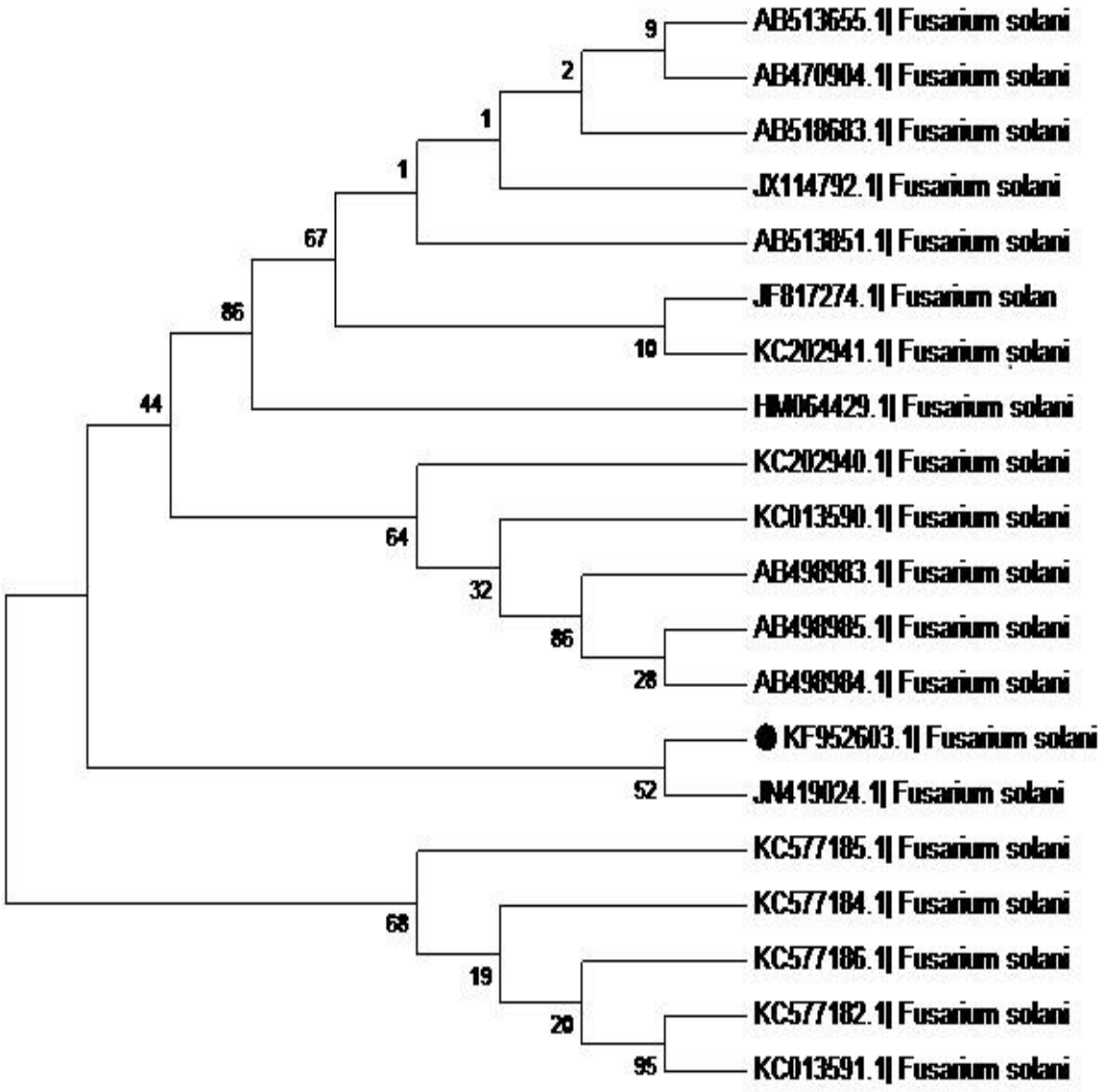


Fig. 32. Phylogenetic placement of *F. solani*(RHS/M532) with other ex-type strain sequences obtained from NCBI GenBank Database

4.10. Molecular characterization of BCA

Among the fungal isolates obtained from mandarin rhizosphere, two important plant growth promoting fungi (PGPF), *T. harzianum* (NAIMCC-F-01961) and *T. asperellum*(NAIMCC-F-01963) were designated as potential PGPF. The identities of these two BCA were further confirmed with the help of 18S r DNA sequences amplified with the help of universal primers.

4.10.1. RAPD-PCR and phylogenetic analysis of *Trichoderma* isolates of mandarin rhizosphere

Genomic DNA of *T. asperellum* and *T. harzianum* were 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. ITS region of rDNA was amplified using genus specific T/ITS1 and T/ITS4 primers. Amplified products of 600bp size was evident in case of *Trichoderma* (Fig. 33). The genetic relatedness among isolates of *T. harzianum* and *T. asperellum* wereanalyzed by random primers to generate reproducible polymorphisms. All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicated the genetic diversity of all isolates. A total of 11 reproducible and scorable polymorphic bands ranging from approximately 1000bp to 2000bp were generated among the isolates of *T. harzianum* and *T. asperellum* (Table 8, Fig. 33 B & C). In theRAPD profiles showed that primer OPD-6 scored highest bands which ranged between 200to 6000bp in all case of *T. asperellum* and *T. harzianum* isolates and random RAPD primer OPD-6 showed 87.5% polymorphism in the case of *T. asperellum* and *T. harzianum.* Relationships among the isolates were evaluated by cluster analysis of the data based on the similarity matrix. The dendogram was generated by un weighted pair-group methods with arithmetic mean (UPGMA) using NTSYSpc software. Based on the results obtained all the four isolates can be grouped into two main clusters. One cluster represents *T. harzianum* and other *T. asperellum*.

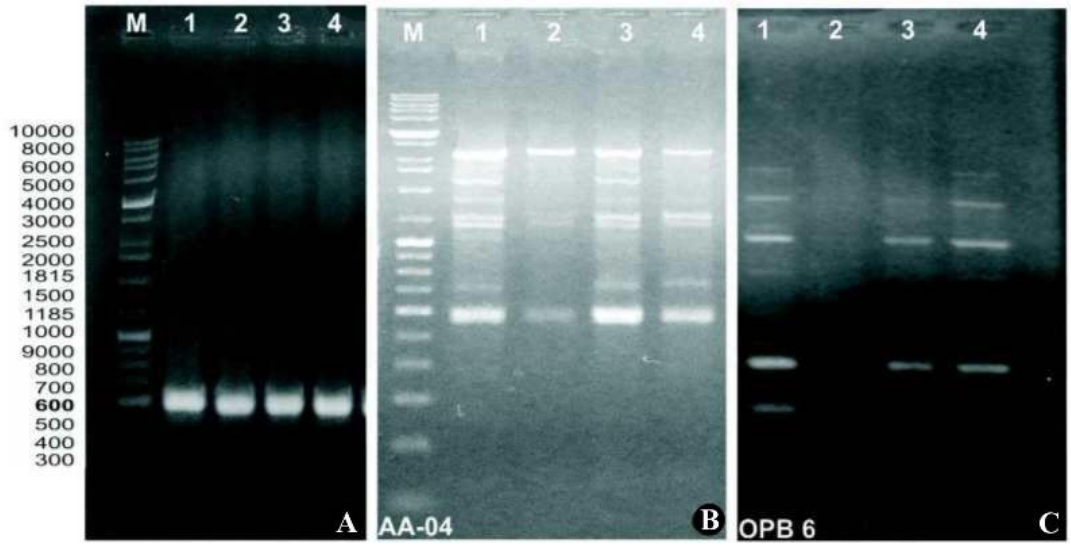


Fig. 33. RAPD-PCR analysis of *Trichoderma* isolates of mandarin rhizosphere (A) Agarose gel (2%) electrophoresis of ITS region. Lane M-high range DNA ladder, lane 1-2, *Trichoderma asperellum* and 3-4 *T. harzianum*; (B-C) Agarose gel (2%) electrophoresis of RAPD, Lane M-High range DNA ladder, lane 1-4 for *T. asperellum* & *T. harzianum* with Primer AA-04 &OPB 6 respectively

4.10.2. 18 S rDNA sequence and Phylogenetic analysis for identification of BCA

4.10.2.1. *T. harzianum*

Genomic DNA of *T. harzianum* (*Hypocrea lixii*) (NAIMCC-F-01961) were suspended in 100µl 1X TE buffer treated with RNAse (60 µg) until further use. Agarose gel electrophoresis of genomic DNA revealed that they were RNA free. Purity of DNA evaluated in terms of the ratio between absorbance of A_{260} and A_{280} showed that genomic DNA was ~1.8. ITS region of rDNA was amplified using genus specific T/ITS1 and T/ITS4 for *Trichoderma*primers where the amplified products of 600 bp size was produced by both the primers.

4.10.2.1.1. Chromatogram

The BLAST query of the 18S rDNA sequence of T/ITS1 and T/ITS4 (for *Trichoderma*) against GenBank database confirmed their identity**.** The sequences have been deposited in NCBI, GenBank database under the accession no. GQ995194 *Hypocrealixii*. The sequence chromatograms of *T.harzianum* have been represented in Fig 34.

4.10.2.1.2 Multiple sequence alignment

A multiple sequence alignment of ITS gene sequences of *T. harzianum* was conducted. Sequences of other strains obtained from NCBI Genbank database showing maximum homology with our strain was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. 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. There were quite a number of gaps that were introduced in the multiple sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Fig. 35).

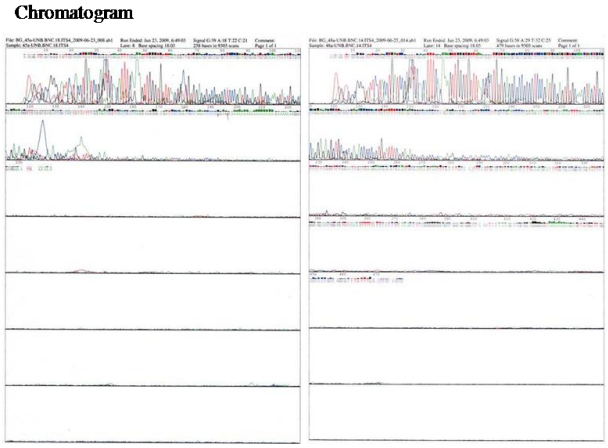


Fig. 34. Chromatogram and sequence deposition of 18S r DNA region of *T. harzianum* strain NAIMCC-F-01961

Phylogenetic analyses were completed using the MEGA package (version 4.01; Institute of Molecular Evolutionary Genetics, University Park, PA). Phylogenetic analysis was carried out with Ex-type strain sequences obtained from NCBI Genbank database which showed maximum homology with *T. harzianum* (*Hypocrea lixii*) (GQ995194) (Table 20).

Table 20. Genbank Accession Numbers and Geographic locations of the Ex-Type strains of *T. harzianum* (*Hypocrea lixii*) (RHS/M511) that showed homology with the isolate

Accession No.	Strain/isolate	rDNA Sequence	Country	Source
GU563350	A25-2	583bp	China	Soil
GU479433	NBAII(N)SOL 3-Th	595 bp	India	Soil
GU479430	NBAII(N)KA 12-Th	575bp	India	Soil
JQ665264	TriH-JSB72	470bp	Japan	Rhizosphere
JQ665263	JQ665263	511bp	Japan	Rhizosphere
JQ665261	TriH-JSB40	497bp	Japan	Rhizosphere
JQ665259	TriH-JSB27	474bp	Japan	Rhizosphere
JN713922	DPNST-4	596bp	India	Soil
JN716383	NBAII-Th32	483bp	India	Soil
JN716382	NBAII-Th31	522bp	India	Soil
JN716381	NBAII-Th30	609bp	India	Soil
JN228900	NBAII-Th23	591bp	India	Soil
GU566265	G36	659bp	Czech Republic	Rhizosphere
GU566195	A24	659bp	Czech Republic	Rhizosphere
GU566194	A23	659bp	Czech Republic	Rhizosphere
HQ637340	JZ-179	593bp	China	Soil
HQ637339	JZ-77	580bp	China	Soil
HQ857115	475/02	591bp	Brazil	Soil
FJ412042	CPK 2712	592bp	Ethiopia	Soil
GQ995194	**RHS/M511**	**194 bp**	**India**	**Rhizosphere**

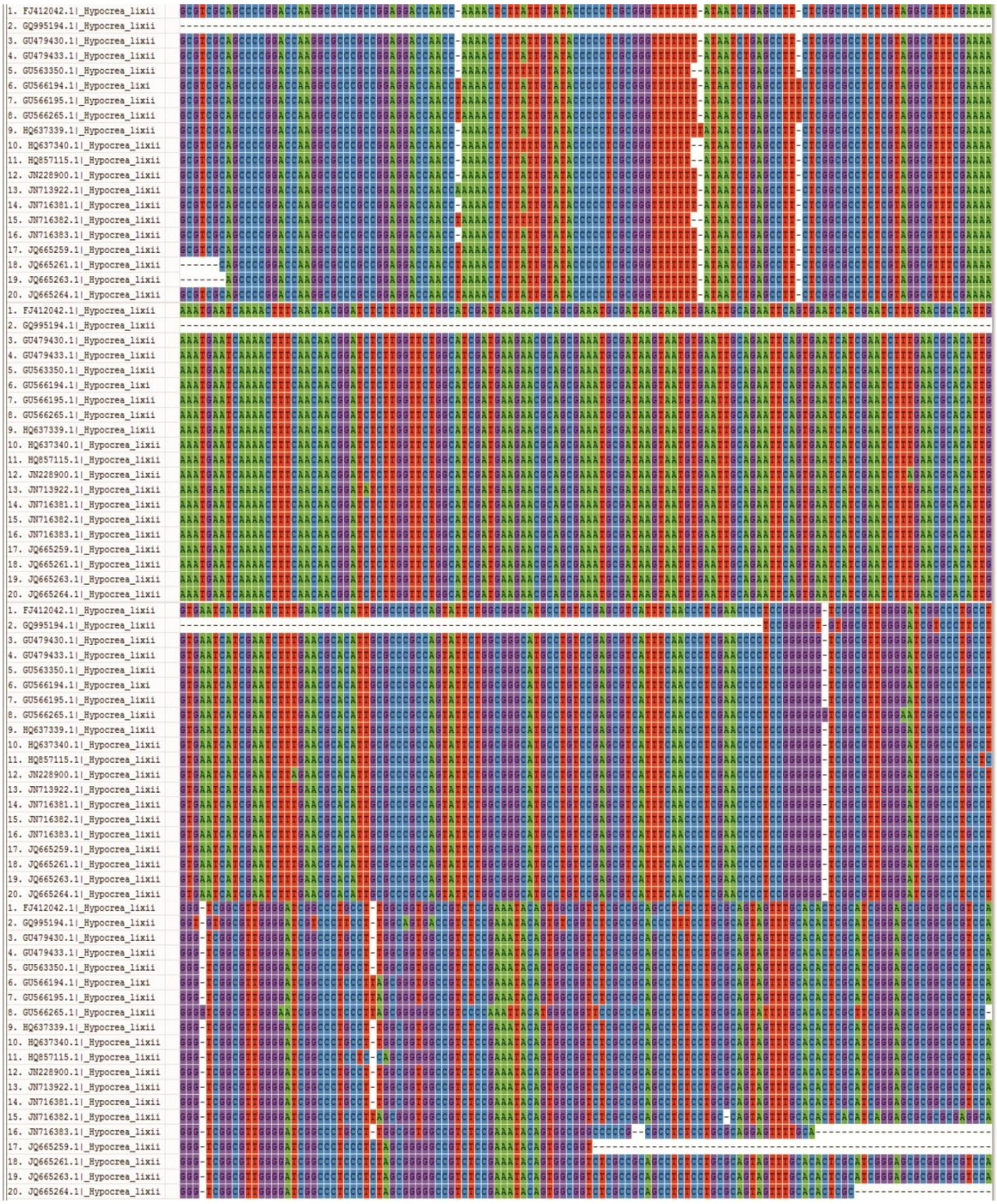


Fig. 35.18S r DNA sequence alignments of *T. harzianum* (NAIMCC-F-01961) with other extypes isolate. The conserved regions of the gene are demonstrated in different colours

4.10.2.2. *T. asperellum*

Genomic DNA of *T. asperellum* were suspended in 100µl 1X TE buffer treated with RNAse (60 µg) until further use. Agarose gel electrophoresis of genomic DNA revealed that they were RNA free. Purity of DNA evaluated in terms of the ratio between absorbance of A_{260} and A_{280} showed that genomic DNA was ~1.8.

ITS region of rDNA was amplified using genus specific T/ITS1 and T/ITS4 for *Trichoderma* primers where the amplified products of 600 bp size was produced by both the primers.

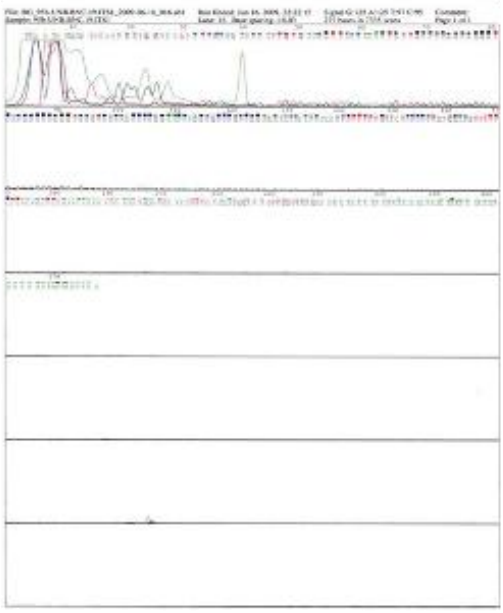
4.10.2.2.1. Chromatogram

The BLAST query of the 18S rDNA sequence of T/ITS1 and T/ITS4(for *Trichoderma*) against GenBank database confirmed their identity**.** The sequences have been deposited in NCBI, GenBank database under the accession no. HQ265418. The sequence chromatograms have been represented in Fig 36.

4.10.2.2.2. Multiple sequence alignment

A multiple sequence alignment of ITS gene sequences of *T. asperellum* was conducted. Sequences of other strains obtained from NCBI Genbank database showing maximum homology with our strain was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. 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. There were quite a number of gaps that were introduced in the multiple sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Fig 37). Phylogenetic analyses were completed using the MEGA package (version 4.01; Institute of Molecular Evolutionary Genetics, University Park, PA). Phylogenetic analysis was carried out with Ex-type strain sequences obtained from NCBI Genbank database which showed maximum homology with *T. asperellum* (GQ995194) (Table 21).

Chromatogram



Partial sequence of 18S ribosomal RNA gene

TACTATTAGAACTCACTCGGTAGTGTATGTCTCTCCCGACCCATTGTTGTAACGTTACCAAACT
GTTGCCTCGGCGGGGTCACGCCCCGGGTGCGTCCCAGCCCCGGAACCAGGCGCCCGCCGGA
GGAACCAACCCAACTCTTTCTGTAATCCCCTCGCGGACGTTTTTCTTACAGCTCTGAACAAAA
ATCAAATGAATCAAACTTTCACAACGGATACTTGGTTCAG

Sequence deposited: NCBI

ACCESSION:**HQ265418**
VERSION:HQ265418.1
GI:315112483
DNA linear: 228 bp

Title: *Trichoderma asperellum* isolate RHS/M 512
18S ribosomal RNA gene, partial sequence; internal transcribed spacer 1,
5.8S ribosomal RNA gene, and internal transcribed spacer 2,
complete sequence; and 28S ribosomal RNA gene, partial sequence.

ORIGIN

1 tactattaga actcactcgg tagtgtatgt ctctcccgac ccattgttgt aacgttacca
61 aactgttgcc tcggcggggt cacgcccccg gtgcgtccca gccccggaac caggcgcccg
121 ccggaggaac caacccaact ctttctgtaa tcccctcgcg gacgttttte ttacagctct
181 gaacaaaaat caaatgaatc aaactttcac aacggatact tggttcag

Fig. 36. Chromatogram and sequence deposition of 18S r DNA region of *T. asperellum* strain NAIMCC-F-01963

Table 21. Genbank Accession Numbers and Geographic locations of the Ex-Type strains of *T. asperellum* (RHS/M512) that showed homology with the isolate

Accession No.	Strain/isolate	rDNA Sequence	Country	Source
FN396553	V5	525 bp	Slovakia	Soil
FN396552	V33	525 bp	Czech Republic	Soil
JX465483	NBAII-Ta102	581 bp	India	Soil
GU318216	ZJPH0810	602 bp	China	Soil
JX513900	Cu4	571 bp	India	Soil
JX465482	NBAII-Ta101	607 bp	India	Soil
JN974006	TNAU-MNT07	582 bp	India	Soil
JN974005	TNAU-06	537 bp	India	Soil
JN716379	NBAII-Ta15	586 bp	India	Soil
GU198318	GJS 05-328	597 bp	USA	Soil
GU198317	GJS 90-7	600 bp	USA	Soil
GU198314	GJS 02-66	599 bp	USA	Soil
GU198307	GJS 06-294	599 bp	USA	Soil
JF798504	T2	600 bp	Italy	Soil
HM246517	A45-1	565 bp	China	Soil
EU264001	GJS 05-328	573 bp	USA	Soil
KC884820	T-63	602 bp	China	Soil
KC859434	TR15	751 bp	India	Soil
KC479819	TR9	603 bp	Indonesia	Rhizosphere
JX677935	AF35	603 bp	Japan	Rhizosphere
HQ265418	**RHS/M 512**	**228 bp**	**India**	**Rhizosphere**

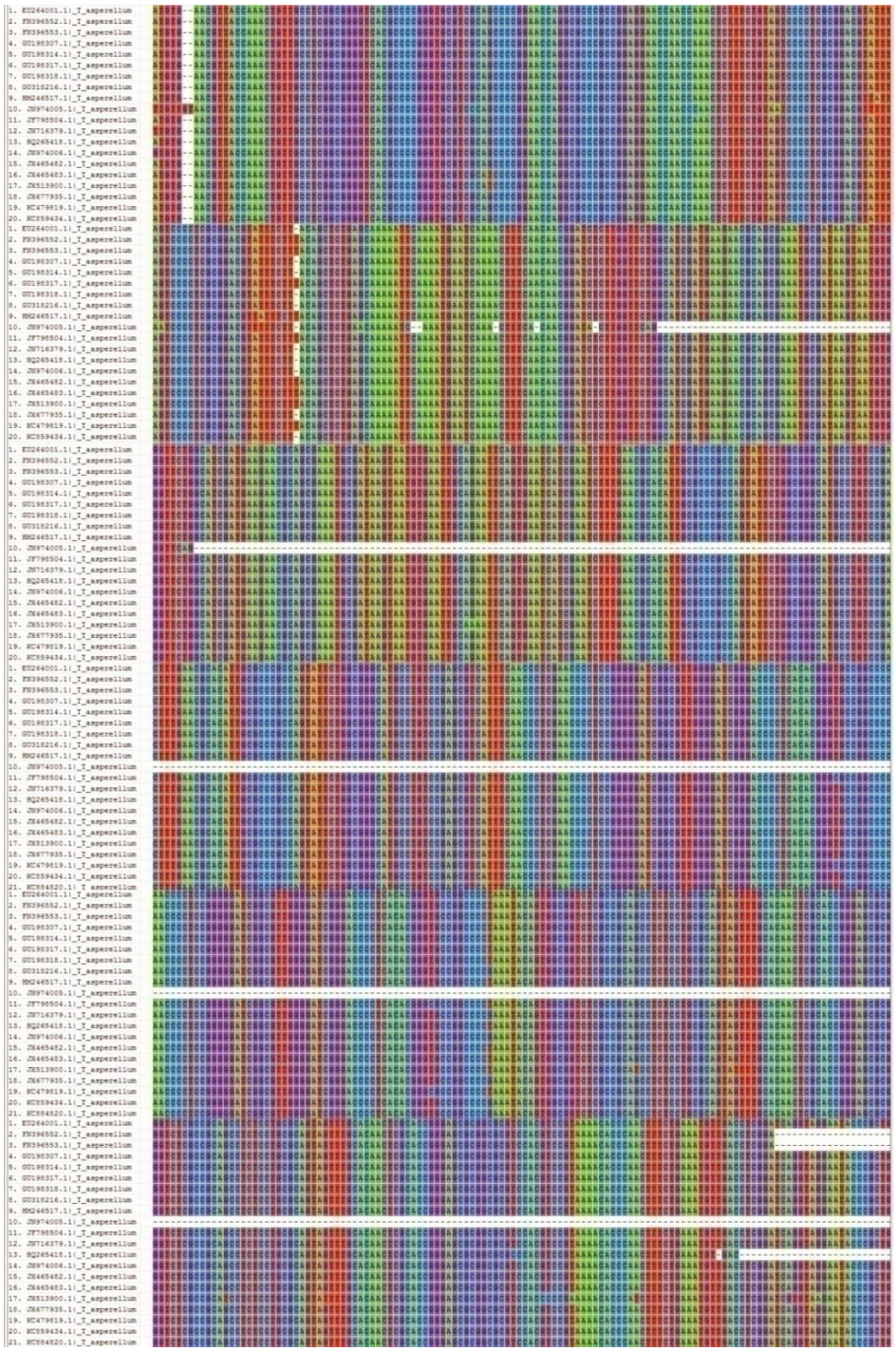


Fig. 37. 18S r DNA sequence alignments of *T. asperellum*(NAIMCC-F-01963) with other extypes isolate. The conserved regions of the gene are demonstrated in different colours

4.10.3. Phylogenetic analysis of *T. harzianum*

The evolutionary history was inferred (Fig 38) using the UPGMA method (Sneath & Sokal, 1973). The optimal tree with the sum of branch length = 37.45168819 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 (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, *et al.*, 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 228 positions in the final datas*et* Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

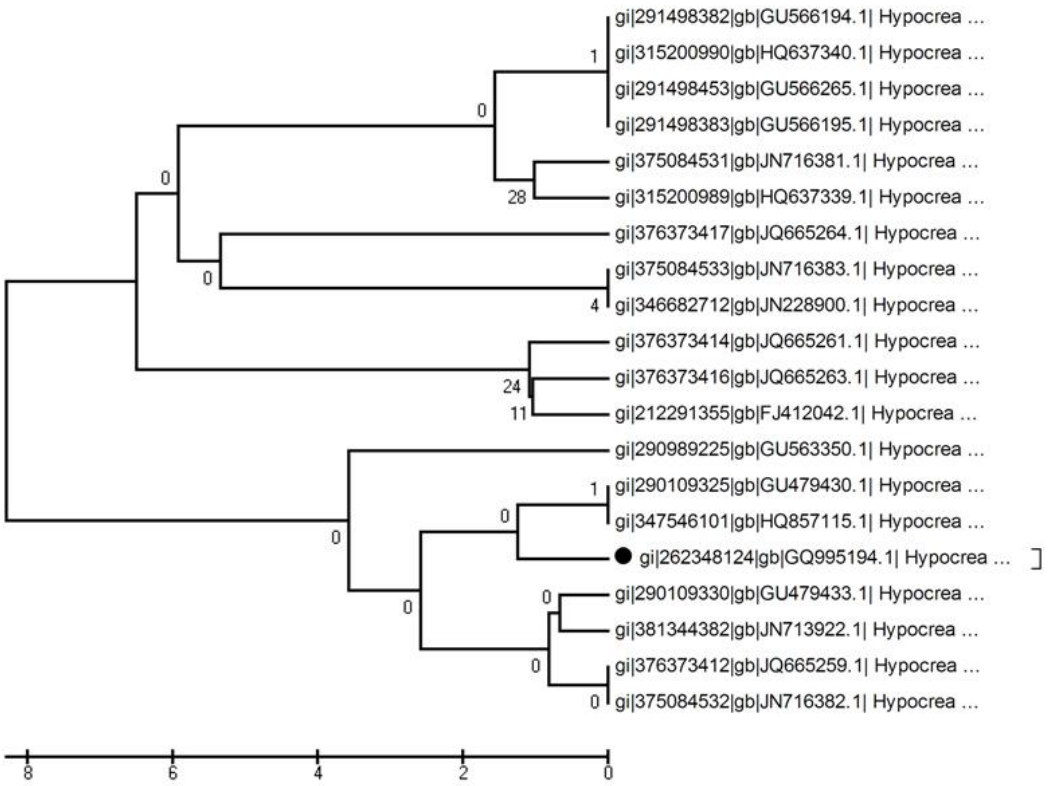


Fig. 38. Phylogenetic tree of *T. harzianum* (*Hypocrea lixii*)strain NAIMCC-F-01961 with other extypes isolate

4.10.4. Phylogenetic analysis of *T. asperellum*

The evolutionary history was inferred (Fig 39) using the UPGMA method (Sneath & Sokal, 1973). The optimal tree with the sum of branch length = 47.89743316 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 (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 *et al.*, 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).

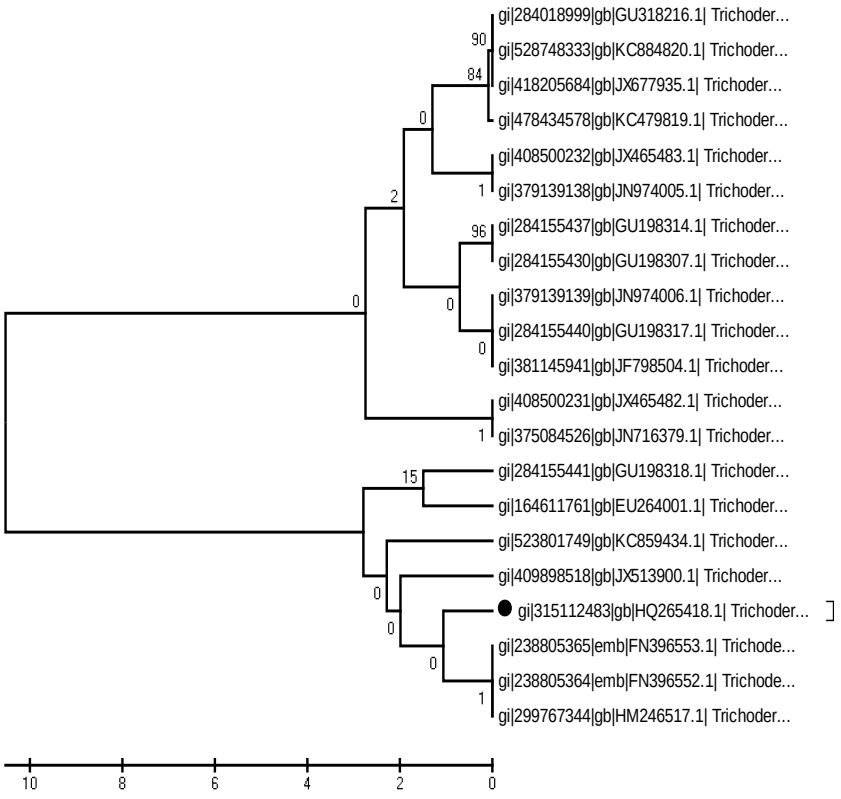


Fig. 39. Phylogenetic tree of *T. asperellum*(NAIMCC-F-01963) with other extypes isolate.

4.11. Molecular characterization of PGPR isolate

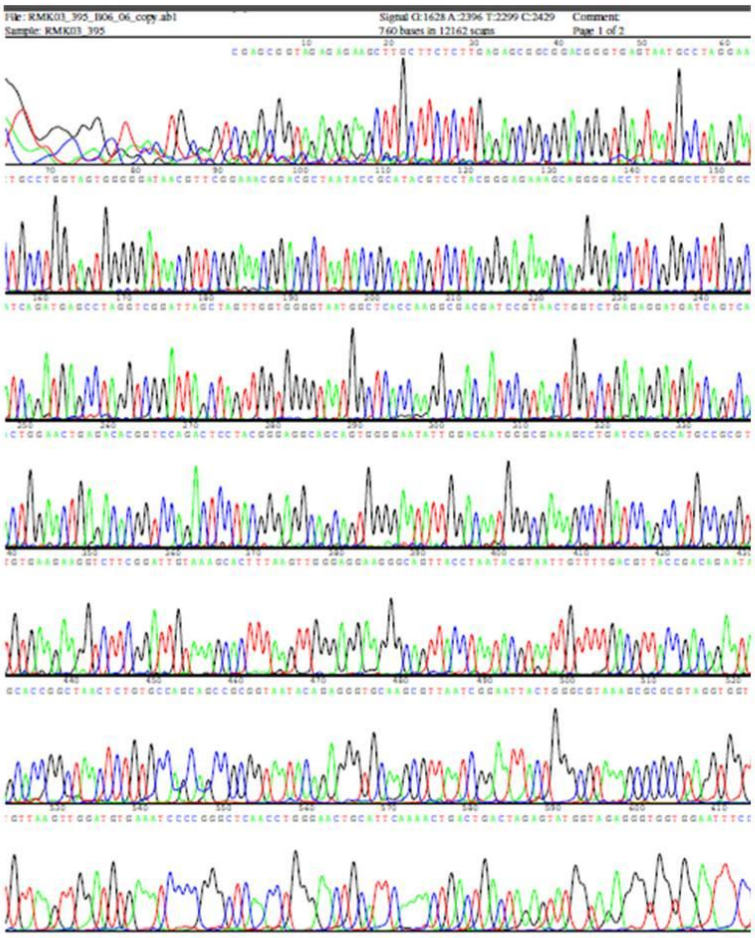
Among the bacterial isolates obtained from mandarin rhizosphere, one important plant growth promoting rhizobacteria (PGPR), *P. poae* (RMK03) was designated as potential PGPF. The identity of this PGPR was further confirmed with the help of 16S r DNA sequences amplified with the help of universal primers. The sequences have been deposited in NCBI, GenBank database under the accession no. KJ917553. The sequence chromatograms have been represented in Fig 40.

4.11.1. 16S rDNA sequences and BLAST analysis

The identity of the isolate was confirmed with the help of 16S rDNA sequences. The BLAST query of the 16S r DNA sequence of the isolates against GenBank database confirmed the identity of the isolate RMK03 as *P. poae.* The sequence chromatogram of the PGPR isolates have been represented in Fig 41.

4.11.2 Multiple sequence alignment and phylogeny

A multiple sequence alignment of ITS gene sequences of *P. poae* was conducted. Sequences of other strains obtained from NCBI Genbank database showing maximum homology with our strain was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. 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. There were quite a number of gaps that were introduced in the multiple sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Fig.40). Phylogenetic analyses were completed using the MEGA package (version 4.01; Institute of Molecular Evolutionary Genetics, University Park, PA). Phylogenetic analysis was carried out with Ex-type strain sequences obtained from NCBI Genbank database which showed maximum homology with *P. poae* (RMK03) (Table 22).



Partial sequence of 18S ribosomal RNA gene

CGAGCGGTAGAGAGAAGCTTGCTTCTCTTGAGAGCGGCGGACGGGTGAGTAATGCCTAGGAATCTGCCTGGTAGTGGGGGAT
AACGTTCGGAAACGGACGCTAATACCGCATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGC
CTAGGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGATGATCAGTCACAC
TGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCAT
GCCGCGTGTGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTTACCTAATACGTAATTGTTTTGA
CGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACT
GGGCGTAAAGCGCGCGTAGGTGGTTTGTTAAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTCAAAACTGACT
GACTAGAGTATGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAG
GCGACCACCTGGACTAATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGT
AAACGATGTCAACTAGCCGTTGGAAGCCTTGAGCTTTTAGTGGCGCAGCTAACGCATTAAGTTGACCGCCTGGGGAGTACGGCC
GCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACC
TTACCAGGCCTTGACATCCAATGAACTTTCTAGAGATAGATTGGTCCCCCTCGGGAACATTGAGACAGGTGCTGCATGGCTGTC
GTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCA
CTCTAAGGAGACTGCCGGTGACAAACCGGAGGAAGGTGGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTGGGCTACAC
ACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCCGGATCGCA
GTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTTCCCGGGCCTTGTAC
ACACCGCCCGTCACACCATGGGAGTGGGTTGCACCAGA

Fig. 40. Chromatogram of 16S r DNA region of *P. poae* (RMK03)

Table 22. Genbank Accession Numbers and Geographic locations of the Ex-Type strains of *P. poae* that showed homology with the isolate

Accession No.	Strain/isolate	rDNA Sequence	Country
EU350370	ST250	1496 bp	China
FJ179369	BIHB 808	1500	India
NR_028986	P 527/13	1492	USA
EU600373	NS12	1337	India
EU600372	RT5RP2	1395	India
GU220067	PB2RP1(2)	1429	UK
KF054938	IARI-NIAW2-1	1470	India
KJ529107	SGS1	796	Finland
KJ529105	FGS11	799	Finland
KJ529101	SGS20-1	798	Finland
KJ024691	ZR4-7_Ps	522	Austria
JX477091	S2_013	680	China
KF719959	KA-5	721	India
HQ256531	NBB19	1502	India
HQ003435	NBGD6	1489	India
HE648159	C1 11139	874	France
JF706533	PDD-37b-2	1400	France
JQ291748	1H7	540	Italy
JX885489	MBC27	607	India
KJ917553	**RMK03**	**1368**	**India**

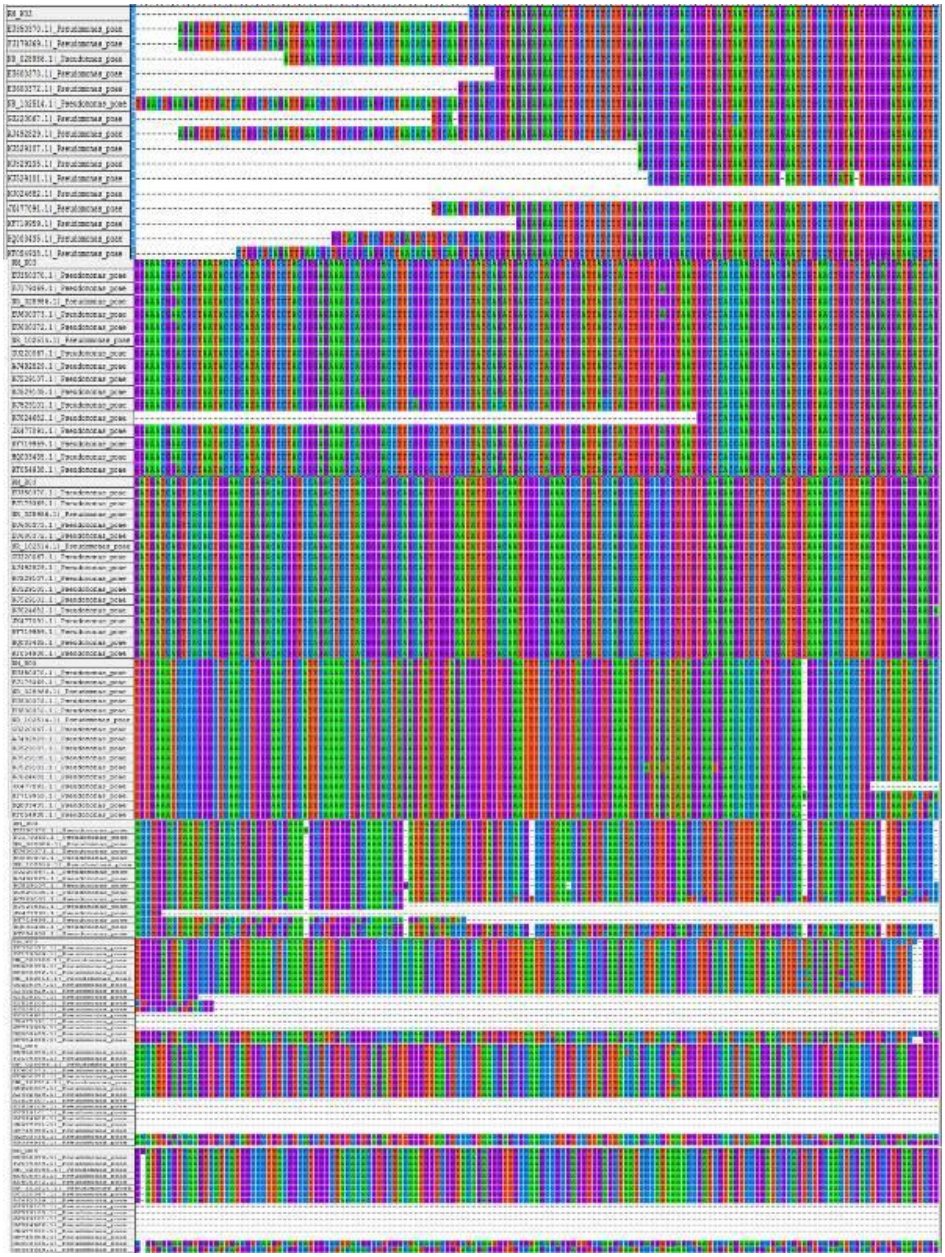


Fig. 41.16S r DNA sequence alignments of *P. poae* (RMK03) with other extypes isolate. The conserved regions of the gene are demonstrated in different colours

The evolutionary history (Fig 42) was inferred using the UPGMA method (Sneath & Sokal, 1973). The optimal tree with the sum of branch length = 0.04505909 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 (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 *et al.*, 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 477 positions in the final dataset Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2007).

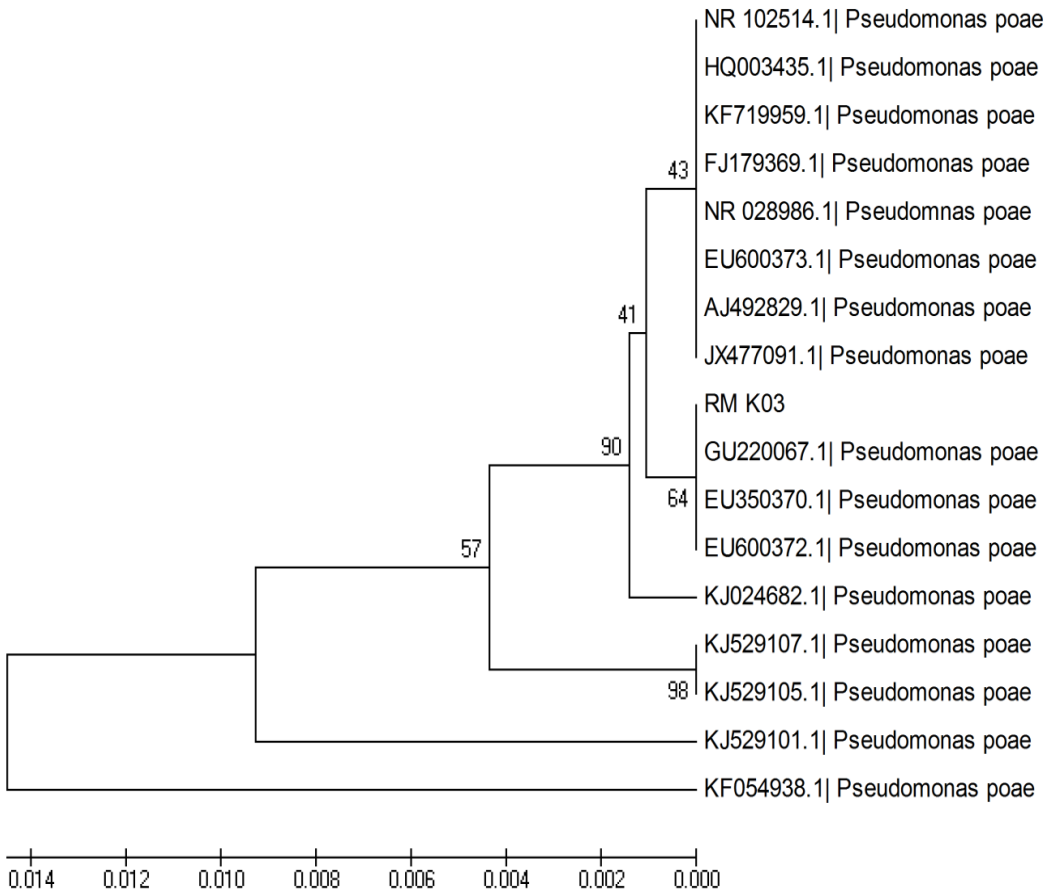


Fig. 42. Phylogenetic tree of *P. poae* (RMK03)with other extypes isolate

4.12. Antioxidative responses of *C. reticulata* following water stress

Response of *Citrus reticulata* plants to water stress was observed. The experimental design was made up of two soil water regimes, flood and drought. Four years old mandarin plants obtained from Kalimpong, Mirik, Bijanbari and Teesta Valley orchards were subjected to flood and drought stress. Mandarin plants were planted in field and flooded with water in order to obtain flooding stress while for drought stress plants were planted in pots and left without water.Morphological and biochemical changes induced by water logging and drought conditions were determined.

4.12.1. Morphological changes

4.12.1.1. Water logging

The plants started showing symptoms of wilting after 24 hrs of treatment. The leaves curled inwards and drooped. Leaves started falling after 72 hours of treatment. Yellowing of leaves began after 48 hours of treatment. The plants brought from Bijanbari was most susceptible to flooding stress. 100% of the leaves had dropped by the third day of treatment.

4.12.1.2. Drought

The first signs of symptoms was leaf drooping. Leaves started drooping after 48 hours of drought stress. Signs of leaf withering showed leaves curling inwards, colour change from green to yellow to brown, became crisp and finally leaf drop. All plants from the four sites showed the same signs of stress at the same time.

4.12.2. Biochemical changes

Antioxidative enzymes such as peroxidase, catalase, ascorbate peroxidase, was observed in the leaves and roots. Antioxidative activity was seen to be more in the leaves than in the roots. Among the antioxidants carotenoid (Table 23) content showed a significant decrease during the flood stress but increased in drought stress. An increase in ascorbate content was observed during stress in comparison to the control. Catalase and ascorbate peroxidase activity (Fig 43) decreased in almost all the samples during both types of stresses but peroxidase activity mostly increased in leaf especially during both drought and flooding stress indicating that peroxidase activity plays a vital role in scavenging of free radicals. PAL activity showed varied trends in samples subjected to flooding stress, the activity increased in some samples during stress in both leaf and root

whereas in some samples the activity decreased with flooding stress (Fig. 44 A). But in case of drought (Fig. 44 B) the activity increased in both the leaf and the root of the samples in comparison to the control plants. Maximum activity was observed in BJ, MR and TV in both the leaf and roots of these samples.

Protein content in all the samples was more in the leaves than in the root during both drought and flooding stress (Table 24). During drought stress proline (Table 25) content increased in all the four samples of mandarin plant. Both leaves and root showed an increase during drought stress but there was more increase in the roots than in the leaves. Mandarin plants subjected to flood and drought stress have been represented in Fig 45.

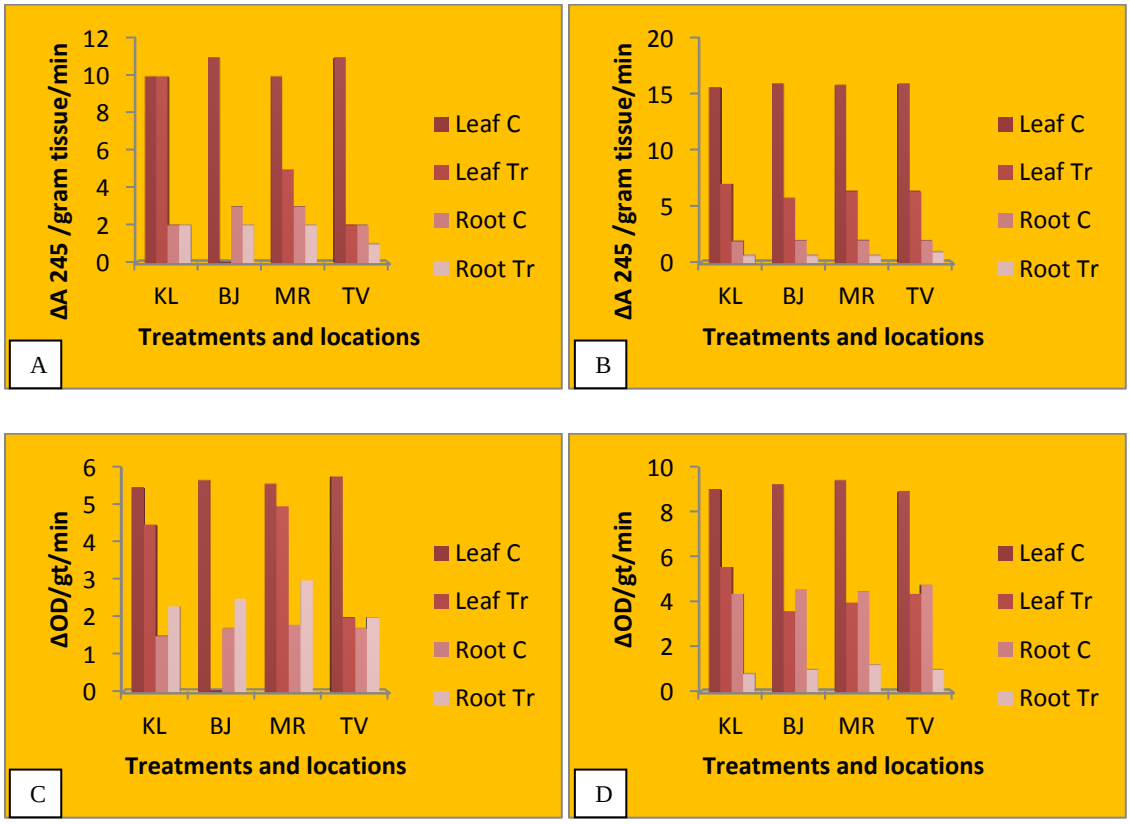


Fig. 43. Catalase(A&B) & Ascorbate peroxidase(C&D) activities during flooding(A&C) and drought(B&D)

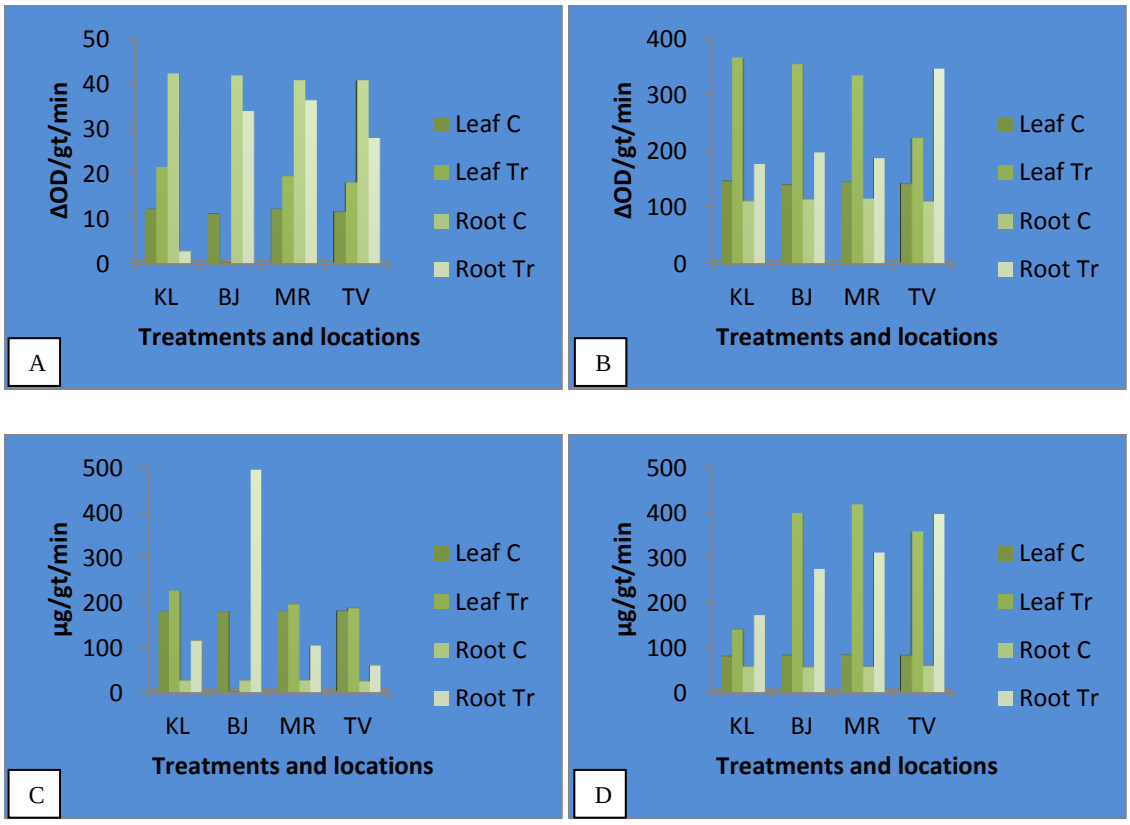


Fig.44 : Peroxidase (A&B) & Phenylalanine Ammonia Lyase (C&D) activities during flooding (A&C) and drought (B&D)

Table 23. Ascorbate and carotenoid content during flooding and drought

Plant	ASCORBATE (µg/g tissue)				CAROTENOID (mg/g tissue)			
	DROUGHT		FLOOD		DROUGHT		FLOOD	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
KL	4	6.50	9.26	10.78	0.006	0.025	0.027	0.026
BJ	4.11	7.75	9.23	0.000	0.005	0.010	0.027	0.000
MR	4.15	7.64	9.11	18.70	0.002	0.017	0.027	0.016
TV	4.19	7.30	9.12	14.32	0.004	0.018	0.027	0.020

Table 24. Protein content of leaf and root during flooding and drought

Plant	PROTEIN (LEAF) (mg/g tissue)				PROTEIN (ROOT) (mg/g tissue)			
	DROUGHT		FLOOD		DROUGHT		FLOOD	
	Control	Treated	Control	Treated	Control	Treated	Control	Treated
KL	150.5	192.0	235	182.5	15.0	26.0	55.0	70.0
BJ	150.5	200.0	235	0.000	15.0	24.0	55.0	75.0
MR	150.5	196.0	235	225.6	15.0	24.0	55.0	62.5
TV	150.5	196.0	235	200.0	15.0	26.0	55.0	50.0

Table 25. Proline content of leaf and root during drought

Plant	PROLINE (LEAF) (mg/g tissue)		PROLINE (ROOT) (mg/g tissue)	
	Control	Treated	Control	Treated
KL	0.56	0.59	0.9	1.34
BJ	0.5	1.23	0.96	1.76
MR	0.52	1.94	0.96	2.12
TV	0.51	1.52	0.94	3.36

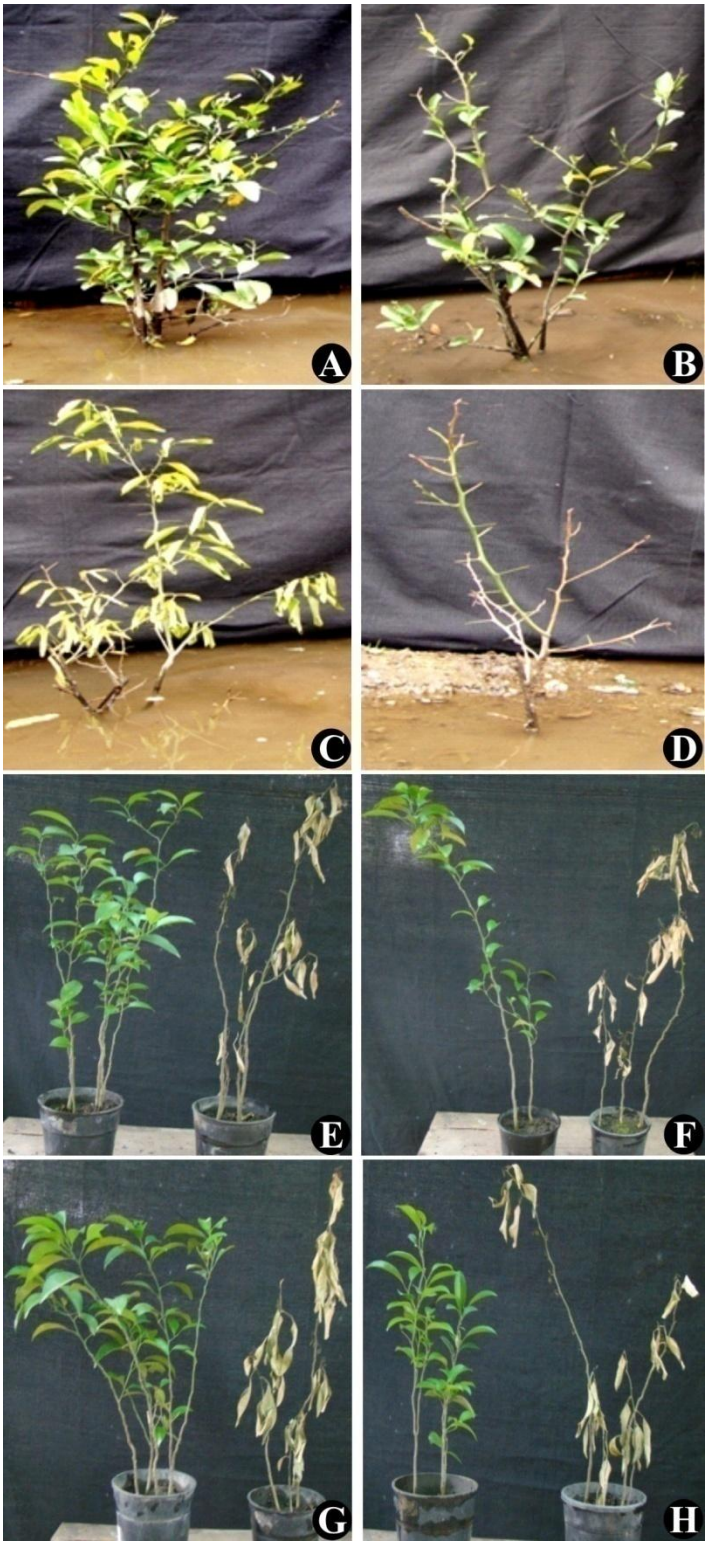


Fig. 45: Abiotic stress to Mandarin plants.
(A) Water loggging, (B) Drought conditions

4.13. Activation of defense response of *C. reticulata* following application of *Trichoderma* and AMF against *F. solani*

4.13.1. Effect of *T. harzianum* and *T. asperellum* against *F. solani*

4.13.1.1. Disease suppression

In the first set of trial, *T. harzianum* and *T. asperellum* were tested for their effect in inhibiting root rot of mandarin seedlings caused by *F. solani* in nursery conditions when applied singly and in combination. *Trichoderma*were inoculated to the rhizosphere of one year mandarin seedlings prior to pathogen challenge. *T. harzianum* and *T. asperellum*alone could effectively reduce disease incidence. However combined inoculation with both species of *Trichoderma* showed better results (Table 26).

Table 26. Disease index of root rot ofmandarin seedlings following *Trichoderma* treatment and pathogen challenge

Disease index	No. of days after inoculation		
Pre treated with*	15	30	45
F. solani	2.1±0.577	3.0±0.577	5.3±0.577
T. harzianum + F. solani	0.9±0.115	1.1±0.057	2.6±0.58
T. asperellum + F. solani	1.2±0.057	2.1±0.057	2.0±0.577
T. asperellum + T. harzianum + F. solani	0.5±0.115	1.0±0.577	1.7±0.333
Rot index: 0- no symptoms; 1- small roots turn brownish and start rotting; 2- leaves start withering and 20-40% of roots turn brown; 3- leaves withered and 50% of roots affected; 4- shoot tips also start withering; 60-70% roots affected; 5- shoots withered with defoliation of lower withered leaves, 80% roots affected; 6- whole plants die, with upper withered leaves still remaining attached; roots fully rotted ±=SE			

4.13.1.2. Growth enhancement

Growth enhancement was evaluated in terms of percent increase in height and leaf number in comparison to a similar increase in control plants. A significant increase in growth and number of leaves in all the mandarin plants treated *T. harzianum*

and *T. asperellum* was observed. Growth enhancement was significantly higher when both the AMF were applied jointly (Fig. 46).

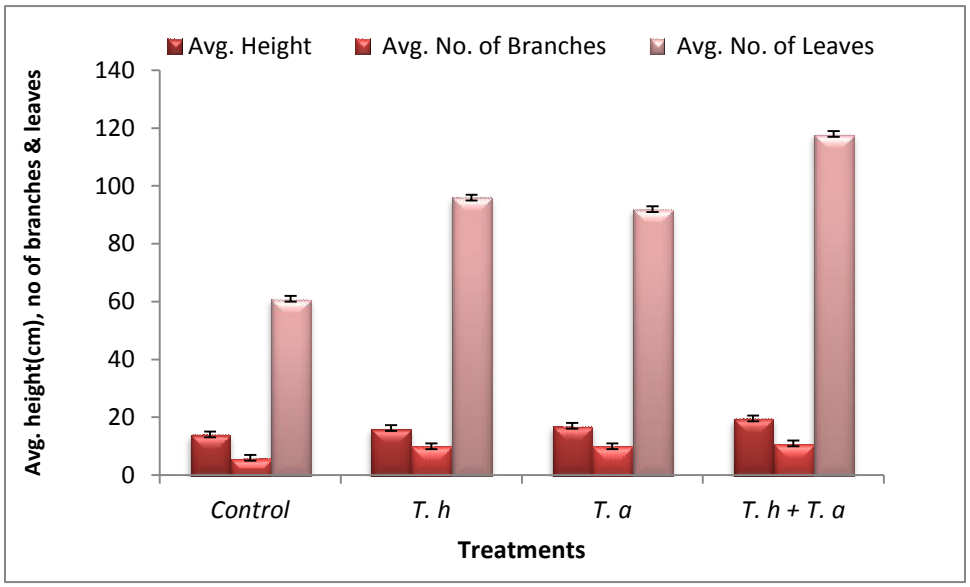


Fig. 46 Growth of manadrin seedlings following treatments with *T. harzianum* and *T. asperellum*

4.13.1.3. Changes in defense enzymes

Significant biochemical changes were observed on application of *T. harzianum* and *T. asperellum* prior to pathogen challenge in mandarin seedlings in nursery conditions. Experiments were conducted to assess the effect of single as well as combined application of *T. harzianum* and *T. asperellum* on biochemical components of mandarin leaves and roots. Multifold increase in activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in roots as well as leaf of mandarin plants was observed after application of *T. harzianum* and *T. asperellum* followed by inoculation with *F. solani***.** Overall results show that the defense enzyme activities were higher in leaves than the roots. (Fig. 47).

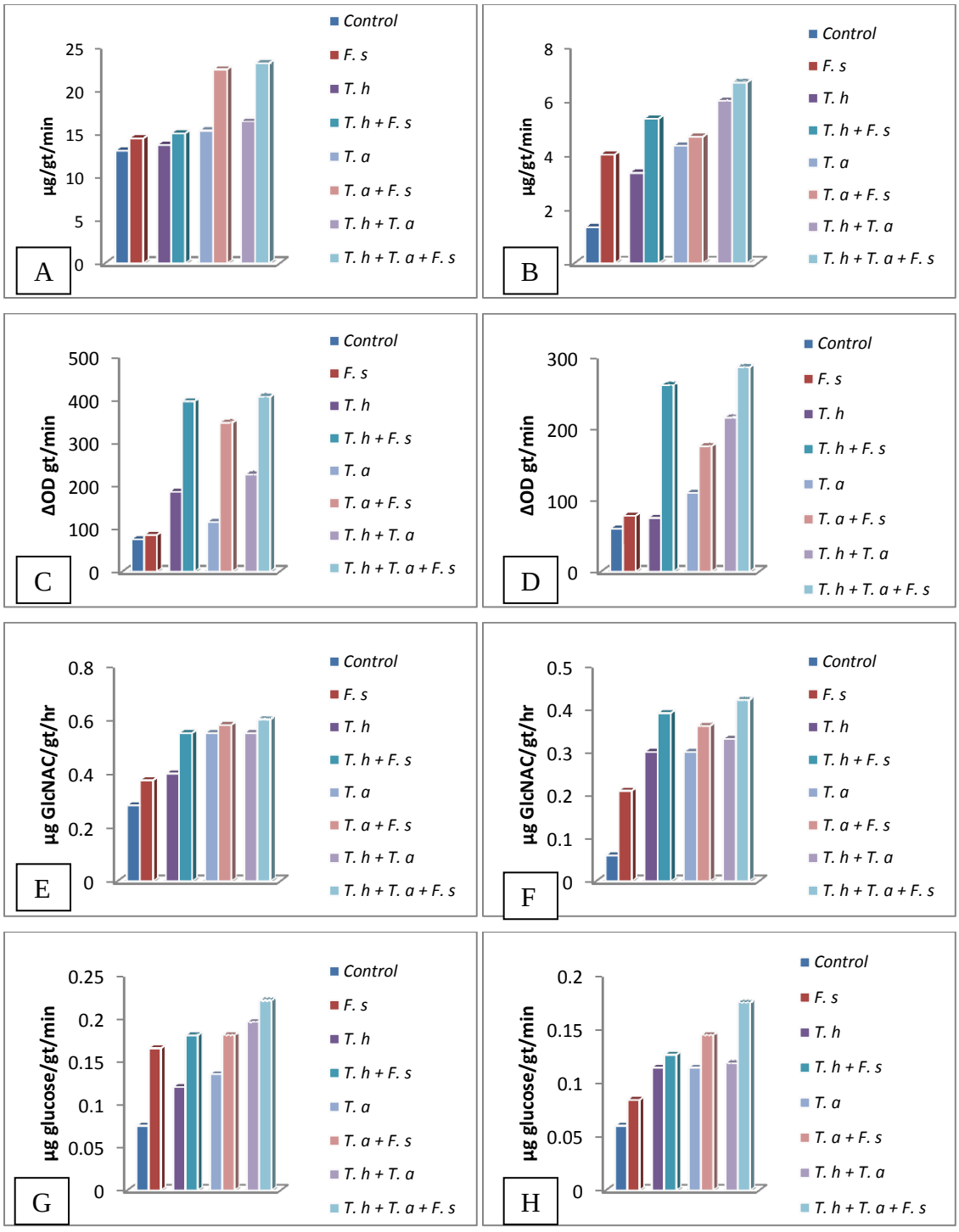


Fig. 47 PAL (A- Leaf, B- Root), Peroxidase (C-Leaf, D-Root), Chitinase (E-Leaf, F-Root) and Glucanase (G-Leaf, H-Root) activities in *Trichoderma* treated mandarin seedlings following inoculation with *F. solani*

4.13.2. Effect of *G. fasciculatum* and *Gi. gigantea* against *F. solani*

4.13.2.1. Disease suppression

In the second set of trial, *G. fasciculatum* and *Gi. gigantea* were tested for their effect in inhibiting root rot of mandarin seedlings caused by *F. solani* in field conditions when applied singly and in combination. AMFwere inoculated to the rhizosphere of one year old mandarin seedlings prior to pathogen challenge. *G. fasciculatum* and *Gi. gigantea* alone could effectively reduce disease incidence. However combined inoculation with both species of AMF showed better results (Table 27).

Table 27. Disease index of root rot ofmandarin seedlings following AMF treatment and pathogen challenge

Disease index	No. of days after inoculation		
Pre treated with*	15	30	45
F. solani	2.0±0.577	2.9±0.057	4.9±0.057
Gi. gigantea + *F. solani*	0.7±0.115	1.0±0.057	2.4±0.115
G. fasciculatum + *F. solani*	1.1±0.057	1.8±0.058	1.9±0.088
Gi. gigantea + *G. fasciculatum* + *F. solani*	0.4±0.057	0.9±0.057	1.6±0.057
Rot index: 0- no symptoms; 1- small roots turn brownish and start rotting; 2- leaves start withering and 20-40% of roots turn brown; 3- leaves withered and 50% of roots affected; 4- shoot tips also start withering; 60-70% roots affected; 5- shoots withered with defoliation of lower withered leaves, 80% roots affected; 6- whole plants die, with upper withered leaves still remaining attached; roots fully rotted ±=SE			

4.13.2.2. Growth enhancement

Growth enhancement was evaluated in terms of percent increase in height and leaf number in comparison to a similar increase in control plants. A significant increase in growth and number of leaves in all the mandarin plants treated with *G. fasciculatum* and *Gi. gigantea*was observed. Growth enhancement was significantly higher when both the AMF were applied jointly(Fig. 48).

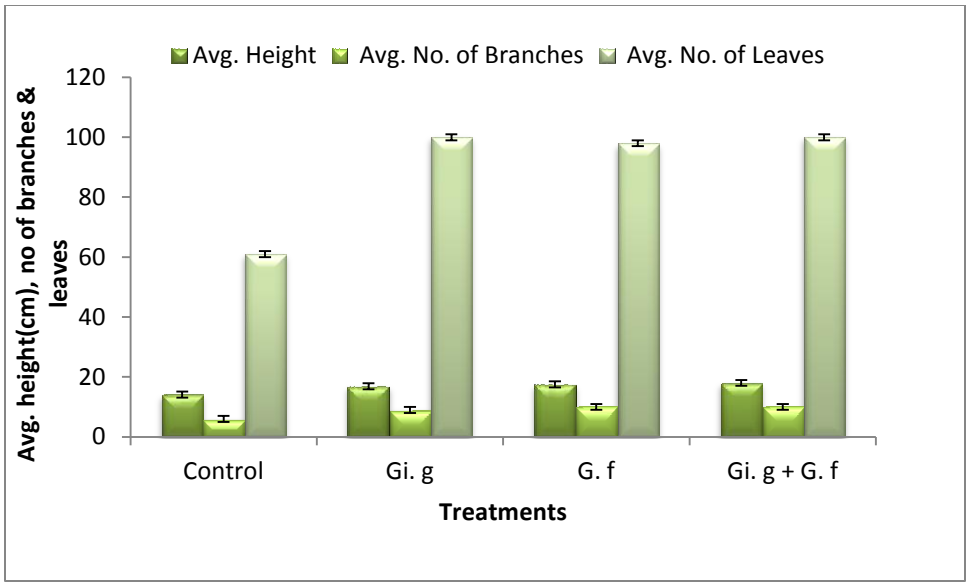


Fig. 48 Growth of manadrin seedlings following inoculation with *G. fasciculatum* and *Gi. gigantea*

4.13.2.3. Changes in defense enzymes

Significant biochemical changes were observed on application of *G. fasciculatum* and *Gi. gigantea* prior to pathogen challenge in mandarin seedlings in field conditions. Experiments were conducted to assess the effect of single as well as combined application of *G. fasciculatum* and *Gi. gigantea* on biochemical components of mandarin leaves and roots. Multifold increase in activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in roots as well as leaf of mandarin plants was observed after application of *G. fasciculatum* and *Gi. gigantea* followed by inoculation with *F. solani***.** Overall results show that the defense enzyme activities were higher in leaves than the roots (Fig. 49).

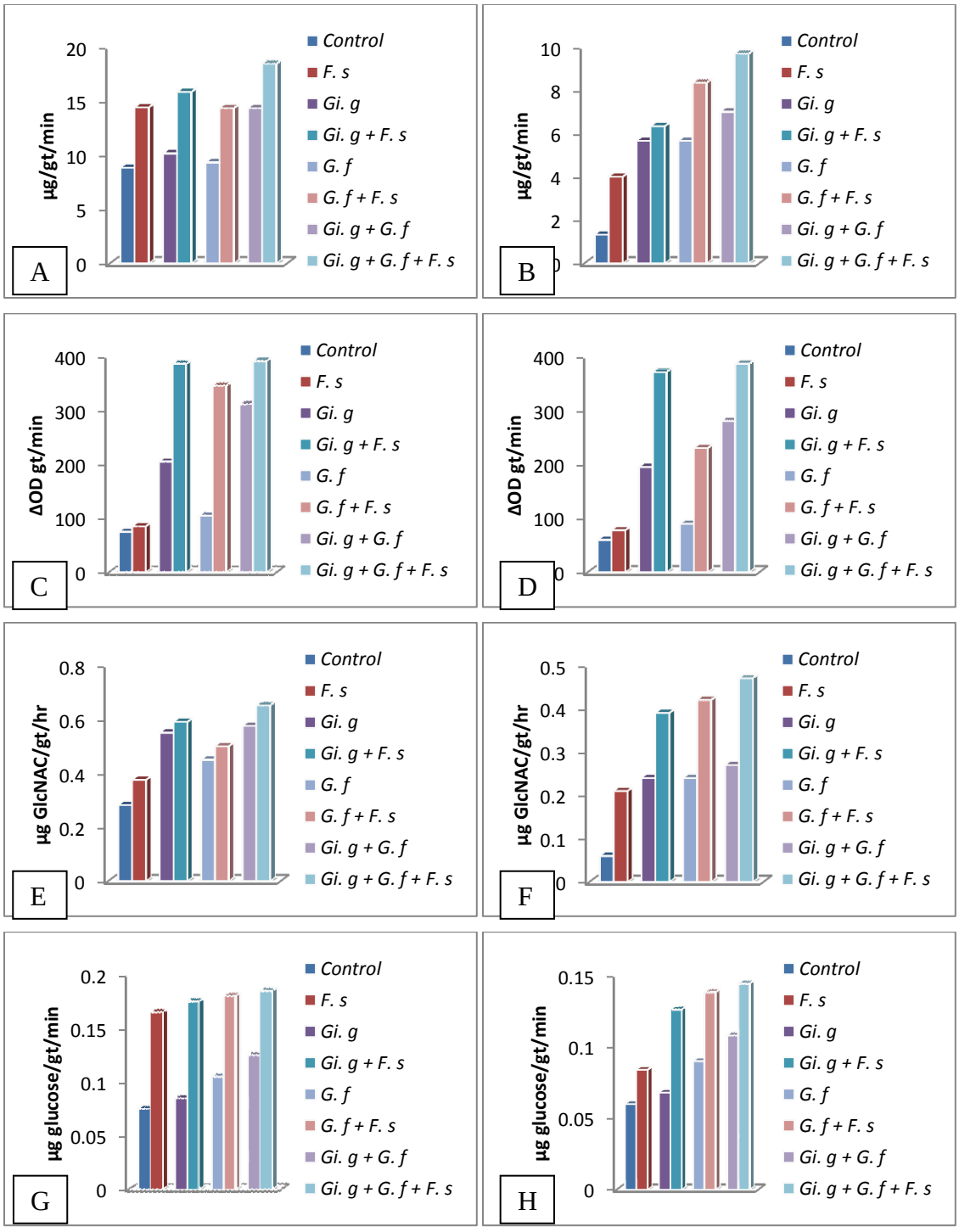


Fig. 49. PAL (A- Leaf, B- Root), Peroxidase (C-Leaf, D-Root), Chitinase (E-Leaf, F-Root) and Glucanase (G-Leaf, H-Root)activities in AMF treated mandarin seedlings following inoculation with *F. solani*

4.13.3. Effect of AMFand *Trichoderma* against *F. solani*

4.13.3.1. Disease suppression

In the third set of trial, *G. fasciculatum, Gi. gigantea, T. harzianum* and *T. asperellum* were tested for their effect in inhibiting root rot of mandarin seedlings caused by *F. solani* in field conditions when applied singly and in combination. AMF and *Trichoderma* were inoculated to the rhizosphere of one year old mandarin seedlings prior to pathogen challenge. Combined inoculation with both AMF and *Trichoderma* showed better results (Table 28).

4.13.3.2. Growth enhancement

Growth enhancement was evaluated in terms of percent increase in height and leaf number in comparison to a similar increase in control plants. A significant increase in growth and number of leaves in all the mandarin plants treated with AMF and *Trichoderma* was observed. Growth enhancement was significantly higher when both the AMF and *Trichoderma* were applied jointly (Fig. 50). There was marked increase in overall plant growth (Fig 51) and induced flowering (Fig 51) and fruiting (Fig 53) after application of AMF and Tricho compost.

4.13.3.3. Changes in defense enzymes

All defense enzymes in both leaves and roots were greatly enhanced on application of AMF and *Trichoderma* prior to pathogen challenge in mandarin seedlings in field conditions. Multifold increase in activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in roots as well as leaf of mandarin plants was observed after application of AMF and *Trichoderma* followed by inoculation with *F. solani***.** Peroxizyme analysis showed the presence of an extra band in AMF and *Trichoderma* jointly inoculated leaf (Fig. 54 B & C)**.** Overall results show that the defense enzyme activities were higher in leaves than the roots (Table 29 & 30).

Table 28. Disease index of root rot ofmandarin seedlings following AMF and *Trichoderma* treatment and pathogen challenge

Disease index Pre treated with*	No. of days after inoculation		
	15d	30d	45d
F. s	2.0±0.471	2.9±0.470	4.9±0.69
Gi. g + T. h + F. s	0.7±0.094	1.7±0.471	1.9±0.72
Gi. g + T. a + F. s	0.7±0.094	1.7±0.471	1.8±0.71
G. f + T. h + F. s	0.8±0.094	1.6±0.577	1.8±0.68
G. f + T. a + F. s	0.7±0.094	1.5±0.580	1.8±0.52
Gi. g + G. f + T. h+ F. s	0.5±0.093	1.1±0.630	1.4±0.55
Gi. g + G. f + T. a + F. s	0.3±0.075	0.9±0.690	1.3±0.59
Gi. g + G. f + T. a + T. h + F. s	0.1±0.027	0.8±0.520	1.1±0.65

Rot index: 0- no symptoms; 1- small roots turn brownish and start rotting; 2- leaves start withering and 20-40% of roots turn brown; 3- leaves withered and 50% of roots affected; 4- shoot tips also start withering; 60-70% roots affected; 5- shoots withered with defoliation of lower withered leaves, 80% roots affected; 6- whole plants die, with upper withered leaves still remaining attached; roots fully rotted

±=SE

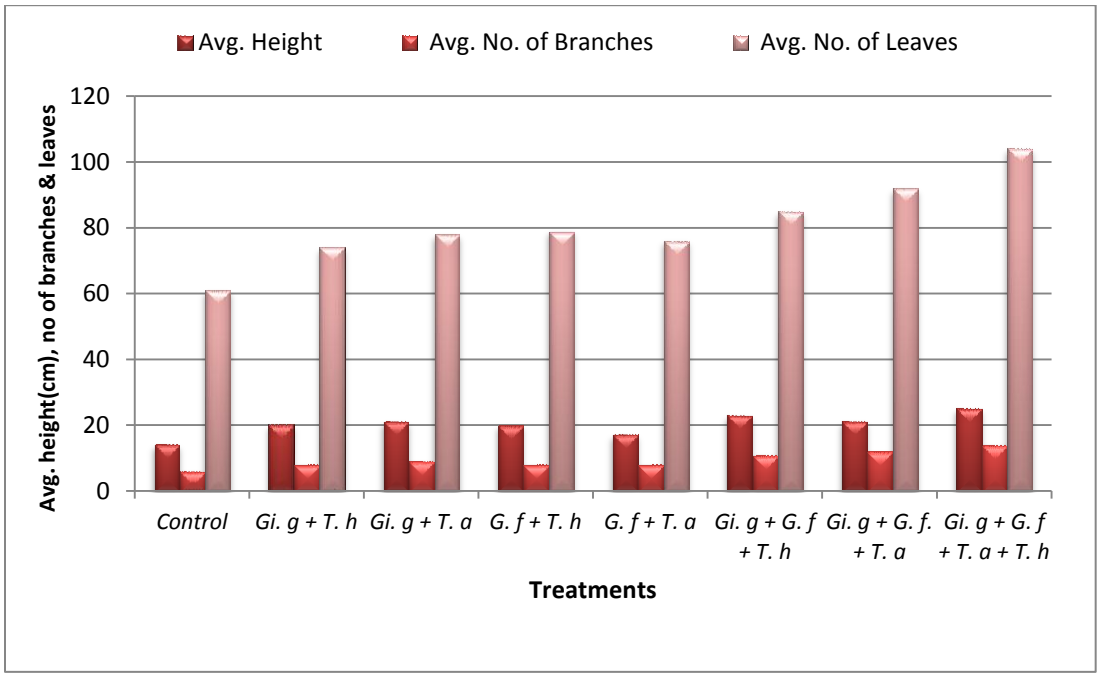


Fig. 50 Growth of manadrin seedlings following joint inoculation with AMF and *Trichoderma*

Fig. 51. Growth of Mandarin plants following colonization with AMF and field application of Tricho compost. (A&B) Untreated Healthy Control ; (C&D) AMF inoculated and Trich compost treated

Fig. 52 Flowering induced in mandarin plants grown in foot hills following following colonization with AMF and field application of Tricho compost

Fig. 53 Developmental stages of mandarin fruits in plants grown in foot hills colonization with AMF and field application of Tricho compost

Table 29. PAL and Peroxidase activities in AMF treated mandarin seedlings following inoculation with *F. solani*

Treatments	PAL(µg cinnamic acid/gt/min)		POX(ΔOD/min/gt)	
	Leaf	Root	Leaf	Root
Control	13.0±0.57	1.33±0.054	75±2.55	60±1.15
F. s	14.4±0.58	4.00±0.067	85±1.15	78±2.24
Gi. g	10.7±0.63	5.66±0.049	205±1.77	195±2.87
G. f	9.33±0.69	5.66±0.048	105±1.15	90±3.12
T. a	15.3±0.74	4.33±0.067	115±2.77	110±2.34
T. h	13.7±0.57	3.33±0.063	185±2.55	75±2.32
Gi. g + T. h	17.0±0.66	6.66±0.069	225±3.12	120±2.21
Gi. g + T. h + F. s	21.0±0.57	7.50±0.073	250±2.23	125±3.21
Gi. g + T. a	16.0±0.72	6.00±0.072	222±2.44	120±2.34
Gi. g + T. a + F. s	17.5±0.73	6.50±0.069	255±2.78	128±1.77
G. f + T. h	15.0±0.69	6.50±0.052	225±2.99	125±1.85
G. f + T. h + F. s	16.5±0.79	7.66±0.097	236±3.22	135±1.98
G. f + T. a	14.5±0.78	6.66±0.099	225±2.23	125±1.23
G. f + T. a + F. s	16.7±0.83	7.88±0.014	235±1.15	134±1.15
Gi. g + G. f + T. h	17.5±0.88	8.22±0.015	240±1.55	154±1.56
Gi. g + G. f + T. h+ F. s	22.0±0.99	8.66±0.051	256±1.73	160±1.58
Gi. g + G. f + T. a	18.0±0.98	8.00±0.017	240±1.77	156±1.98
Gi. g + G. f + T. a + F. s	21.0±0.92	8.66±0.023	258±1.84	165±2.23
Gi. g + G. f + T. a + T. h	23.0±0.45	9.50±0.021	267±2.21	171±2.45
Gi. g + G. f + T. a + T. h + F. s	25.7±0.58	10.66±0.018	275±2.45	179±2.54

±=SE Average of three replicates

Table 30. Chitinase and Glucanase activities in AMF treated mandarin seedlings following inoculation with *F. solani*

Treatments	CHT(mg GlcNAc/gt/min)		GLU(µg glucose/gt/min)	
	Leaf	Root	Leaf	Root
Control	0.28±0.57	0.06±0.057	0.075±0.056	0.060±0.052
F. s	0.37±0.77	0.21±0.21	0.165±0.072	0.084±0.032
Gi. g	0.55±0.58	0.24±0.15	0.085±0.063	0.068±0.034
G. f	0.45±0.57	0.24±0.20	0.105±0.069	0.090±0.036
T. a	0.55±0.63	0.30±0.18	0.135±0.072	0.114±0.063
T. h	0.40±0.69	0.30±0.20	0.120±0.054	0.114±0.069
Gi. g + T. h	0.56±0.66	0.31±0.22	0.169±0.062	0.117±0.072
Gi. g + T. h + F. s	0.57±0.62	0.36±0.25	0.185±0.063	0.144±0.077
Gi. g + T. a	0.56±0.59	0.32±0.31	0.171±0.062	0.118±0.073
Gi. g + T. a + F. s	0.57±0.56	0.36±0.34	0.180±0.061	0.138±0.072
G. f + T. h	0.56±0.33	0.34±0.39	0.174±0.051	0.118±0.081
G. f + T. h + F. s	0.65±0.69	0.39±0.41	0.183±0.052	0.138±0.080
G. f + T. a	0.58±0.63	0.33±0.62	0.172±0.050	0.119±0.089
G. f + T. a + F. s	0.66±0.74	0.38±0.69	0.187±0.049	0.135±0.091
Gi. g + G. f + T. h	0.68±0.73	0.38±0.63	0.190±0.069	0.140±0.090
Gi. g + G. f + T. h+ F. s	0.71±0.84	0.39±0.72	0.221±0.078	0.186±0.067
Gi. g + G. f + T. a	0.68±0.81	0.38±0.70	0.210±0.092	0.150±0.065
Gi. g + G. f + T. a + F. s	0.72±0.80	0.39±0.72	0.222±0.093	0.192±0.069
Gi. g + G. f + T. a + T. h	0.74±0.82	0.41±0.68	0.234±0.081	0.198±0.078
Gi. g + G. f + T. a + T. h + F. s	0.77±0.79	0.45±0.66	0.246±0.083	0.209±0.083

±=SEAverage of three replicates

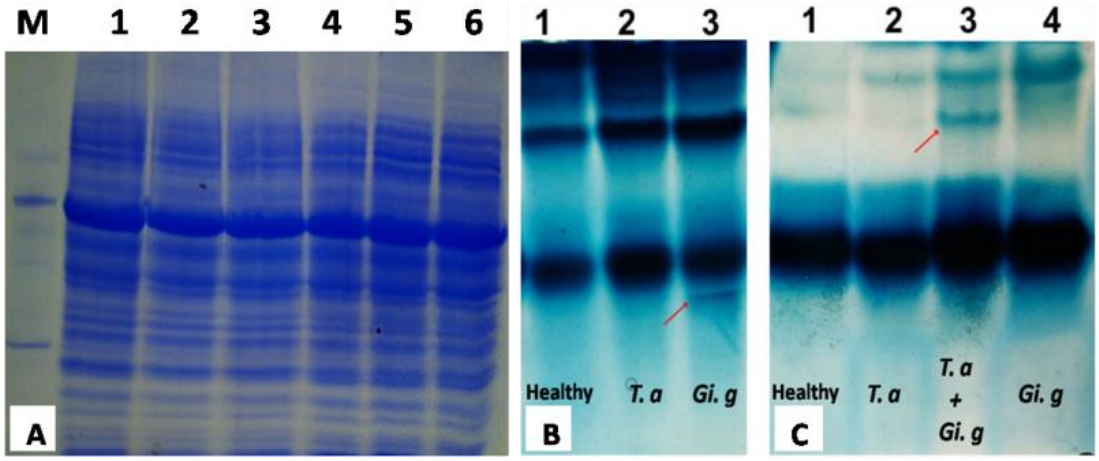


Fig 54: SDS-PAGE of leaf proteins of mandarin plants following inoculation with *Gi. gigantea* and *T. asperellum* (A), Peroxizyme analysis of mandarin roots and leaves following inoculation with *Gi. gigantea* and *T. asperellum* Root (B) and Leaf (C)

4.14.Activation of defense response of *Citrus reticulata* following application of bioinoculants against *F. oxysporum*

4.14.1. Screening of PGPR for plant growth promotion

4.14.1.1. Application of PGPR in mandarin plants

Plant growth promoting rhizobacteria *Pseudomonas poae* (RMK03),isolated from rhizosphere of *Citrus reticulata*, *Bacillus stratosphericus* (RHS/CL-01) isolated from rhizosphere of *Citrus limonia*, *Ochrobactrumanthropi*, *Paenibacillus lentimorbus*, *Bacillus pumilus*, *Bacillus megaterium* and *Bacillus amyloliquefaciens* isolated from rhizosphere of *Camellia sinensis* were taken for the experiment. Aqueous suspension was of the isolates were applied as a foliar spray as well as a soil drench, @ 100 ml/plant to the rhizosphere of mandarin plants one month after transplantation. Application was done at an interval of one month and three applications were done.

4.14.1.2. Growth of mandarin seedlings

Growth promotion was studied in terms of increase in height and number of leaves in comparison to control in potted conditions. Enhanced growth of mandarin seedlings was observed markedly by all the isolates but better growth enhancement was noticed by *P. poae, B. stratosphericus, O. anthropi* and *B. pumilus* (Fig. 55). For each treatment 10 replicates were taken and average of the 10 replicate plants were analysed.

Percent increase in height and number of leaves in mandarin plants were recorded after 1 and 4 months of application of seven isolates as a soil drench as well as foliar spray.

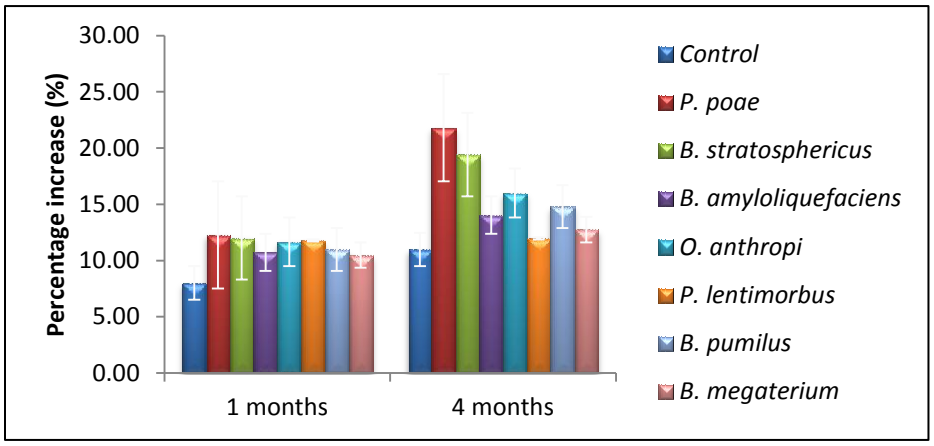


Fig 55 Growth of mandarin seedlings following application of PGPR

4.14.1.3. Protein content

Protein content also increased significantly in plants following bacterial application compared to the untreated control plants (Table 31). SDS-PAGE analysis of proteins revealed that the intensity of bands was comparatively higher in treatments in comparison to control. Leaf protein exhibited bands in SDS-PAGE ranging in molecular weight (12,14, 20,22, 29, 40, 43, 66, 68, 95, 99 KDa) (Fig 56) and bands were of varying intensities.

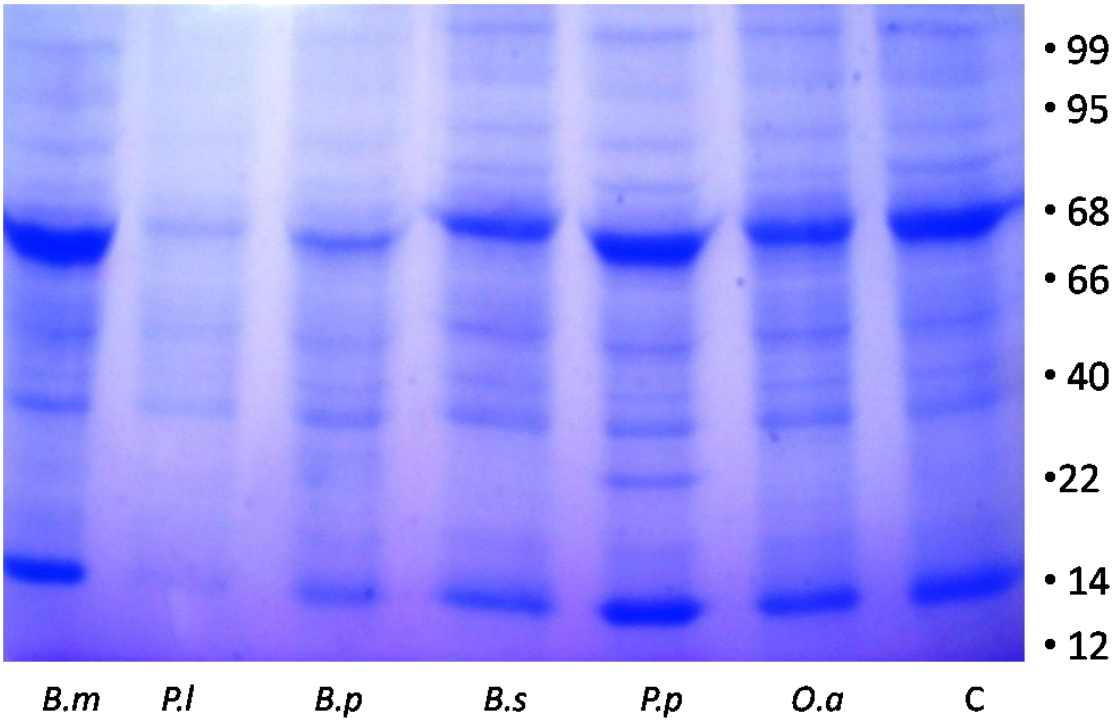


Fig 56: SDS- PAGE of mandarin leaves after inoculation with PGPR isolates

Table 31. Protein content in leaf and root after application of bacterial isolates

Treatments	Protein content	
	Leaf	Root
Control	63.0±0.57	2.68±0.397
Pseudomonas poae	136.8±1.18	8.58±0.881
Bacillus stratosphericus	106.4±1.16	6.37±0.867
Bacillus amyloliquefaciens	107.7±1.74	4.04±1.15
Ochrobactrumanthropi	104.2±1.15	6.29±1.15
Paenibacilluslentimorbus	104.2±0.58	5.77±1.73
Bacillus pumilus	76.4±1.16	5.81±0.57
Bacillus megaterium	86.8±1.19	2.88±0.57

±= SE

4.14.1.4. Chlorophyll and phenol contents

When biochemical tests were performed to evaluate the changes brought about by seven PGPR isolates. Enhanced accumulation of chlorophyll and total phenol were observed (Fig 57)**.**

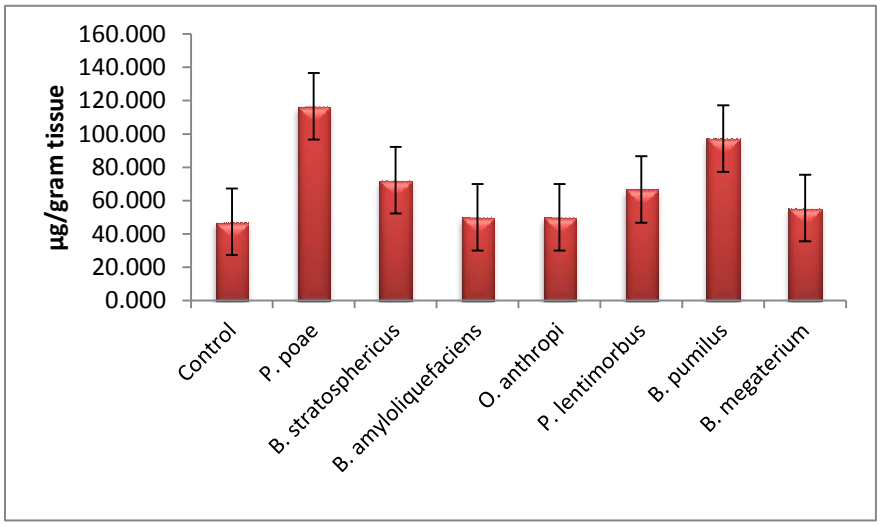


Fig. 57 Chlorophyll (A) and total phenol content (B) in mandarin plants following appilcation of PGPR

4.14.1.5. Defense enzymes

There was considerable increase in the activities of all four defense enzymes tested- Phenylalanine ammonia lyase, peroxidase, chitinase andβ-1,3-glucanase. Protein and chitinase content increased with increase in hours in both leaves and roots whereas peroxidase activity (Fig 58) decreased with increase in hours. Leaves showed a significant drop in peroxidase activity within 96 hours of inoculation (Fig. 59). Native PAGE analysis of peroxidase showed that there was clear distinction between the treated and untreated plants as far as intensities were considered. Maximum intensity of bands was noticed in the treated plants with Rm values of 0.884,0.574,0.347, 0.256. No significant difference was noticed in glucanase (Fig. 59) and phenylalanine lyase (Fig. 60) activity in both leaves and roots.

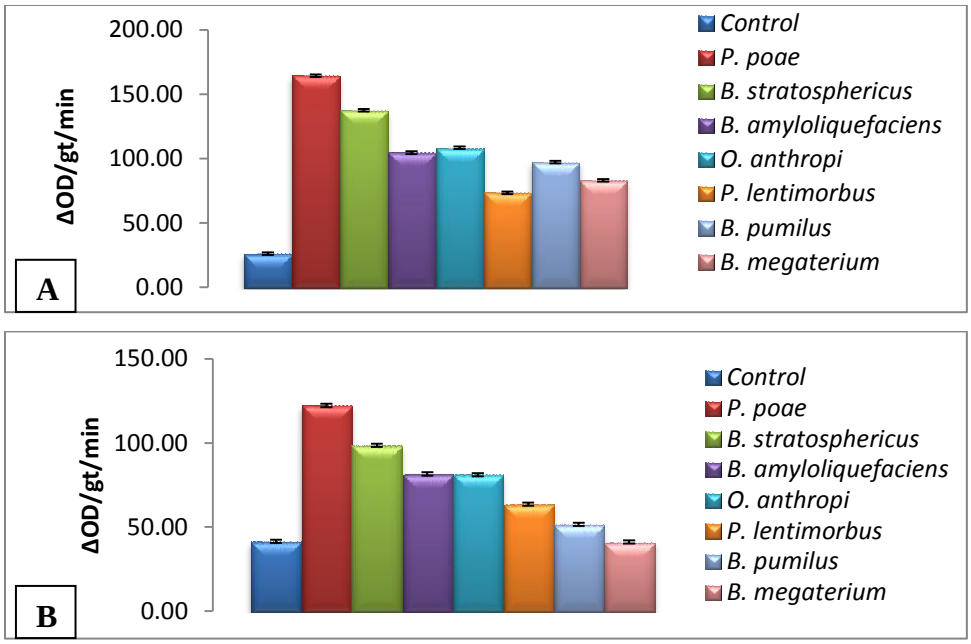
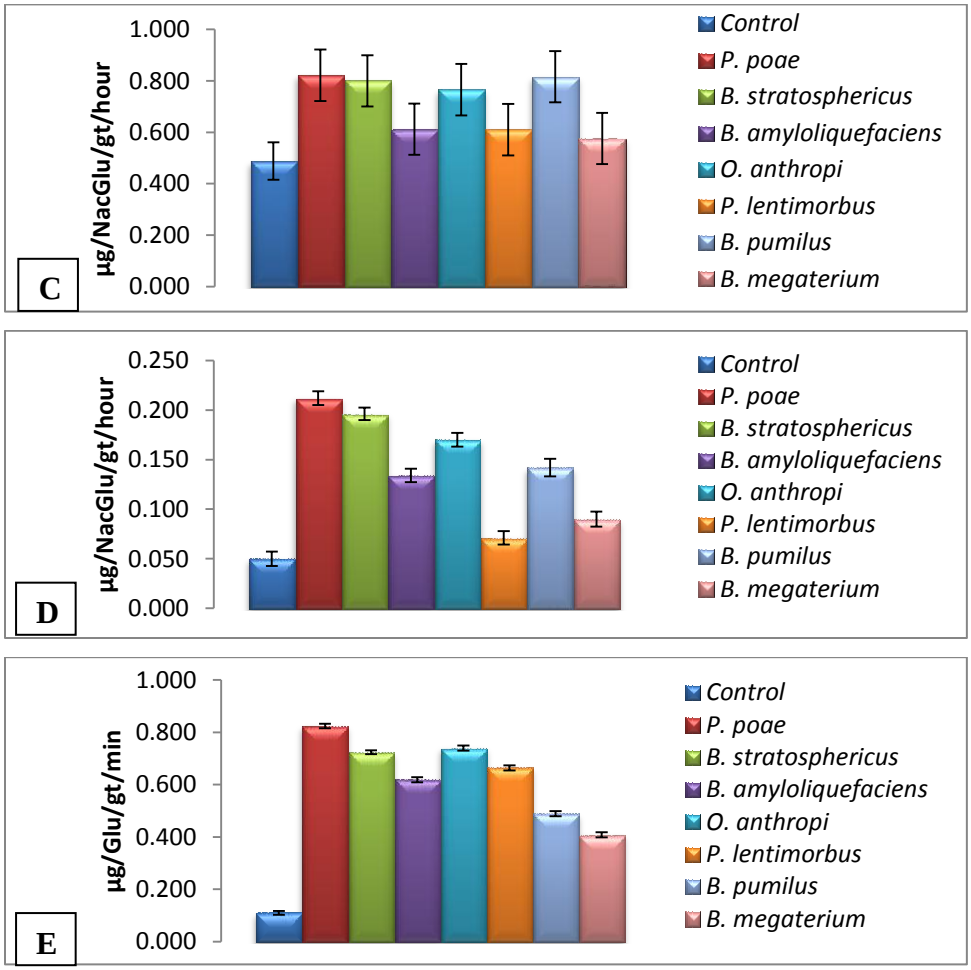


Fig. 58 Peroxidase activity in mandarin leaves (A) and roots (B) following application of PGPR



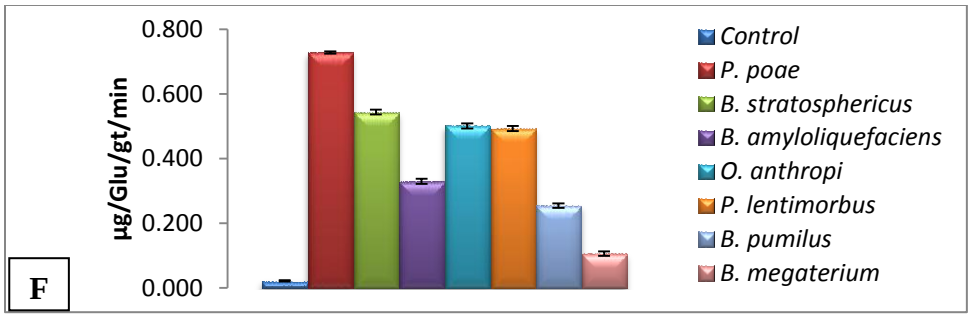


Fig. 59 Chitinase (C&D) and glucanase (E&F) activity in mandarin leaves and roots following application of PGPR

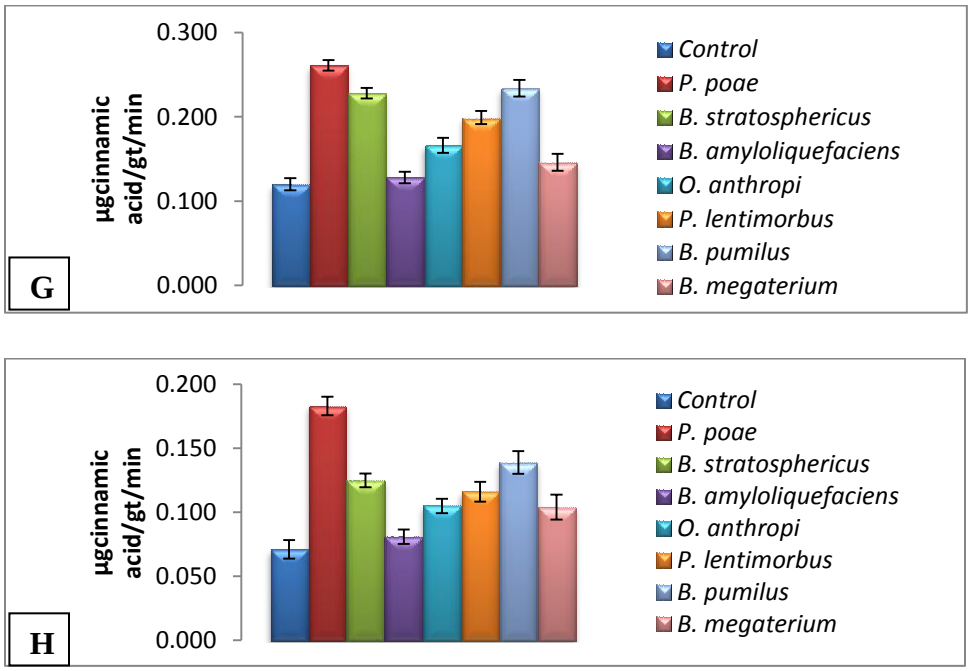


Fig. 60 PAL activity in mandarin leaves (G) and roots (H) following application of PGPR

4.14.2. Biochemical changes following application of AMF and PGPR

4.14.2.1. Growth enhancement

Based on the above experimental results, *P. poae* was found to be the most potential PGPR and hence was selected for further experimental test against *F. oxysporum* which is a causal organism of root rot of mandarin. Since *Gi. gigantea* is one of the most abundant AMF found in the rhizosphere of mandarinit was selected for further antagonistic activity against *F. oxysporum*. *Gi. gigantea* (AMF) and *P. poae* (PGPR) was selected to test the activation of defense response of mandarin seedlings

both singly and in combination in nursery conditions. Growth enhancement was evaluated in terms of percent increase in height and leaf number over similar increase in control plants after one month and two months of treatment. Results revealed enhancement of plant growth by single as well as joint application of PGPR and AMF, however the growth of mandarin seedlings grown under same environmental and physical conditions were enhanced to a greater extent when both *Gi. gigantea* and *P. poae* were applied jointly. (Fig.61).

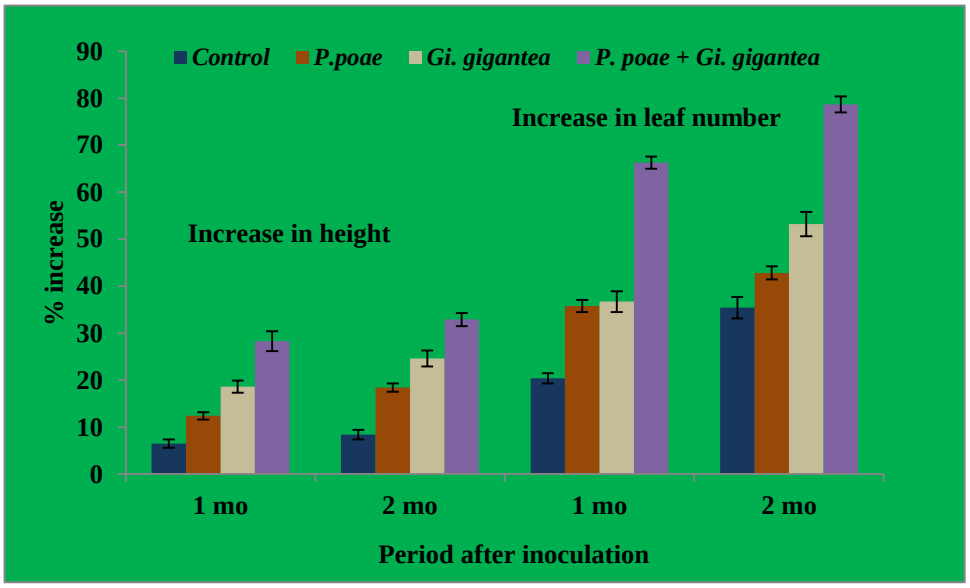


Fig 61. Effect of *Gi.gigantea* and *P. poae* on growth of mandarin seedlings in pot condition measured in terms of percent increase in height and number of leaves

4.14.2.2. Total phosphate content of soil

The growth enhancement in mandarin seedlings in nursery conditions was also evaluated in terms of total phosphate mobilized by *Gi. gigantea* and *P. poae*. Total phosphate content in soil was determined after application of the microorganisms. The overall results reveal that the total soil P-content decreased following application of *Gi. gigantea* and *P. poae* singly or jointly which indicated efficient uptake of soil phosphate which had been solubilized by both PGPR and AMF (Table 32).

Table 32. Soil phosphate content in rhizosphere of mandarin plants after application of microorganisms

Treatment	Soil phosphate (µg/g tissue)
Control	49.37^{a}±1.18
P. poae	33.12^{b}±0.57
G. fasciculatum	31.25^{b}±3.60
P. poae + *G. fasciculatum*	23.75^{c}±0.33

Average of 3 replicates

±= Standard Error

Difference between values significant at P=0.01where superscript is different; not significant where superscript is same

4.14.2.3. Disease assessment

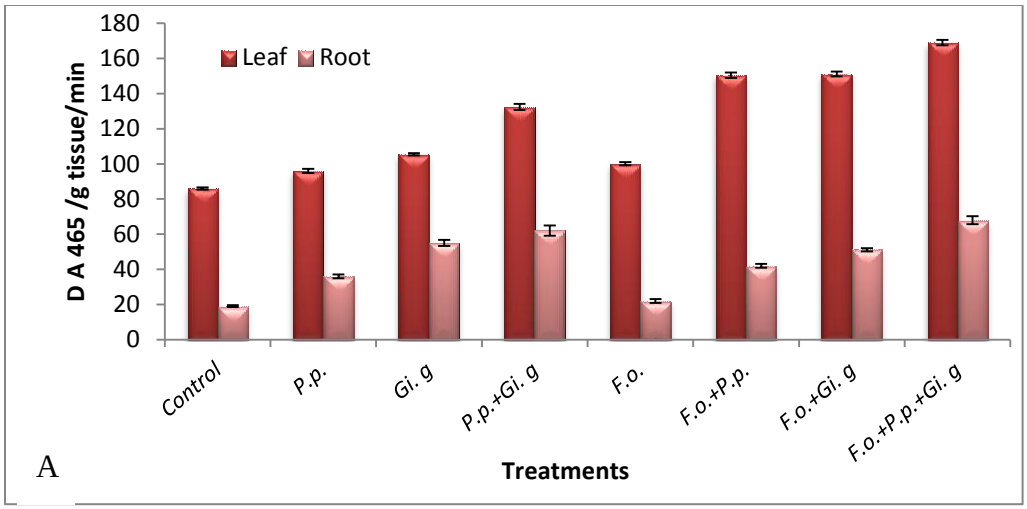
Rhizosphere of mandarin was inoculated by *Gi. gigantea* and *P. poae* prior to challenge inoculation with *Fusarium oxysporum*. Development of root rot was determined after 15, 30 and 45 days of inoculation on the basis of root rot index. Results revealed that both microorganisms could reduce root rot, but maximum suppression of disease was due to joint inoculation (Table 33)

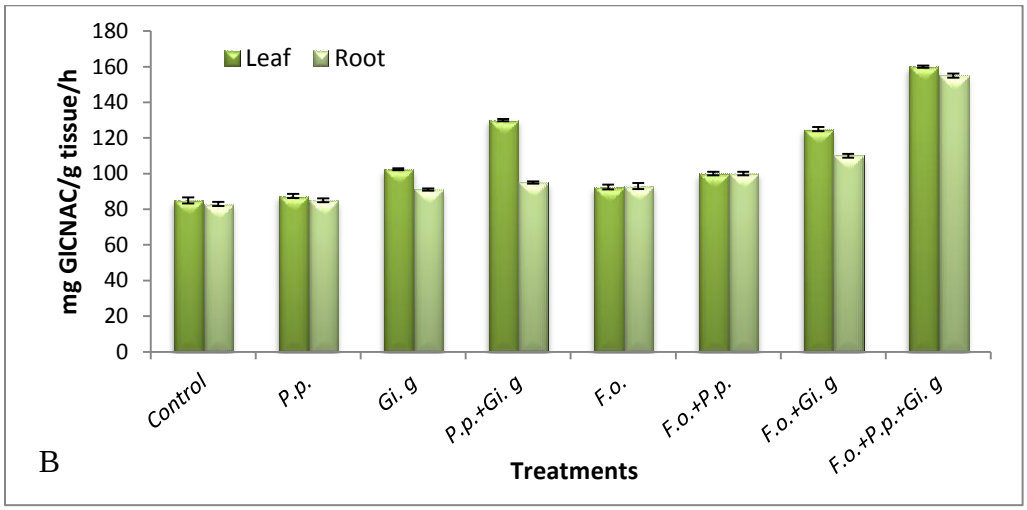
Table 33. Disease index of root rot of mandarin seedlings following AMF and PGPR treatment and pathogen challenge in nursery condition

Days of inoculation	Disease index Pre treated with			
	F. oxysporum	***F. oxysporum*** + ***P. poae***	***F. oxysporum*** + ***Gi. gigantea***	***F. oxysporum*** + ***Gi. gigantea***+ ***P. poae***
15	2.3±0.145	6±0.145	1.1±0.088	0.6±0.088
30	3.4±0.115	1.2±0.115	2.5±0.145	1±0.057
45	5.9±0.057	2.8±0.115	4.3±0.057	1.8±0.057
Rot index: 0- no symptoms; 1- small roots turn brownish and start rotting; 2- leaves start withering and 20-40% of roots turn brown; 3- leaves withered and 50% of roots affected; 4- shoot tips also start withering; 60-70% roots affected; 5- shoots withered with defoliation of lower withered leaves, 80% roots affected; 6- whole plants die, with upper withered leaves still remaining attached; roots fully rotted				

4.14.2.4. Changes in activities of defense enzymes in mandarin leaves and roots

Activities of defense enzymes- phenylalanine ammonia lyase, chitinase, β-1,3 glucanase and peroxidase were assayed after 24 hours and 96 hours of application in leaves and roots of mandarin seedlings subjected to various treatments- ie., *P. poae*, *Gi. gigantea*, *F. oxysporum*, *Gi. gigantea*+ *P. poae*,*F. oxysporum*+*P. poae*,*F.oxysporum*+*Gi. gigantea*, and*F.oxysporum*+*P. poae*+*Gi. gigantea*. Activities of all 3 enzymes, in both leaves and roots, were significantly enhanced due to the various treatments (Figs. 62). 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.





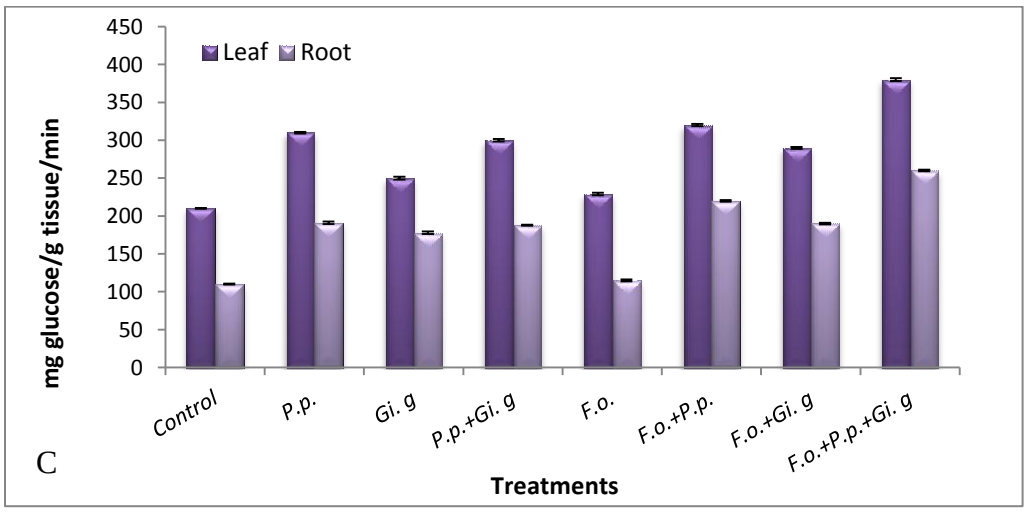


Fig. 62 Activity of defense enzyme on application of *P.poae* and *Gi.gigantea* in Citrus seedlings (A) Peroxidase, (B) Chitinase and (C) Glucanase following inoculation with *F. oxysporum*

4.14.2.5. Immunological tests with PAbs raised against defense enzymes

In order to confirm the induction of enhanced activities of defense enzymes due to treatment with *P. poae*, *Gi. gigantea* 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 Blot were carried out. Results revealed that ELISA values of reaction of PAbs of chitinase and glucanase with enzyme extracts from leaves grown in treated soil were higher than the control values. Similarly, in Dot-Blot, more intense dots were observed in treated plants (Table 34).

Table 34. ELISA and Dot-Blot values of reactions between PAbs of defense enzymes and enzyme extracts of leaves from treated mandarin plants

Antigen source*	PAb of chitinase		PAb of β1,3-glucanase	
	A 405 ELISA	Colour intensity# Dot-Blot	A 405 ELISA	Colour intensity# Dot-Blot
Control	0.034	+	0.049	+
F.oxysporum	0.036	+	0.052	+
F.oxysporum + *P. poae*	0.420	++	0.368	++
F.oxysporum + *Gi. gigantea*	0.468	++	0.345	++
F.oxysporum + *P. poae*+*Gi. gigantea*	0.982	+++	0.865	+++

* Enzyme extracts from leaves of plants treated as mentioned;

Colour Intensity - += Light pink; ++ = Dark pink ; +++ = Deep purplish

4.15. Bacteria associated with spores of the AMF (*Gi. gigantea*)

One of the dominant spore associated with the roots of mandarin plant was *Gigaspora gigantea*. The application of *Gi. gigantea* alongwith other PGPF have been proved that it helps in plant growth promotion as well as induction of resistance towards the pathogen (*F. solani*). Bacterial population associated with AMF, called mycorrhiza helper bacteria, have beneficial effects on AMF growth.They not only improve the mycorrhizal root colonization and stimulate extraradical hyphal growth but also by facilitate AMF spore germination.

Spores of the arbuscular mycorrhizal fungus – *Gi. gigantea* were harvested from mandarin rhizosphere followed by single-spore-derived cultures with *Sorghum bicolor* as host plant. One bacteria (MHB) was successfully isolated from *Gi. gigantea* spore originally obtained from mandarin root rhizosphere. The bacteria isolated was rod shaped and gram positive (Fig. 63). Biochemical characterizations of all the bacterial isolates have been presented in Table 35.

Table 35. Morphology and biochemical tests of MHB obtained from mandarin plant

Code	Shape	Gram reaction	IAA	Phosphate solubilization	Protease production	Catalase activity	production	HCN production	H_2S production	Urease activity
MHB	Small rod	+	+	-	+	-	-	-	-	-

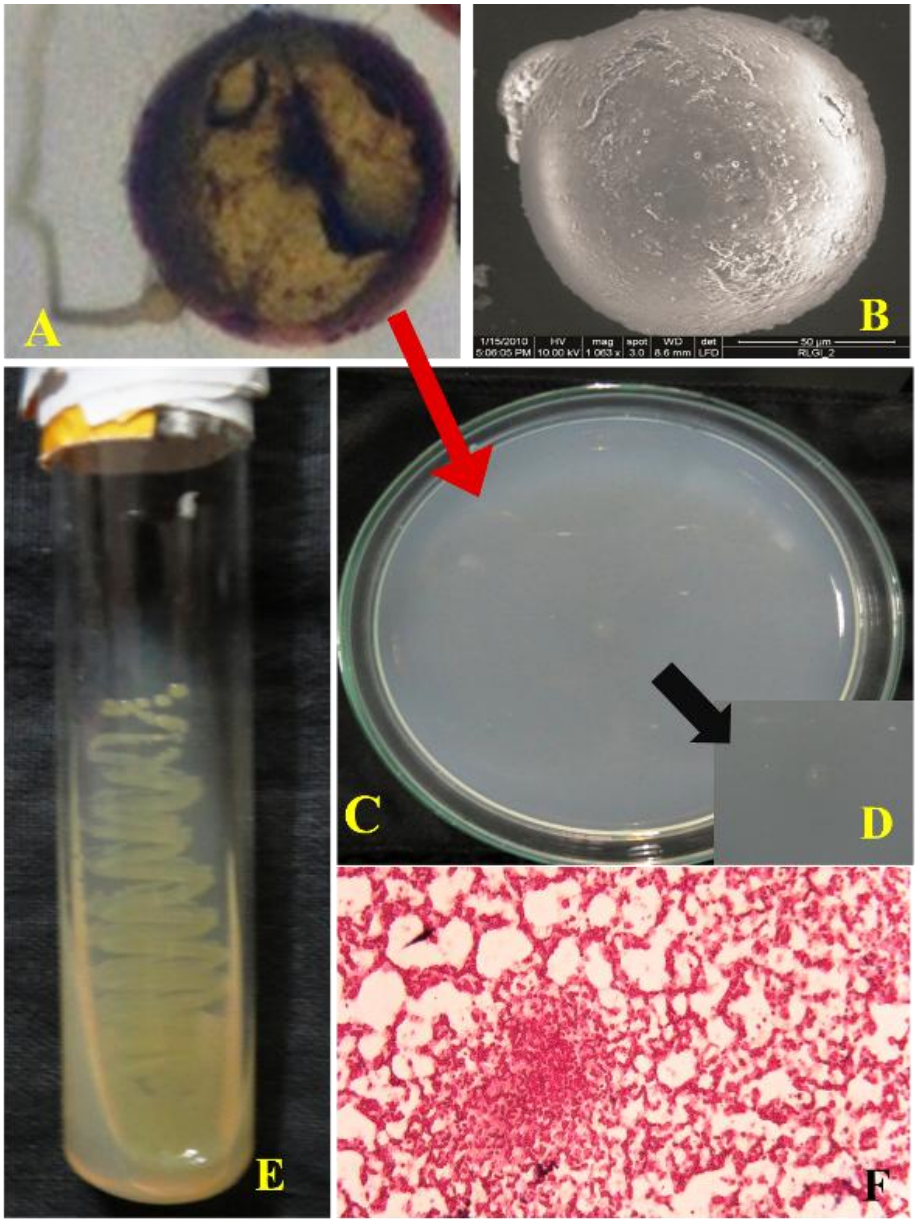


Fig. 63. Bacteria associated with AMF spore (A) *Gi. Gigantea* spore, (B) SEM micrographs of *Gi. gigantea* showing characteristic hyphal attachment and spore wall structures, (C&D) Isolation of mycorrhiza helper bacterium (MHB) on agar medium, (E) Pure culture maintained on nutrient agar slants, (F) Gram staining and microscopic characters of MHB isolated from *Gi. gigantea*

Among the bacterial isolates obtained from mandarin rhizosphere, one important plant growth promoting rhizobacteria (PGPR), of *B. mycoides* (MHB) was designated as potential PGPF. The identity of this PGPR was further confirmed with the help of 16S rDNA sequences amplified with the help of universal primers.

4.15.1. 16S rDNA sequences and BLAST analysis

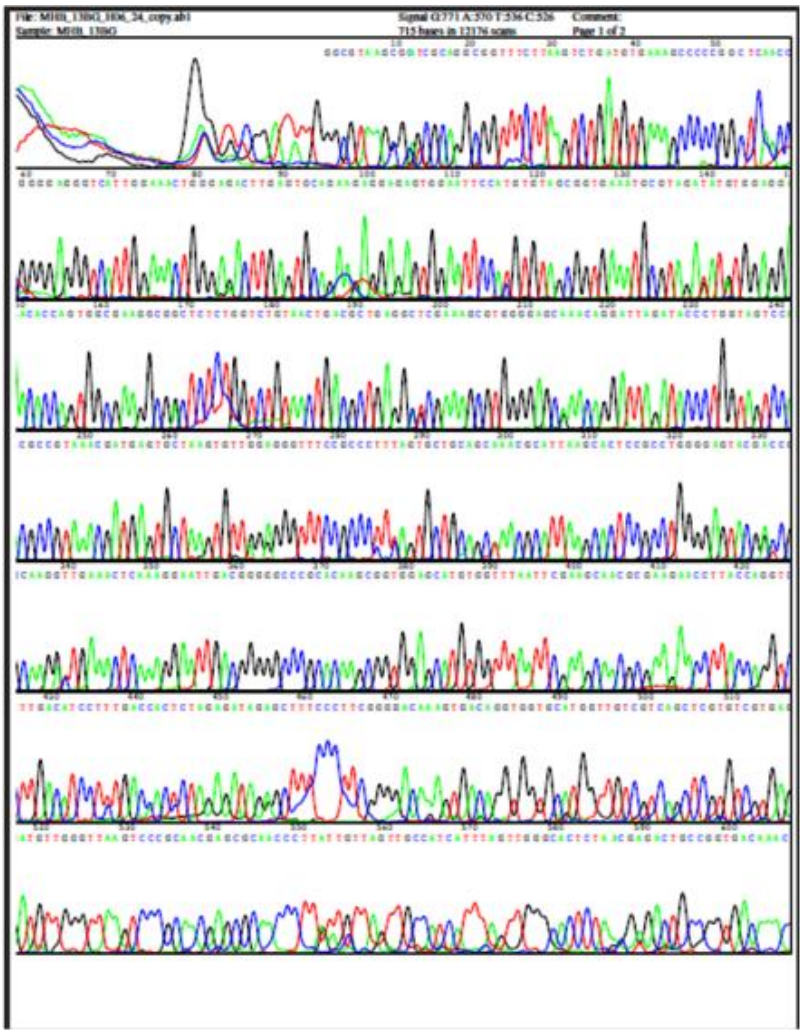
The identity of the isolate was confirmed with the help of 16S rDNA sequences. The BLAST query of the 16S r DNA sequence of the isolates against GenBank database confirmed the identity of the isolate MHB as *B.mycoides*. The sequence chromatogram of has been represented in Fig 64.

4.15.2 Multiple sequence alignment and phylogeny

A multiple sequence alignment of ITS gene sequences of *B.mycoides* was conducted. Sequences of other strains obtained from NCBI Genbank database showing maximum homology with our strain was conducted using CLUSTAL-W algorithm which is a general purpose multiple sequence alignment program for DNA of MEGA-4.1 software. 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. There were quite a number of gaps that were introduced in the multiple sequence alignment program within the region that were closely related and similar sequence indicated the relationship among the isolates. The differences in these highly conserved regions are shown in different colours (Fig. 65). Phylogenetic analyses were completed using the MEGA package (version 4.01; Institute of Molecular Evolutionary Genetics, University Park, PA). Phylogenetic analysis was carried out with Ex-type strain sequences obtained from NCBI Genbank database which showed maximum homology with *B.mycoides* (MHB) (Table 36).

The evolutionary history (Fig. 65) was inferred using the UPGMA method (Sneath & Sokal, 1973). The optimal tree with the sum of branch length = 0.60305778 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 (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 *et al.*, 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 3 positions in the final dataset Phylogenetic analyses were conducted in MEGA4 (Tamura *et al.*, 2004).



GAGGCCACGTTTCTTGCCTGCCCATAAGACTGGGATCGGTTATAAAAAACCGGGGC
TAATACCGGATAACATTTTGCGCCGCATGGCGCGAAATTGAAAGGCGGCTTCGGCT
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AGGCAACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGAC
ACGGCCCAGACTCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAA
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AAAGCCCACGGCTCAACCGTGGAGGGTCATTGGAAACTGGGAGACTTGAGTGCAG
AAGAGGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGTAGAGATATGGAGGAA
CACCAGTGGCGAAGGCGACTTTCTGGTCTGTAACTGACACTGAGGCGCGAAAGCGT
GAGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTA

Fig. 64. Chromatogram of 16S r DNA region of *B. mycoides*

Table 36. Genbank Accession Numbers and Geographic locations of the Ex-Type strains of *B. mycoides* that showed homology with the isolate

Accession No.	**Strain/isolate**	**rDNA Sequence**	**Country**
AJ305310	HN51	483	Vietnam
AM910408	BGSC1	1493	India
Z84591	MWS5303-1-4	2177	Germany
HG794253	HC28	781	Belgium
HF584834	BD17-B03	1077	Italy
HG008800	B0210-14M1	1052	Spain
HE648111	C1 11146	768	France
AM747229	CIP 103472	1663	France
AM747228	SDA NFMO448	1663	France
AY373357	c2	1506	China
AJ841874	DSM 2048	543	Germany
AJ344516	SFLB6	325	Germany
Z84583	MWS5303-2-51	1474	Germany
Z84582	MWS5303-1-4	1474	Germany
AB547222	NCCP-37	1436	Pakistan
EF210313	BGSC 6A20	1461	Canada
KJ917554	**MHB**	**724**	**India**

Fig. 65.16S r DNA sequence alignments of *B. mycoides* (MHB) with other extypes isolate. The conserved regions of the gene are demonstrated in different colours

Fig. 66. Phylogenetic tree of *B. mycoides* with other extypes isolate

4.16. Serological detection of AMF (*Glomus* and *Gigaspora)* in mandarin root tissue using indirect immunofluorescence and immunogold labelling following induction of resistance

PAbs of *Glomus mosseae* and *Gigaspora gigantea* goat antisera specific to rabbit globulins conjugated with FITC and RITC separately were used for indirect immunofluroscence study to detect the spores and AMF hyphae colonization in root tissues of mandarin plants. Spores were sieved and washed. Selected spores were taken in grooved slides and left overnight in antibacterial solution. The spores were washed thrice with PBS (phosphate buffer saline solution) pH 7.2. Antigen was given with a dilution of 1:40 and left overnight. The next day the spores were again washed thrice with PBS. FITC was given at a dilution of 1:10 and again left overnight. Finally, the next day, spores were mounted in grooved and simple slides in 10% glycerol and observed under fluorescence microscope.

Roots of mandarin seedlingswere carefully separated, cleaned and inoculated with AMF spores extracted from the rhizosphere of mandarin plant. The present investigation was designed to locate colonization of AMF (*Glomus* and *Gigaspora*) in the rhizosphere as well as their cellular localization in root tissues of mandarin.

AMF spores showed a bright apple green fluorescence which was distributed throughout the spore wall (Fig 67 A-H). Subtending hyphae also gave off the green fluorescence (Fig 67 I-N). Spores with their hyphae were more prominent in maize root than in roots of mandarin plant. Single spore of *Gigaspora* sp. and aggregate of *Glomus* sp. clearly showcased the apple green fluorescence in case of FITC and in case of RITC labelled fluorescent staining a red coloured fluorescence was observed. It was observed that the fluorescence was more intense on young spore walls (Fig 68 & 69). It was noticed that spores were distributed throughout the length of the root including the tips of the root which indicates how quickly AMF colonize in the root cells. The hyphal proliferation within the root tissues of maize and feeder roots of mandarin were also specifically located with the help of FITC. On observation under UV- microscope bright apple green fluorescence of the hyphae, vesicles and arbuscules within the host tissue were shown. Fluorescence was more prominent towards the cortex layer in most of the

tissues which tells us their successful colonization in mandarin roots leading towards induction of resistance.

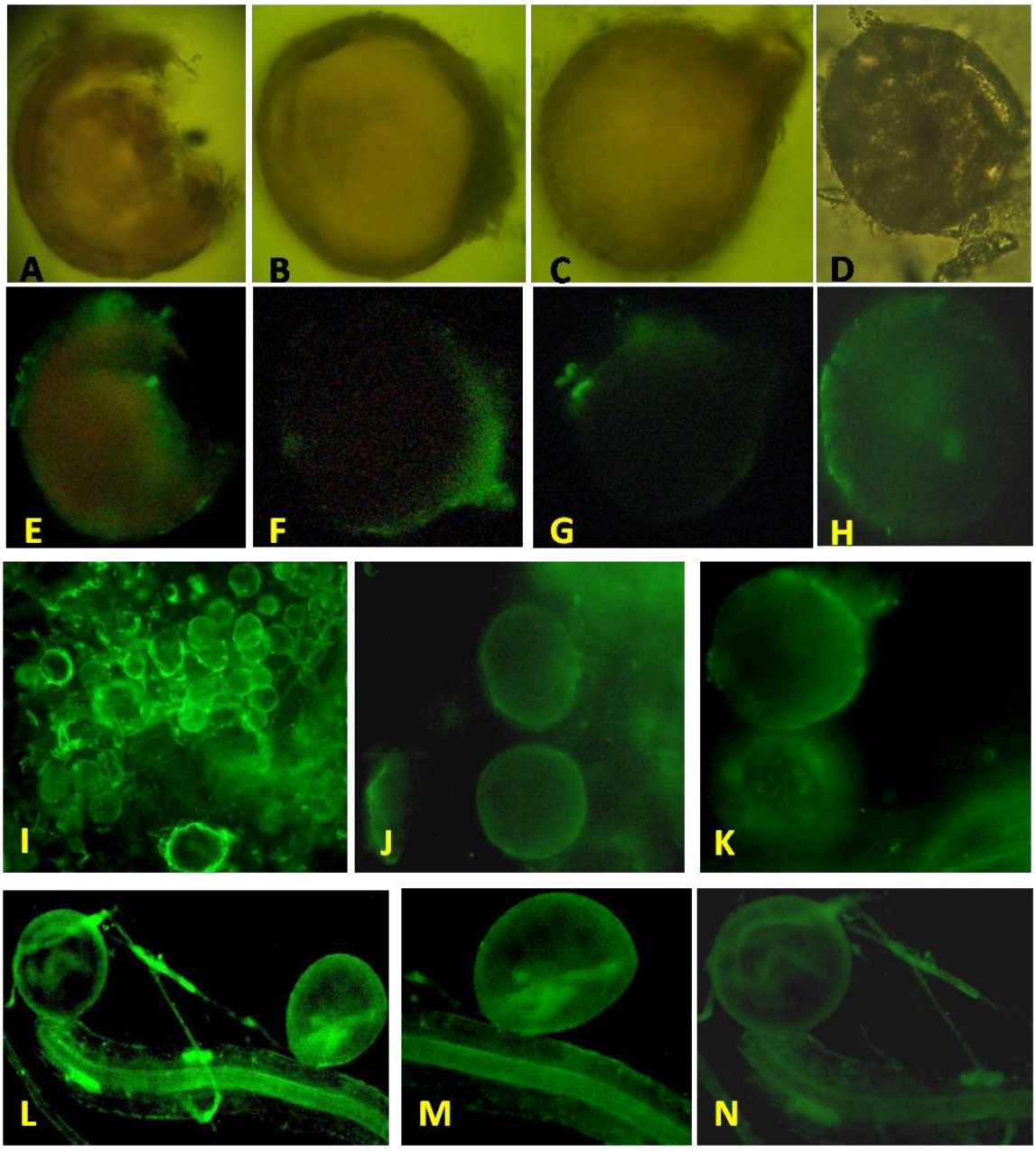

Fig. 67. Indirect imunofluorescence of AMF associated with mandarin roots. AMF spores under bright field (A-D), Indirect-immunofluorescent labelling of AMF spores (E-H) and spores adhered to mandarin roots (I-K) treated with PAb of *G. mosseae* and reacted with FITC, spores adhered to mandarin roots (L-N) treated with PAb of *Gi. gigantea* and reacted with FITC

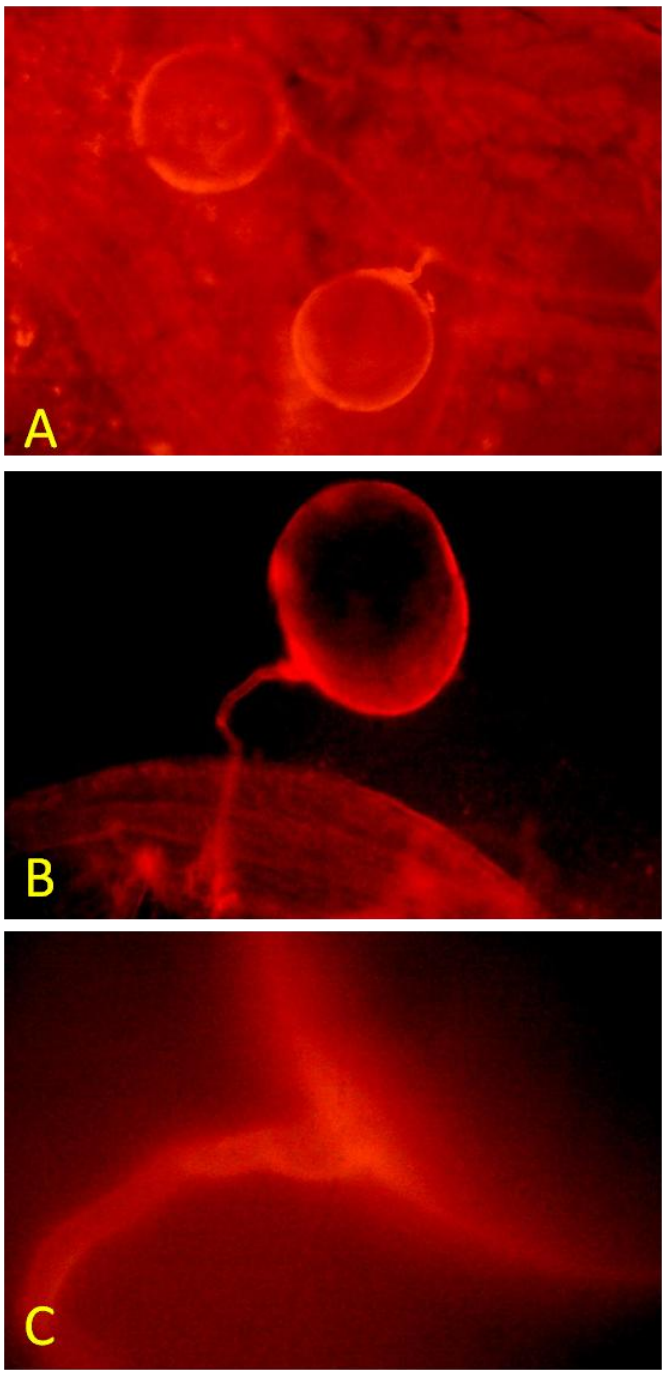

Fig. 68. Indirect-immunofluorescent labelling of AMF spores adhered to mandarin roots and treated with PAb of *Gi. gigantea* and reacted with RITC.
Gigaspora sp. spores adhered to mandarin roots (A), single spore of *Gigaspora sp.*attached to mandarin root (B) and close up of spore showing bulbous suspensor (C)

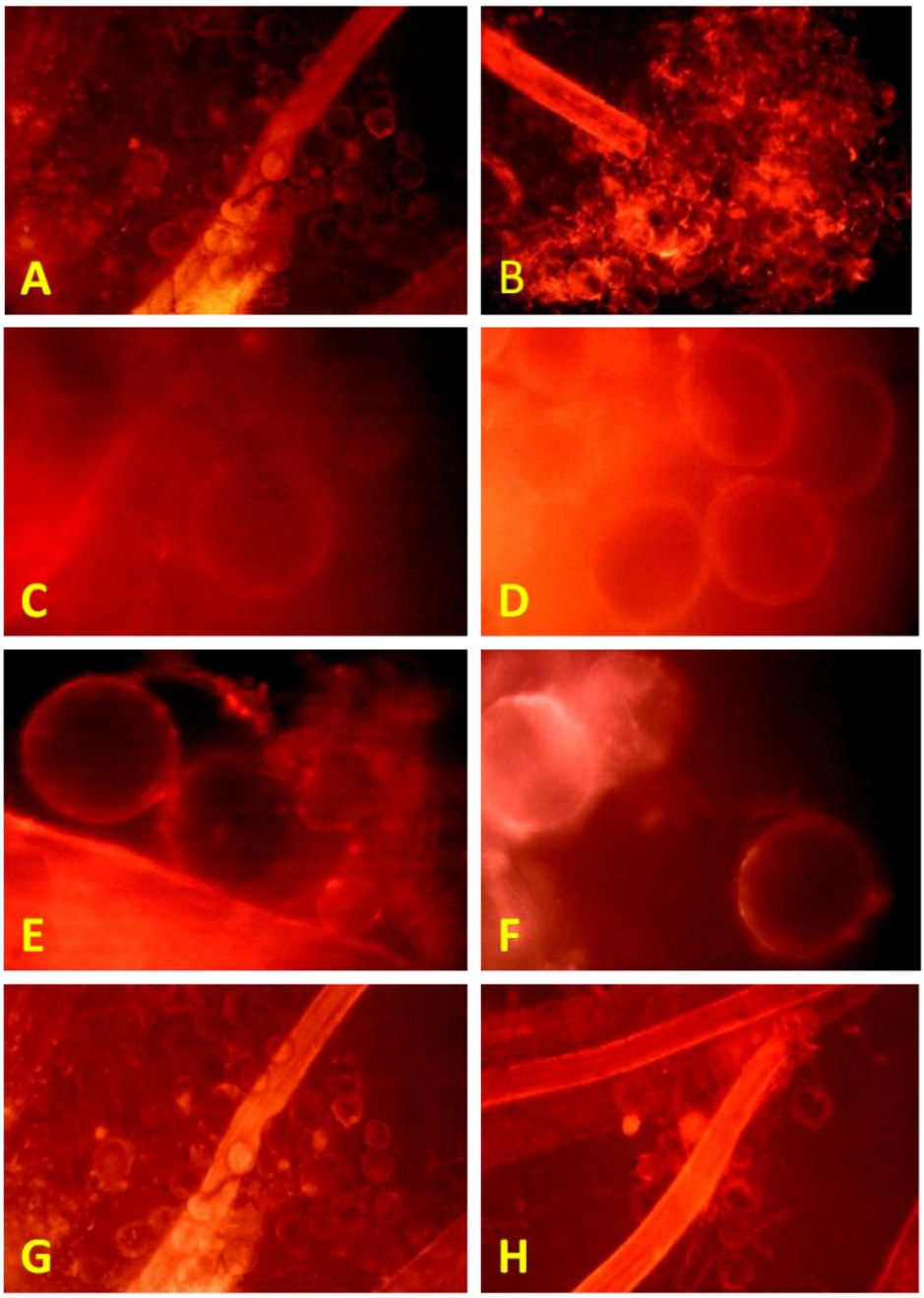

Fig. 69. Indirect-immunofluorescent labelling of *Glomus* spores adhered to mandarin roots and treated with PAb of *G. mosseae* and reacted with RITC

Labeling of *G. mosseae* and *Gi. gigantea* treated root segments was performed on sections of LR-white embedded tissues, previously fixed with 0.1 M sodium phosphate buffered-glutaraldehyde (2.5%) and using PAb of *G. mosseae* and *Gi. gigantea* and labeled with antirabbit-IgG (whole molecule) gold conjugate (10nm). AMF treated roots were labeled with two separate antibodies. One set was treated with PAb of *G. mosseae* and the other set with PAb of *Gi. gigantea*. In both the sets, labeling was profuse but gold labeling was more intense in roots treated with PAb of *G. mosseae*. Many clusters of particles were scattered around the cell wall. Gold particles were concentrated mostly near the cell wall and interfacial matrix (Fig 70).

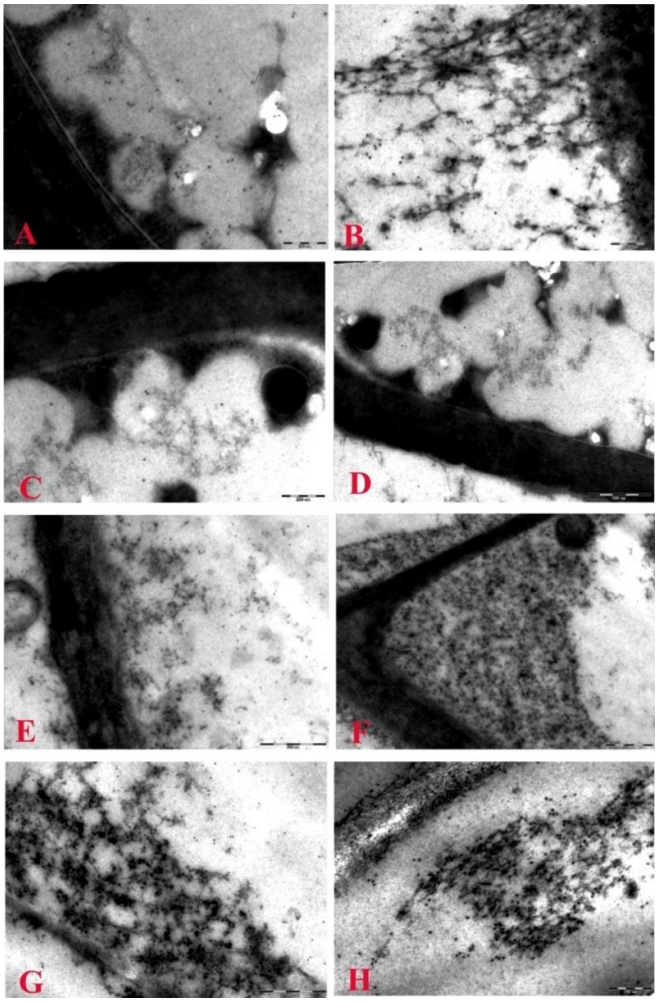

Fig 70. Transmission electron micrographs of immunogold labelled AMF colonized mandarin roots using PAbs of *G. mosseae* and *Gi. gigantea*. (A-D) AMF (*G. mosseae*) colonized roots probed with homologous PAb (E-F) AMF (*Gi. gigantea*) colonized roots probed with homologous PAb

4.17. Cellular location of chitinase in root and leaf tissues of *C. reticulata* following induction of resistance

Root rot incidence in mandarin plants was successfully reduced after application of *T. asperellum* in the rhizosphere of mandarin saplings prior to pathogen challenge. Disease reduction was found to be brought about by enhanced activities of key defense enzymes like chitinase, glucanase, phenylalanine ammonia lyase and peroxidase which increased significantly after pathogen challenge.

Cellular localization of chitinase in mandarin leaf and root tissues was determined following indirect immunofluorescence test using FITC binding and treatment with PAb raised against chitinase. Leaf and root sections from untreated control, untreated pathogen (*F. solani*) inoculated, *T. asperellum* inoculated, AMF inoculated, AMF and *T. asperellum* inoculated and treated with *Fusarium* plants were taken. Immunolocalization of chitinase in treated as well as pathogen inoculated mandarin leaves and roots were observed using FITC labeling and treatment with PAb of chitinase. Positive reaction with FITC was observed in cellular localization which gave indication of the induction of chitinase in mandarin leaf and root tissues (Fig. 71). Strong fluorescence was observed in treated leaves and roots. Inoculation with AMF and *T. asperellum* following challenge inoculation with the pathogen showed maximum bright apple green fluorescence which was homogenous in root cells and the mesophyll tissues in leaf (Fig. 72).

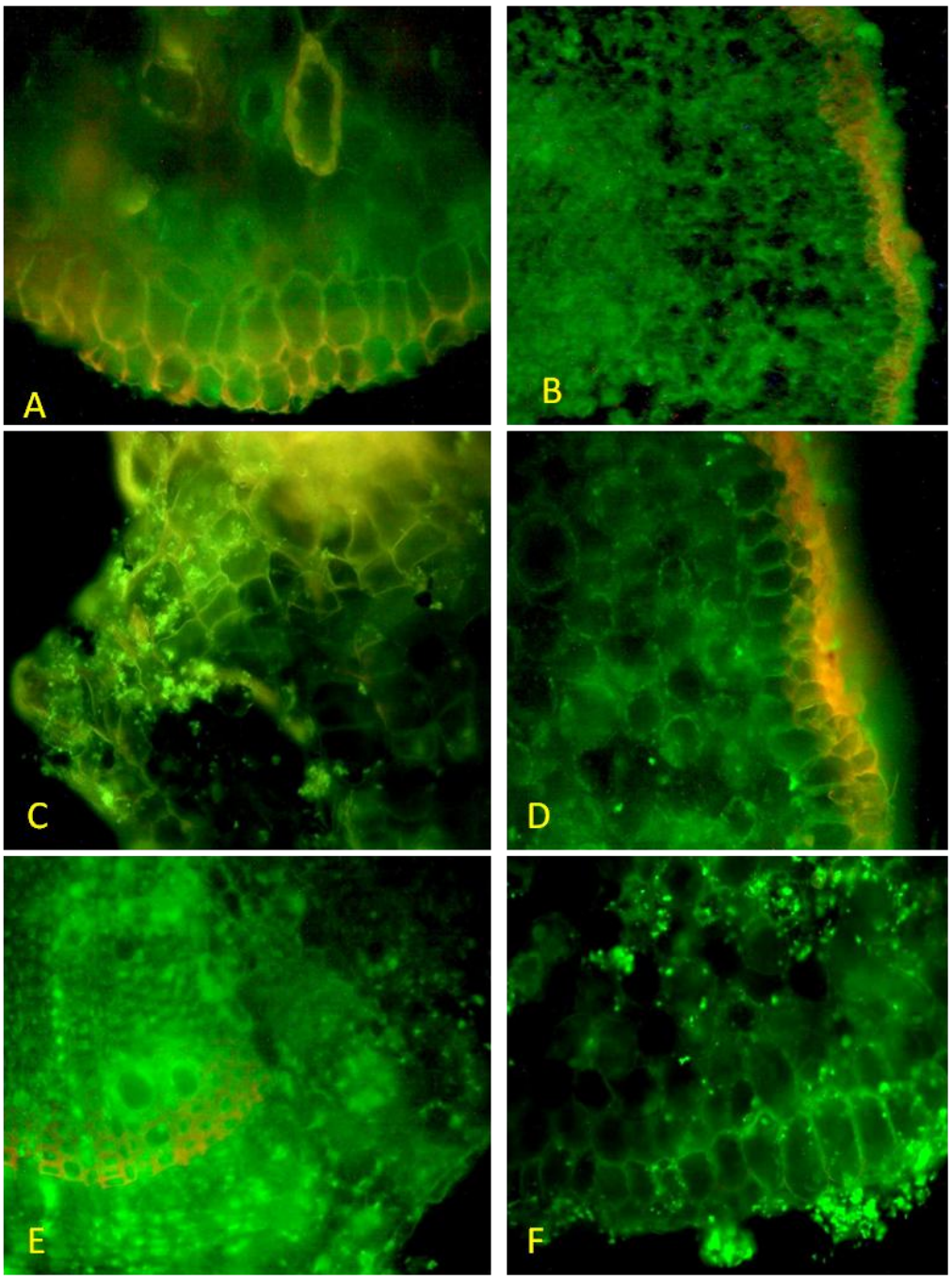

Fig 71. Cellular localization of chitinase in mandarin roots following treatments with bioinoculants. Healthy root (A), AMF inoculated (B) *T. asperellum* inoculated (C&D) AMF + *T. asperellum* treated (E) AMF + *T. asperellum* treated and inoculated with *F. solani* (F). All the samples probed with PAb of chitinase and labelled with FITC

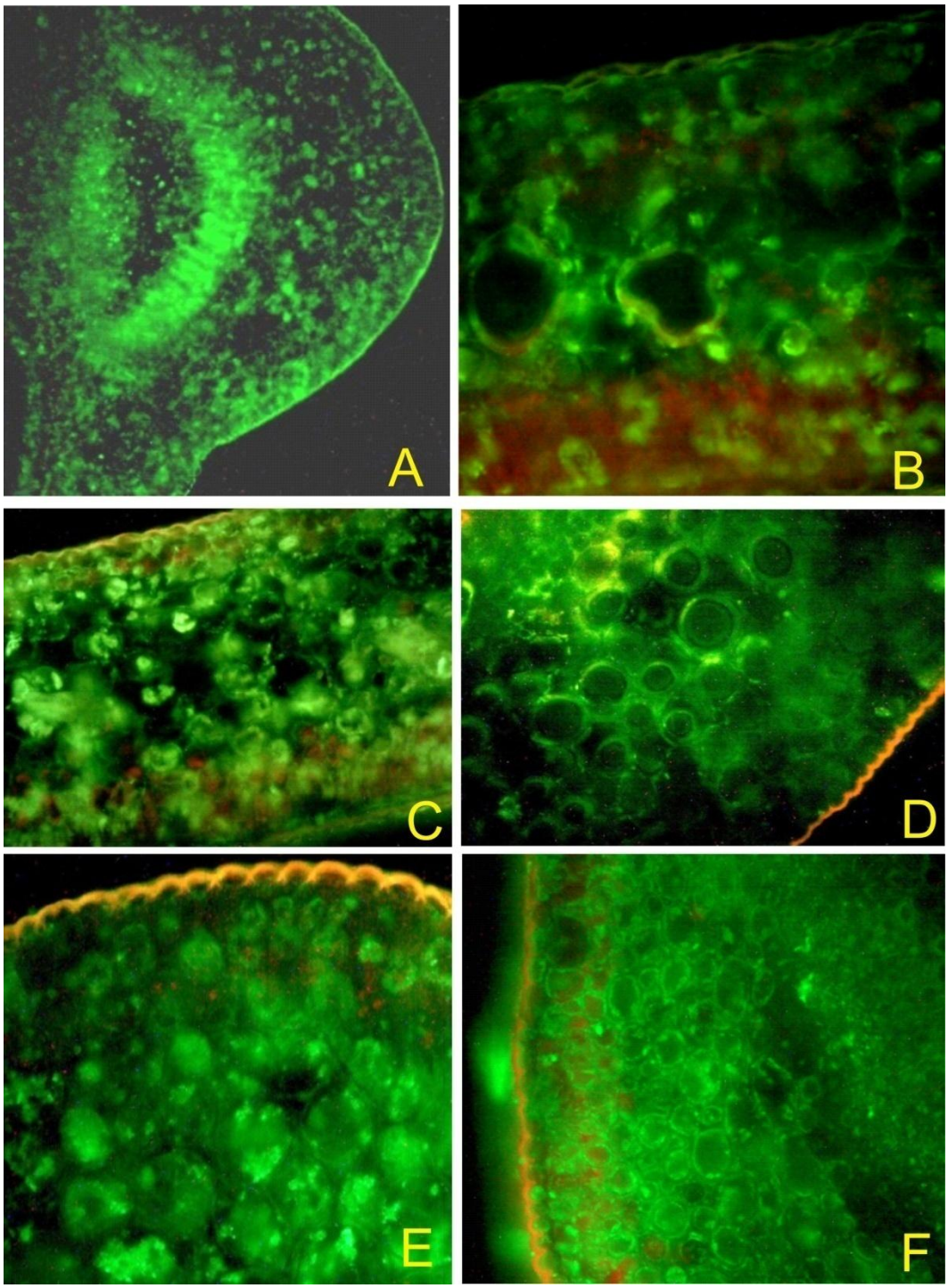

Fig. 72 Cellular localization of chitinase in mandarin leaves following treatments with bioinoculants. Healthy leaf (B), AMF inoculated (A) *T. asperellum* inoculated (C&D) AMF + *T. asperellum* treated (E) AMF + *T. asperellum* treated and inoculated with *F. solani* (F). All the samples probed with PAb of chitinase and labelled with FITC

4.18. Immunogold localization of chitinase following treatment with bioinoculants and challenge inoculation with *F. solani*

Labeling of control and *F. solani*, AMF and *T. asperellum* treated root segments was performed on sections of LR-white embedded tissues, previously fixed with 0.1 M sodium phosphate buffered-glutaraldehyde (2.5%) and using PAb of chitinase and labeled with antirabbit-IgG (whole molecule) gold conjugate (10nm). Control plant, in one set, was labeled with pre-immune serum instead of primary antibody and another set was labeled with PAb of *G. mosseae*. No gold labeling was observed in sections treated with pre-immune serum whereas very weak gold labeling was observed on the cell wall of control root treated with *G. mosseae*. This indicates that we cannot rule out the infection caused naturally in the soil.

4.18.1. Induced resistance following AMF colonization

Ultrathin sections of AMF colonized mandarin roots showed the presence of fine arbuscule branches within the root cells (Fig. 73). Sites of AMF infection in treated roots were detected by the presence of black precipitates of colloidal gold. However in the infected tissue, gold particles were predominantly recognized. The gold particles observed on the surface appeared as either individual spherical particles or were found in closely associated groups or clusters of particles (Fig 74). Gold particles were mostly concentrated near the cell wall.

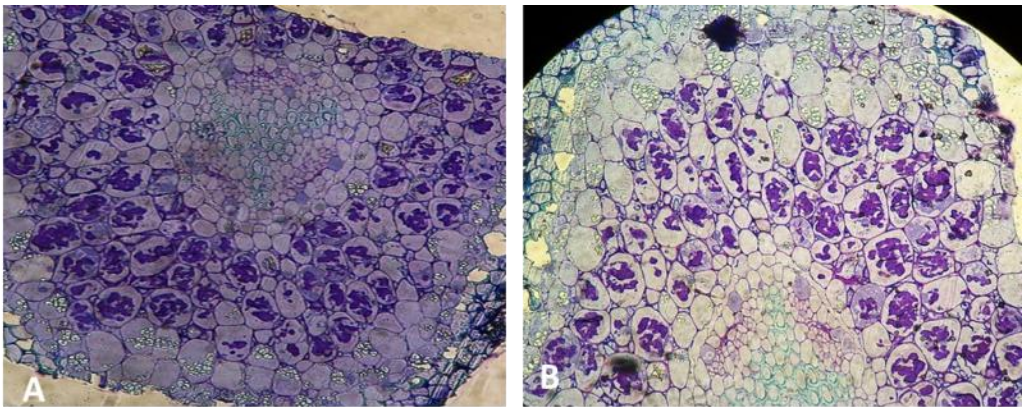

Fig. 73 Ultrathin section of AMF colonized mandarin roots stained with toluidine blue

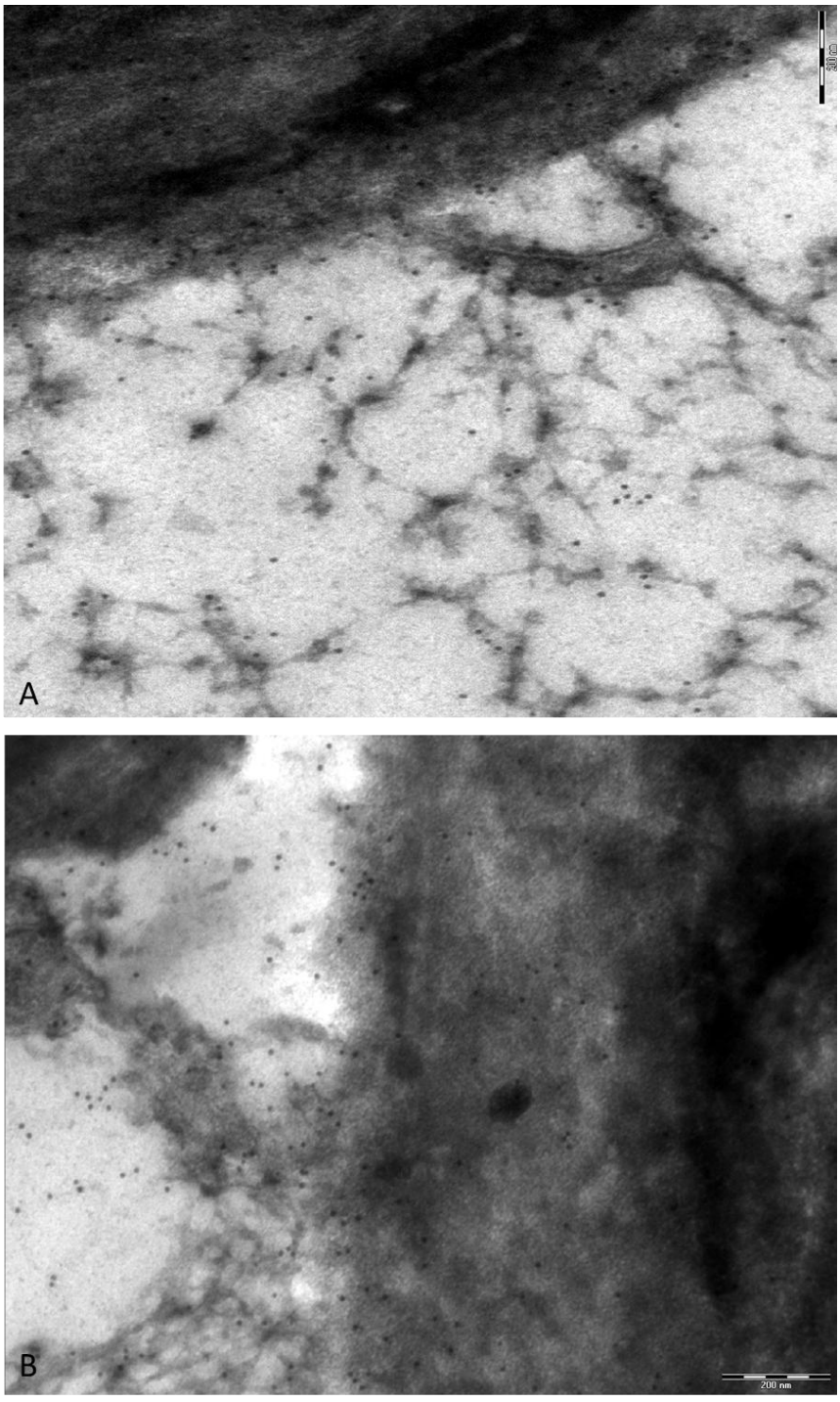

Fig. 74(A&B) Transmission electron micrographs showing immunogold localization of chitinase in AMF colonized mandarin roots using PAb of chitinase

4.18.2. Induction of resistance following treatment with *T. asperellum*

T. asperellum treated roots were labeled with PAb of chitinase. Many clusters of particles were scattered around the cell wall. Gold particles were concentrated mostly near the cell wall and interfacial matrix. Gold labeling was was observed in host cytoplasm. Junction of cortical cells showed heavy labeling in the middle lamella. Primary cell walls of cortical cells were labeled slightly (Fig. 75).

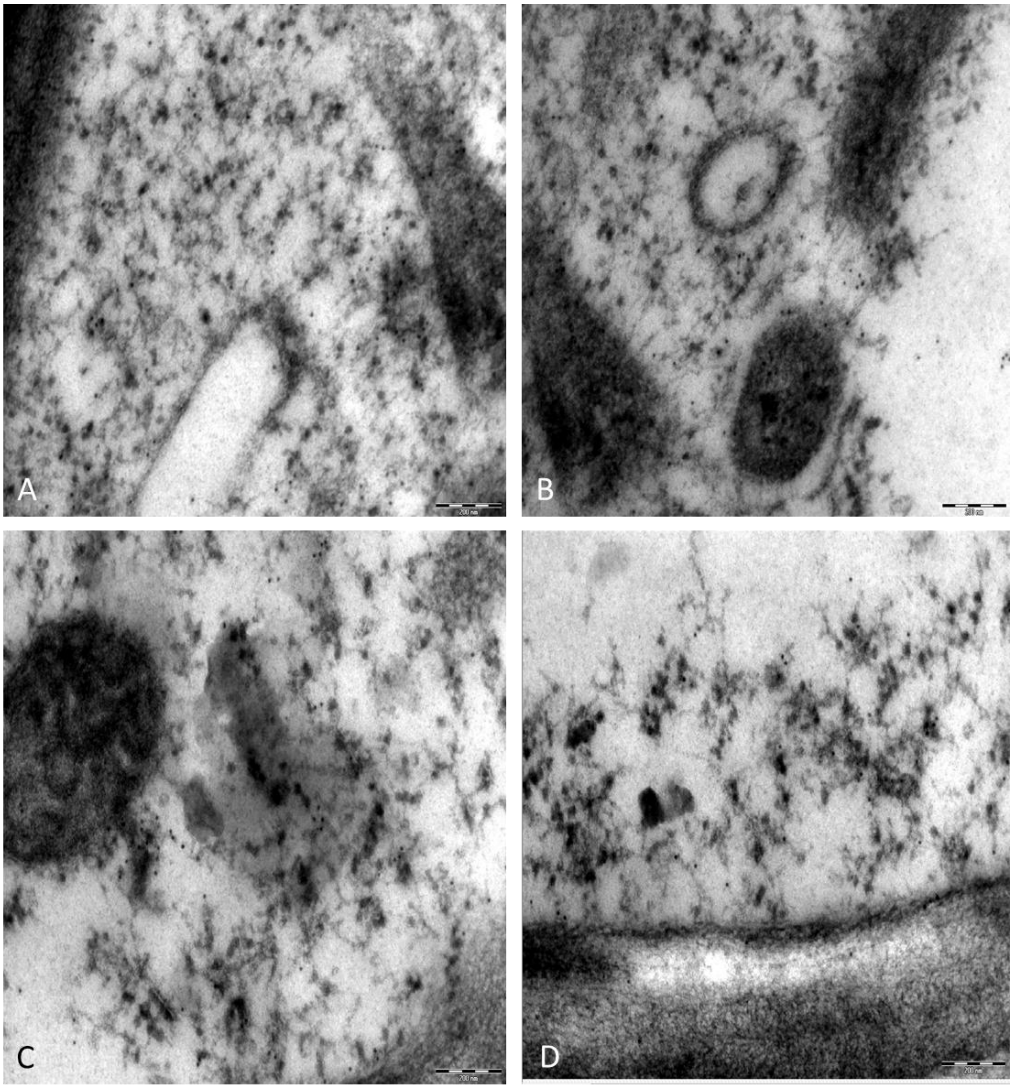

Fig. 75 (A-D).Transmission electron micrographs showing immunogold localization of chitinase in *T. asperellum* treated mandarin roots using PAb of chitinase

4.18.3. Activation of defense in mandarin roots following treatment with AMF and *T. asperellum* and challenge inoculation with *F. solani*

Intense black particles of gold was distributed throughout the cell structure in mandarin roots treated with AMF and *T. asperellum* followed by challenge inoculation with *F. solani*. Application of PAb of chitinase resulted in the heavy deposition of gold particles over the host cell walls (Fig 76). The immunolocalization study confirmed the immunoflorescence results and precisely showed the sites of chitinase expression which was detected by the formation of black precipitates of colloidal gold

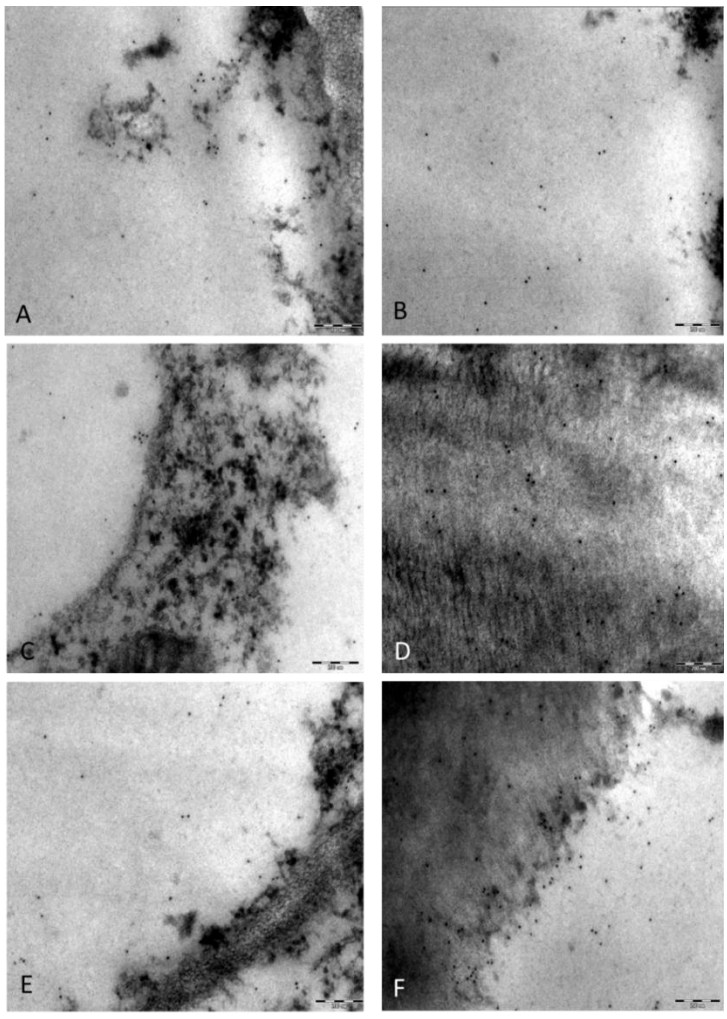

Fig. 76 (A-F) Transmission electron micrographs showing immunogold localization of chitinase in mandarin roots colonized with AMF and treated with *T. asperellum* following challenge inoculation with *F. solani* using PAb of chitinase

DISCUSSION

Mandarin is a perennial plant and is subjected to various environmental stresses including both biotic and abiotic factors. Various parts of the plant are attacked by pests and pathogens. Fungus is a common miscreant in mandarin plants which often targets roots and leaves. The symbiosis between arbuscular mycorrhizal fungi and plant is increasingly being recognized as an important and integral part of natural ecosystems throughout the world (Chakraborty *et al..,* 2014a) . Arbuscular mycorrhizal symbiosis is often associated with improved plant growth. This enhanced growth has been attributed to nutritional and non-nutritional effects of AM fungi. AMF hyphae form a network within the soil and between plants. This network distributes carbon and nutrients. It supplies energy for soil processes, extending the rhizosphere to the much mycorrhizosphere. Bacteria associated with AMF spores usually colonize only the outer layer of the wall and rarely penetrate into the inner layers. Nevertheless, some bacteria have been found in the cytoplasm of AMF spores. The role of AMF spore associated bacteria is not clear but they have been estimated to stimulate spore germination by eroding spore walls, by producing stimulatory compounds such as CO2 and other volatiles, or by influencing AMF phosphorus acquisition. Keeping the above in mind, our present study was undertaken to identify potential Arbuscular Mycorrhizal Fungi (AMF), Plant Growth Promoting Fungi (PGPF) and Plant Growth Promoting Rhizobacteria (PGPR) from rhizosphere of mandarin so they can be used as beneficial microorganisms. Mandarin seedlings were brought from Kalimpong, Mirik, Bijanbari and Kurseong of Darjeeling hill to study their growth behavior, mycorrhization and other experimental purposes. Soil and root samples were also brought from Rangli Rangliot, Sukhia Pokhari and Gorubathan for various experimental purposes.

Population of different species of AM fungi isolated from the rhizosphere of mandarin from different locations both from hill region as well as foot hills was determined. Sites selected were hilly regions of Kalimpong, Mirik, Bijanbari and Kurseong of Darjeeling hill. Spores were identified upto the species level using the help

of standard keys (Walker, 1992) and website of INVAM. Among the AM fungi, *Glomus mosseae* could be determined as the most predominant, followed by other genera such as *Gigaspora, Acaulospora* and *Scutellospora* (Allay *et al.*. 2012). Percentage of AM spores determined from different regions showed maximum of different *Glomus* sp., followed by *Gigaspora* sp., *Acaulospora* and *Scutellospora* during the summer months. Presence of *Gigaspora* sp. was profuse in monsoon and winter season but their spore population decreased in summer season. During the summer, *Glomus* sp. flourished in all the locations. This was evident as the spores of *Gigaspora* sp. was less common in the plant rhizosphere of the foothills as compared to the hills. Spore population of *C. limonia* and *C. medica* also proved the same fact by showing more population of *Glomus* sp.. In *C. limonia* diversity of *Gigaspora* sp. was evident but their population was less. *C. medica* showed variation in spore diversity. Observation has it that plant roots emit a volatile signal that stimulates directional growth of the fungi towards them (Koske, 1982). AM fungi have weak cellulose and endopolygalacturonase activities which have the capacity to catalyze to the release of oligosaccharides or oligosaccharins from the plant cell wall (Fry *et al.,* 1993). The latter could trigger the colonization and spread of the fungus which are all controlled by the host. Scanning Electron Microscopy (SEM) of the spores revealed the spore wall morphology. *Glomus fasciculatum* has a rough outer surface. The outer layer is sloughed. Spores of *G. fasciculatum* are found in aggregates of 2-6. The surface is rough and shape is subglobose. The wall of *Gigaspora gigantea* is smooth walled with the typical bulbous suspensor. The polygonal reticulum on the spore wall surface is characteristic of *Acaulospora reticulata*.

Root colonization in citrus plants varied according to the age of the plant. Colonization percent was more in mature plants than young seedlings. Presence of abundant vesicles was evident. Organisms of AMF have a bimodal pattern of differentiation (Morton, 1990). The vegetative thallus consists of arbuscules, intraradical vesicles (shared only by species in the suborder Glomineae), extraradical auxiliary cells (shared only by species in the suborder Gigasporineae), and intraradical and extraradical hyphae (Smith and Read, 1997; Morton and Benny, 1990). Arbuscules are finely branched structures in close contact with the cell plasma membrane, functioning in

exchange of nutrients between host and fungal cells (Smith and Read, 1997). Hyphae are important in nutrient acquisition and as propagules to initiate new root colonization (Graham *et al.,* 1982; Friese and Allen, 1991). Vesicles are globose structures arising from swelling of the hyphae and filled with glycogen granules and lipids and are considered to be storage structures (Bonfante-Fasolo, 1984; Brundrett, 1991). AM effects the evolution of the plant, microbial communities, soil nutrient status and structure at long term. Direct, short-term AM influences such as pathogen antagonism, alleviation of drought and heavy metal stresses, competition and enhancement of photosynthetic rates and phytohormone levels are well-established (Allen *et al.,* 1980). Frequently observed is an increased uptake of less mobile nutrients, especially P, but also ammonium (NH4+), Cu and zinc (Zn), potassium (K), calcium (Ca) and sulfur (S) (Bethlenfalvay *et al.*, 1989). AM also promotes symbiotic N fixation (Barea *et al.,* 1987) even if this may be related to improved P nutrition. Enhanced uptake of P is most often responsible for the growth increase of plants due to mycorrhization (Abbott and Robson, 1984). Up to 80% of the plant P, 60% of Cu and 25% of Zn can be delivered by external AMF hyphae extending as much as 12 cm from the root surface (Li *et al.,* 1991; Marschner and Dell, 1994).

Citrus prefer well drained soil with pH ranging between 5.5 to 7 and low salinity. At lower pH values, trees can suffer from aluminium and other heavy metal ion toxicity as well as restricted phosphorus availability. At pH values above 7.5, nutrient availability in particular phosphorus and micronutrients can be reduced even though high total amounts of these elements may be present in the soil. Keeping this fact in mind soil from Kurseong, Kalimpong block I and block II and Gorubathan have ideal soil for nutrient availability and fruit production. Clay particles tend to retain or fix phosphorus in soils. Fine textured soils such as clay-loam soils have a greater capacity of fixing phosphorus than sandy coarse textured soils. On isolation of mandarin rhizosphere soil, various fungi and bacteria have been obtained. Fungi mainly belonged to the genera *Aspergillus, Trichoderma, Fusarium, Gongronella Sporotrichum* and *Emericella*. Besides, AMF spores isolated from *C. reticulata* were mass multiplied in maize, turf grass and sorghum but the spores isolated from *C. limonia* and *C. medica* were mass multiplied only in maize plant. Spores mass multiplied in maize had a good proliferation rate than those

multiplied in turf grass and sorghum. Turf grass is used not only for mass multiplication of spores but also for storing spores as it is a perennial grass. Since AM spores need a living host to survive it can be stored for long period of time in turf grass.

Phosphorus is an essential nutrient both as a part of several key plant structure conpounds and as a catalysis in the conversion of numerous key biochemical reactions in plants. The specific growth factors that are associated with phosphorus are stimulated root development, increased stalk and stem strength, improved flower formation and seed production, more uniform and earlier crop maturity, improvement in crop quality and increased resistance to plant diseases. Phosphate solubilizing activity of fungal and bacterial isolates were also tested in Pikovaskaya's solid and liquid media. Four fungal isolates and one bacterial isolate showed phosphate solubilizing activities. Maximum phosphate solubilization was observed by *A. fumigatus* whereas *A. oryzae* showed least phosphate solubilization activity. Out of the 14 bacteria isolated from rhizosphere of mandarin, only one showed positive for phosphate solubilization. All bacterial isolates showed positive for IAA test but no reaction was present for HCN, H_2S and urease production. Many reports on phosphorus solubolization by bacteria, fungi and actinomycetes have been provided (Whitelaw, 2000; Oberson *et al.*., 2001; Hamdali *et al.*, 2008 and Minaxi *et al.*, 2010). One bacterial isolate *P. poae* (RMK03) which showed positive for most of the plant growth promoting characters viz. IAA, phosphate solubilization, catalase and siderophore production was tested against mandarin root pathogens *F. solani* and *F. oxysporum*. The bacterial isolate RMK03 could inhibit the root pathogens efficiently in dual culture test.

In the present investigation three pathogenicity characters of *Fusarium* spp. causing root rot of mandarin plants were assessed: phytotoxicity of fungi excretions, capacity to colonize the surface of citrus host plant and ability to colonize the internal tissues. Screening for resistance against these fungal pathogens were attempted using water culture method and young seedlings as well as sand-maize meal culture for one year old seedlings in pot conditions. All trials were carried out on Darjeeling mandarin saplings collected from nurseries from eight orchards. Symptoms of wilting were assessed over two months in phytotoxicity of culture filtrate trial. The results highlighted

a significant correlation between phytotoxicity excretions and invasion ability. Nevertheless, no correlation was found between these two measures and the ability to colonize the surface of citrus seedlings. Despite the large variation existing among strains within the species, strains belonging to *F. solani* showed a higher aggressiveness than *F. oxysporum* among all the characters tested. Due to the fact that all the tested isolates were collected from orchards known to have dry root rot symptoms, and according to our finding regarding the higher aggressiveness of *F. solani* isolates on citrus seedlings, a correlation might be suggested between Fusarium wilt disease affecting citrus seedlings and dry root rot disease observed on scaffold roots of trees in the orchards (Yaseen and D'Onghia, 2012). A study was conducted to characterize fungal and oomycet pathogens associated with root diseases of acid lime and sweet lime. A survey over 166 farms in Oman showed prevalence of dieback symptoms in 8.8% of acid limes and 15.9 % of sweet limes and on isolation it was found that *F. solani* was one of the pathogen alongwith *Lasiodiplodia hormozganensis* and *L. theobromae* (Al-Sadi *et al.*, 2014).

Agar gel double diffusion test was performed using the mycelial antigens of *F. oxysporum* and *F. solani* and homologous PAb. Strong precipitin reactions occurred in homologous reactions in immunodiffusion test. PAbs raised against *F. solani* and *F. oxysporum* were tested with homologous and heterologous antigens of mandarin roots. Among the root antigens of mandarin plants of eight different locations tested against PAbs of *F. solani*, strong and positive reactions were noticed in root antigens of four specific locations locations (Sukhia pokhari, Mirik, Kalimpong Block I and block II) and in the case with PAbs of *F. oxysporum*, the four specific locations where strong and positive reactions were noted are Bijanbari, Mirik, Kalimpong Block I and block II. Dot immunobinding assays confirm the effectiveness of raising antibodies against *F. solani* and *F. oxysporum*. 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. Doubling dilutions of *F. solani* and *F. oxysporum* ranging from 1:125 to 1:4000 were initially tested. ELISA values decreased

with the dilution. IgG dilution of 1:125 was selected for further assay. Dilutions of antigen conc 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 levels. Concentrations as low as 25ug/L could be easily detected by ELISA at both antisera dilutions. PTA-ELISA could readily detect reaction between root antigen and PAb of pathogens (*F. solani* and *F. oxysporum*). Antigens extracted from healthy and artificially inoculated with *F. solani* and *F. oxysporum* were tested against PAbs of the pathogens separately. 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 2mg/l. Kitagawa *et al.* (1989) successfully used a competitive ELISA technique to develop an assay to identify *F. oxysporum* f.sp. *cucumerinum* among other *Fusarium* sp. Deep violet colour developed in homologous reactions in dot blot indicating a positive reaction suggestive of effectiveness of mycelial antigen in raising PAb against the pathogen. However, faint violet colour was observed in heterologous reactions. Sharp bands were produced on SDS-PAGE by the antigens of *F. oxysporum* and *F. solani*.

Amplification of target DNA through PCR with sequence specific primers are potentially more sensitive and rapid than microbiological techniques, as a number of constrains 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; Moukhamedove *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 18S small subunit (SSU), 5.8S, and 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* and *Trichoderma* spp. ITS region of rDNA was amplified using genus specific T/ITS1 and T/ITS4 (for *Trichoderma*) and Fcg17F and Fcg17R (for *Fusarium*) primers. Amplified products of 550bp size was produced by *Fusarium*, while 600bp size was evident in case of *Trichoderma.* The primer pairs Fcg17F and Fcg17R were highly specific for *Fusarium* genus. These results are also in accordance with several workers who observed the amplified rDNA fragment of approximately 600 bp by ITS-PCR in *Trichoderma* (Ospina *et al.,* 1999: Muthumeenakshi *et al.,* 1994; Lieckfiledt *et al.,* 1999; Mukherjee *et al.,* 2002, Venkateswarlu *et al.,* 2008, Chakraborty *et al.,* 2010).

In order to determine the abiotic stress effect of flooding and drought on mandarin plants, these were subjected to water logging condition in the field and in pots for drought condition. Biochemical and morphological changes induced by water logging and drought conditions were determined. The plants showed slight wilting and leaf dropping by the third day of flooding whereas in drought the leaves curled up, became crisp and later dried out. Marked changes in antioxidative enzymes such as peroxidase, catalase and ascorbate peroxidase was observed during stress in comparison to control plants. Antioxidative activity was seen to be more in the leaves than in the roots. Among the antioxidants carotenoid content showed a significant decrease during the flood stress but increased in drought stress (Allay *et al.,* 2013). Accumulation of osmolytes such as proline, helps in maintaining cell water status, sub-cellular structures and protecting membranes and proteins from the denaturing effects of the osmotic stress (Ashraf and Foolad, 2007). With the increase in intensity of drought there was an increase in both proline and ascorbate content in all varieties of maize (Lama and Chakraborty, 2012). An increase in ascorbate content was observed during stress in comparison to the control. It is known that plant adaptation to stress conditions requires additional material and energy resources, making processes such as photosynthesis and respiration more significant (Semikhatova, 2000). Soil water logging and submergence (collectively termed flooding)

and drought are abiotic stresses that influence species composition and productivity in numerous plant communities, world-wide. Hydrological patterns can determine the vegetation in natural and man-made wetlands, since this is dependent on ecophysiological responses of species to flooding (Voesenek *et al*., 2004). For most other crops, excess water is a major constraint which adversely affects grain yields and growth of pasture species (Gibberd and Cocks, 1997; Gibberd *et al.,* 2001). Drought triggers a wide variety of plant responses, ranging from cellular metabolism to changes in growth rates and crop yields. Understanding the biochemical and molecular responses to drought is essential for a holistic perception of plant resistance mechanisms to water-limited conditions (Anjum *et al.,* 2011). Water stress in citrus reduces stomatal conductance, transpiration rate and net assimilation of carbon dioxide (Arbona *et al*., 2005; Garcia-Sanchez *et al.,* 2007). Waterlogging has a profound effect on root metabolism, mainly through the deficiency of oxygen (Drew, 1997). In tropical and subtropical regions, severe crop losses are caused by prolonged seasonal rainfall. Previous work has shown that there is potential for water damage to citrus trees if roots are submerged in water for four days or more during frequent extended summer rains. During the cooler months of December through February, citrus trees can tolerate flooded conditions for longer periods than during the hot summer months (Boman and Tucker, 2002). Sour odour in roots indicate an oxygen deficient environment. The presence of hydrogen sulfide (a rotten egg odour) is an indication that fresh feeder roots are dying. Anaerobic bacteria (which grow only in the absence of oxygen) develop rapidly in flooded soils and contribute to the destruction of citrus roots. Root damage symptoms include leaf yellowing, chlorosis, wilting, fruit drop, leaf drop and dieback. Excess water produces anoxic soil conditions within a few hours (Gambrell and Patrick, 1978). Flooding stress is usually less when water is moving than when water is stagnant for anaerobics cannot multiply if oxygen is present. Drought stress is characterized by reduction of water content, diminished leaf water potential and turgor loss, closure of stomata and decrease in cell enlargement and growth .Severe water stress may result in the arrest of photosynthesis, disturbance of metabolism and finally the death of plant (Jaleel *et al.,* 2008).

Two isolates of *Trichoderma harzianum* and one isolate of *T. asperellum* isolated from mandarin rhizosphere were initially taken up for *in vitro* evaluation of their antagonistic effects against the fungal pathogens (*F. solani* and *F. oxysporum*). *T. harzianum* isolate no RHS/M 511 and *T. asperellum* RHS/M 512 showed maximum inhibitory activities. *Trichoderma* are successful colonizers in all diversified habitats. They efficiently utilize the substrate and their secretion capacity for antibiotic metabolites and enzymes (Schuster and Schmoll, 2010) and enhance systemic resistance in plants (Shoresh *et al*., 2010). *T. harzianum* isolates have shown effective results against plant pathogens such as *Fusarium* spp., *R. solani*, *G. graminis* and *D. sorokiniana* (Kucuk and Kivanc, 2003). Anand and Reddy (2009) studied the biocontrol effects of forty two strains of *Tricoderma* spp. against *S. rolfsii* and *F. ciceri* where disease incidence was found to be nil compared to pathogen check in four of the isolates. Study of antagonistic property of *Trichoderma* and *Pseudomonas* against *S. rolfsii* have been done by many researchers (Dev and Dawande, 2010) as well as the biocontrol capacity of wild and mutant *T. harzianum* strains on *S. rolfsii* with regard to their effect of temperature and soil type during storage (Montealegre *et al.*, 2009).

Activation of defense response of mandarin plants was observed after application of bioinoculants against *F. solani* and *F. oxysporum*. One of the potential biocontrol fungus- *Trichoderma asperellum* isolated from mandarin rhizosphere showed *in vitro* as well as *in vivo* antagonistic reaction against the root rot pathogen-*F. solani* (Allay and Chakraborty, 2013). Application of *G. fasciculatum*, Gi. *gigantea* and *T. asperellum* singly or jointly suppressed root rot of mandarin. Induction of major defense enzymes such as chitinase, β, 1-3 glucanase and peroxidase by treatment with AMF and *T. asperellum* was evident. One induced isoform of peroxizymes following AMF inoculation as well as application of *T. asperellum* was confirmed in PAGE analyses. Concomitant increase in defense enzymes following inoculation with *F. solani* was correlated with the induction of resistance in mandarin plants using bioinoculants (Allay and Chakraborty, 2013). Induction of flower and fruit was enhanced on application of bioinoculants *Trichoderma* and AMF. 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). *Trichoderma* spp. has been widely applied as against several soil-borne pathogens. The spores especially the conidia of *Trichoderma* are more tolerant to adverse environmental conditions during product formulation and field use, in contrast to their mycelial and chlamydospores forms (Amsellem *et al.*, 1999). But the presence of mycelia is also a key component for the production of antagonistic metabolites (Yedida *et al*,. 2000). Generally liquid fermentation is more suitable method over solid state for large scale production. Sharma *et al..*, (2014) discussed the exploitation of *Trichoderma* on various cereal, pulse, oilseed, cash, vegetable, fruit, spices and other crops. *T. harzianum* and *T. viride* have been mostly used by researchers along with other combinations such as fungicides, insecticides and other biocontrol agents. Three species of *Trichoderma* viz. *T. harzianum*, *T. erinaceum* and *T. asperellum* were tested for their *in vitro* effects against *S. rolfsii* and the inhibition percentage was up to 75 percent in *T. harzianum* which was taken up for further *in vivo* assay and was effective in suppressing sclerotial rot of *Glycine max* caused by *S. rolfsii* (Sunar *et al.*, 2014).

Plant growth promoting rhizobacteria isolated and identified from plantation and horticultural crops were evaluated for their efficacy in mandarin plants. *P. poae*, isolated from mandarin rhizosphere gave the best results in plant growth as well as biochemical enzymatic assays. Hence, it was selected for further experiments to suppress root rot of mandarin caused by *F. oxysporum* with *Gi. gigantea*. Joint inoculation of *P. poae* with *Gi*. *gigantea* gave better results in suppression of root rot disease. Mandarin roots when inoculated with *G. mosseae* alone and in combination with *B. pumilus* which was applied as soil drench increased growth of the plants but most significant increase was obtained when both were co-inoculated. Similarly, root rot of mandarin caused by *F. 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* (Chakraborty *et al.*, 2011). 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. 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 *et al.,* 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. 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.

T. harzianum and *Bacillus subtilis* have been proved as effective biocides for controlling Fusarium root rot of certain citrus rootstocks (El-Mohamedy, 2009). All citrus plants infected with *Pythium ultimum* were sprayed on leaves as well as added to rhizosphere soil every month (metalaxyl-10g/20L of water in combination with chemical fertilizers, Chaetomium- 20g/20 L of water and Trichoderma-20g/20L of water in combination with biocompost). Result showed that all treated citrus trees recovered significantly within 3-4 months of applications with growth of new flashes of leaves and root. It is proved that the biological products of *Chaetomium* and *Trichoderma* gave significantly disease control as equal as the chemical fungicide (metalyxyl) when compared to the non-treated control (Kean *et al.,* 2012). In soil infested with *F. solani* and *M. phaseolina*, the treatment with compost fortified with *T. harzianum* in combination with Top.Zn decreased the average number of total bacterial counts in the rhizosphere of orange 85.04 and 78.92% respectively and 59.32 and 92.74 % respectively in the rhizosphere of mandarin (El-Motty *et al.,* 2010).

Levels of disease protection was significantly increased by combined inoculation of *G. mosseae* with *P. simplicissimum* and *T. harzianum* and combined inoculation of *G. mosseae* with *T. harzianum* synergistically enhanced plant growth (Chandanie *et al.,* 2009). Allay *et al,*. (2010) also showed that joint inoculation of *G. mosseae* with *T.*

hamatum gave better results in plant growth and suppressed root rot of mandarin caused by *F. solani*. *G. mosseae* showed a bioprotective effect of mycorrhization and difference in their susceptibility to AMF and *F. oxysporum* f. sp. *lycopersici* independent of cultivar age of tomato plants (Steinkellner *et al.*, 2011). Morsey *et al.* (2009) observed that infection caused by *F. solani* on tomato plants decreased and survival rate increased by dual treatment with *T. viride* and *B. subtilis*. These treatments also favoured greater proliferation of rhizosphere microflora and higher dehydrogenase activity in the rhizosphere. Dual inoculation gave the best results of growth parameters, fruit yield and plant nutrient content than individual ones.

The next phase of investigation was analysis of rDNA sequences of a selected PGPR and a bacterium associated with spores of *Gigaspora* sp (AMF). Their identity was confirmed with the help of 16S rDNA sequences. Bacteria associated with *Gi. gigantea* was sequenced and identified as *Bacillus mycoides* and deposited in NCBI with accession no. KJ917554. Many bacteria are attached to the hyaline layer of *G. mosseae* spores and upto 107 CFU/g chitinolytic microorganisms were present on the sporocarp surface (Filippi *et al.*, 1998). Spores of *Glomus geosporum* and *Glomus constrictum* were harvested from single-spore-derived pot cultures with either *Plantago lanceolata* or *Hieracium pilosella* as host plants. PCR-denaturing gradient gel electrophoresis (DGGE) analysis revealed that the bacterial communities associated with the spores depended more on AMF than host plant identity. The composition of the bacterial populations linked to the spores could be predominantly influenced by a specific spore wall composition or AMF exudates rather than by specific root exudates. The majority of the bacterial sequences that were common to both *G. geosporum* and *G. constrictum* spores were affiliated with taxonomic groups known to degrade biopolymers (*Cellvibrio, Chondromyces, Flexibacter, Lysobacter,* and *Pseudomonas*). Scanning electron microscopy of *G. geosporum* spores revealed that these bacteria are possibly feeding on the outer hyaline spore layer. The process of maturation and eventual germination of AMF spores might then benefit from the activity of the surface microorganisms degrading the outer hyaline wall layer (Roesti *et al.*, 2005). Based on 16S rRNA gene sequences, the endobacteria in *Gi. margarita* was identified to belong to the genus

Burkholderia. The strains belonging to *Gi. margarita* may be difficult to grow *in vitro* outside the spores.

Fluorescent antibody labelling with FITC / RITC is known to be one of the best techniques to detect a number of microorganisms in particular sample. In the recent time study of AMF by fluorescent antibody labelling has been done successfully by many scientists (Vierheiliga *et al*., 2005, Besserer *et al*., 2006). In the present study polyclonal antibody raised against *Gigaspora gigantea* and *G. mosseae* were used to detect their presence and their nature of colonization in the host root. Citrus roots following colonization with AMF alone or in combination with PGPR / PGPF could induce resistance against the pathogen. The present investigation was designed to locate the presence of AMF in rhizoplane as well as their cellular localization in root tissues using PAbs of the AMF (*Gi. gigantea* and *G. mosseae*). In the AMF treated citrus plants, when PAb of *Gi. gigantea* was used in indirect immunofluorescence test, bright apple green fluorescence developed in the spore wall. The green fluorescent was also prominent in the subtending hyphae. In case of RITC labelled fluorescent staining of AMF spores, a red coloured fluorescence was observed and was distributed throughout the outer spore wall. It was observed that the fluorescence was more intense on young spore walls. Similarly feeder roots of citrus were also treated with PAbs of *Gi. gigantea* and *G. mosseae* and labelled with FITC to specifically locate the hyphal proliferation inside the tissues. Observations of treated root tissues under UV- microscope showed bright apple green fluorescence of the hyphae. Fluorescence was more prominent towards the cell wall in most of the tissues clearly indicate the successful colonization of citrus roots with AMF leading towards induced resistance against the pathogen.

Induction of resistance in mandarin against root rot pathogen and cellular localization of defense enzymes mainly chitinase was attempted. Roots and leaves of mandarin plants treated with *T. asperellum* were reacted with PAb of Chitinase (Chakraborty *et al.*, 2004) followed by labeling with FITC. Strong bright apple green fluorescence was observed in the epidermal and homogenously in mesophyll tissues in leaves and homogenously in cortical cells and epidermal cells in roots. Enhancement of chitinase was revealed in both leaves and roots following induction.

The last phase of the present investigation was to study immunogold localization of defense enzyme (chitinase) in mandarin roots following colonization with AMF, treated with bioinoculant (*T. asperellum*) singly and joint inoculation and subsequently challenge inoculation with *F. solani* through Transmission Electron Microscopy. In our present study we examined the systematic response of induced resistance in mandarin plants using PAb of chitinase following successful colonization with AMF as well as treated with *T. asperellum*. Heavy deposition of gold particles was observed near the cell wall of inoculated roots. Yedida *et al.* (1999), observed that application of *Trichoderma* to rhizosphere of cucumber seedlings initiated a series of morphological and biochemical changes. Gold labeling was done with β-1,4-exoglucanase and β-1,3-glucanase where intense gold labeling was observed over both the cell walls and wall appositions. Wisneiwska and Golinowski (2011) located the NtEXPA5 protein at different stages of arbuscule development in mycorrhizal tobacco roots. Labeling of plant cell wall was abundant at the point of penetration, where there was an accumulation of electron-dense material. A few gold particles were also seen in the parts of cell walls that adhered firmly to intercellularly growing fungal hyphae. When plants were colonized with AMF and then treated with *T. asperellum* followed by challenge inoculatio with *F. solani,* elicitation of chitinase was evident as intense gold labeling over both cell walls and wall appositions which activate the defense response in the plants against root rot pathogens. Observed plant health improvement and disease suppression in mandarin plants may be due to a combination of at least three mechanisms- direct inhibition of the pathogen in the soil, induction of resistance in the host or better nutrient availability.

Mycorrhization of plants coupled with bacterization would be more meaningful to 'biotize' *in vitro* propagated plantlets for transient transplant shock during acclimatization. The modern 'hairy-root technology' developed by TERI has played a significant role in establishment of AMF with transgenic host plants. The use of dual technology along with application of PGPF either in soil or foliar application is promising as micropropagated plantlets are suitable platform for understanding the mystery of host-endophyte interaction excessive production of secondary metablolites, heavy metal tolerance, bioprotection, bioremediation and growth promoting activity.

This will also help to unravel impediments for the growth and development of AMF in culture Chakraborty *et al.*, 2014a). Besides, this technology offers an opportunity to apply molecular approaches to understand host- symbiont interactions, secretion of flavanoid and signal transduction pathways. This may result in a more efficient and at the same time agriculturally and environmentally sustainable use of soil microorganisms for crop production (Chakraborty *et al.*, 2014b).

CONCLUSION

- *C. reticulata*, *C. limonia* and *C. medica* were taken for AMF isolation. Depending on their abundance two AMF spores were selected, i.e. *Glomus fasciculatum* and *Gi. gigantea* were taken up for all the *in vivo* studies. Spores isolated were mass multiplied in maize, sorghum and turf grass.
- Scanning electron microscopic observation was made for the AMF spores. Differences in their wall characters and ornamentations were observed.
- Screening for resistance of mandarin against *F. solani* and *F. oxysporum* was undertaken. Seedlings from Mirik and Kalimpong Block II were found to be highly susceptible to the pathogen.
- Three important PGPF viz. one isolate of *T. asperellum* and two isolates of *T. harzianum* were isolated from the rhizosphere of mandarin. *T. asperellum* (RHS/M/511) and one isolate of *T. harzianum* (RHS/M/511) showed better *in vitro* antagonistic activity as a result they were taken up for further studies.
- Among all the bacterial isolates RMK03 showed positive for most of the PGPR tests.
- Strong precipitin reactions occurred in homologous reactions in immunodiffusion test of *F. solani* and *F. oxysporum.* Western blot analyses using polyclonal antibody of *F. solani* and *F. oxysporum* revealed that the PAb could show different levels of homologous reactions with the antigens of *F. solani* and *F. oxysporum* respectively. Sharp and intense bands were produced on the nitrocellulose membrane after enzymatic reaction with NBT-BCIP. Efficacy of polyclonal antibodies raised against the mycelial proteins used as antigen source was further tested with the help of indirect immuno fluorescence of young mycelia of *F. solani* and *F. oxysporum.* The mycelia treated with PAbs and labeled with FITC showed apple green fluorescence.
- RAPD-PCR and Phylogenetic analysis of *Fusarium* and *Trichoderma* isolates was carried out. The genetic relatedness among isolates of *Fusarium* and *Trichoderma* were analyzed separately by random primers to generate reproducible polymorphisms. All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicated the genetic diversity of all isolates.

- The fungal and bacterial isolates designated as potential PGPF and PGPR were confirmed with the help of 16S rDNA sequences. The BLAST query of the 16S rDNA sequence of the isolates against GenBank database confirmed the identity of the isolate RHS/M/511 as *T. harzianum*, RHS/M 512 as *T. asperellum* and RMK03 as *Pseudomonas poae*. The sequences have been deposited in NCBI, GenBank database under the accession no. GQ995194 for *T. harzianum*, HQ265418 for T. *asperellum* and KJ917553 for *P. poae.*
- The BLAST query of the 16S rDNA sequence of the isolates against GenBank database confirmed the identity of the isolate RHS/M534 as *F. oxysporum* and RHS/M532 as. *F. solani*. The sequences have been deposited in NCBI, GenBank database under the accession no. KF952602 for *F. oxysporum* and KF952603 for *F. solani.*
- A multiple sequence alignment of ITS gene sequences of the above sequenced isolates was also conducted. Phylogenetic analysis of the isolates was carried out with the Ex-type strain sequences obtained from NCBI Genbank Database which showed maximum homology with their respective isolates.
- Abiotic stress was observed in mandarin plants in drought and flood condition. Enhanced changes in antioxidative enzymes such as peroxidase, catalase and ascorbate peroxidase was observed. Carotenoid showed a significant decrease during flood stress but increased in drought stress.
- Activation of defense responses in mandarin after inoculation of AMF and PGPF resulted in growth of plants along with increase in number of branches and leaves. Enhanced increase of defense enzymes after pathogen inoculation was observed especially after joint inoculation of AMF with PGPF respectively. Dual application of *Gi. gigantea* and *T. asperellum* induced additional isozyme in native PAGE. Induction of flower and fruit was also enhanced on application of the bioinoculants, *Trichoderma* and AMF.
- *P. poae* isolated from mandarin rhizosphere proved to be a potential PGPR on screening with other PGPR isolated from horticultural and plantation crops. Activation of defense responses in mandarin after inoculation of *Gi. gigantea* with *P. poae* resulted in growth of plants along with increase in number of branches and leaves. Enhanced increase of defense enzymes after pathogen inoculation was observed especially after joint inoculation. Immunological tests also confirmed the efficacy of joint inoculation of *Gi. gigantea* with *P. poae*

- One bacteria (MHB) was successfully isolated from *Gi. gigantea* spore originally obtained from mandarin root rhizosphere. The bacteria isolated was rod shaped and gram positive. The identity of the isolate was confirmed with the help of 16S rDNA sequences. The BLAST query of the 16S r DNA sequence of the isolates against GenBank database confirmed the identity of the isolate MHB as *B. mycoides*. The sequences have been deposited in NCBI, GenBank database under the accession no. KJ917554.
- Strong apple green fluorescence was observed in case of FITC and bright red in case of RITC in spores of AMF. The fluorescence was distributed throughout the spore wall. Fluorescence was more intense in wall layer of young spores.
- Strong apple green fluorescence was observed in the epidermal and mesophyll tissues in leaves and homogenously in cortical cells and epidermal cells in roots. Enhancement of chitinase was revealed in both leaves and roots following induction.
- The systematic response of induced resistance in mandarin plants using PAb of chitinase following successful colonization with AMF as well as treated with *T. asperellum* was observed through Transmission Electron Microscopy. Heavy deposition of gold particles was observed near the cell wall of inoculated roots.

BIBLIOGRAPHY

Abbott LK and Robson AD. Colonization of root system of subterranean clover by three species of vesicular arbuscular mycorrhizal fungi. *New. Phytol.* **96:** 275-281, 1984.

Adholeya A. Mycorrhiza: A friendly association with plants towards sustainable food production. J. Mycol. Plant. Pathol. **42(1):** 2012.

Akhtar MS and Siddiqui ZA. *Glomus intraradices, Pseudomonas alcaligenes, Bacillus pumilus* as effective biocontrol agents for the root-rot disease complex of chickpea (*Cicer arietinum* L.). *Journal of General Plant Pathology.* **74:** 53-60, 2008.

Akrami M, Golzary H and Ahmadzadeh M. Evaluation of different combinations of *Trichoderma* species for controlling *Fusarium* rot of lentil. *African Journal of Biotechnology.* **10 (14):** 2653-2658, 2011.

Al-Askar AA and Rashad YM. Arbuscular mycorrhizal fungi: A biocontrol agent against common bean *Fusarium* root rot disease. *Plant Pathology Journal*, **9(1):** 31-38, 2010.

Alba APC and Devay JE. Detection of cross-reactive antigens between *Phytophthora infestans* (Mont.) de Bary and *Solanum* species by indirect enzyme-linked immunosorbent assay. *Phytopathology*. **112:** 97-104, 1985.

Alizadeh H, Behboudi K, Ahmadzadeh M, Javan-Nikkhah M, Zamioudis C, Pieterse CMJ and Bakker PAHM. Induced systemic resistance in cucumber and *Arabidopsis thaliana* by the combination of *Trichoderma harzianum* Tr6 and *Pseudomonas* sp. Ps14. *Biol. Cont.* **65:** 14-23, 2013.

Allay S and Chakraborty BN. Activation of defense response of mandarin plants against *Fusarium* root rot disease using *Glomus mosseae* and *Trichoderma hamatum*. *Journal of mycology and plant pathology*. **40(4)**: 499-511, 2010.

Allay S and Chakraborty BN. Induction of resistance in *Citrus reticulata* against *Fusarium solani* by dual application of AMF and *Trichoderma asperellum*. *Int. J. of Bio-resource and Stress Management.* **4(4):** 588-592, 2013.

Allay S, De UK and Chakraborty BN. Root colonization of mandarin plants grown in orchards of Darjeeling hills and plains with arbuscular mycorrhizal fungi and their effects on plant growth. *NBU. J. Plant. Sci.* **6(1):** 41-47, 2012.

Allay S, Lama R, Chakraborty U and Chakraborty BN. Antioxidative responses of mandarin plants to water stress. *NBU. J. Plant. Sci.* **7(1):** 21-28, 2013.

Allen MF, Swenson W, Querejeta JI, Egerton-Warburton LM and Treseder KK. Ecology of mycorrhizae: a conceptual framework for complex interactions among plants and fungi. *Ann Rev Phytopathol*. **41:** 271–303, 2003.

Allen MF. Mycorrhizal Functioning: An Integrative Plant-Fungal Process. Routledge, NY: Chapman & Hall, pp. 534. 1992.

Al-Sadi AM, Al-Ghaithi AG, Al-Fahdi N and Al-Yahyai R. Characterization and Pathogenicity of Fungal Pathogens Associated with Root Diseases of Citrus in Oman. *Int. J. Agric. Biol*. **16:** 371− 376, 2014.

Amsellem Z, Zidack NK, Quimby Jr, PC and Gressel J. Long-term dry preservation of viable mycelia of two mycoherbicidal organisms. *Crop Prot.* **18:** 643-649, 1999.

Anand S and Jayaram R. Biocontrol potential of *Trichoderma* sp. against plant pathogens. *International Journal of Agricultural Sciences* **1(2):** 30-39, 2009.

Anjum AS, Xie X, Wang L, Saleem FM, Man C and Lei W. Morphological, physiological and biochemical responses of plants to drought stress. *African J. Agri. Research.* **6(9):** 2026-2032, 2011.

Anzlovar S, Serra MD, Dermastia M and Menestrina G. Membrane permeabilizing activity of pathogenesis-related protein lunusitin from flax seed. *Mol. Plant-Microbe Interact*. **11:** 610-617, 1998.

Arbona V, Iglesias, DJ, Jacas, Primo-Millo E, Talon M and Gomez-Cadenaz A. Hydrogel substrate amendment alleviates drought effects on young citrus plants. *Plant soil.* **270:** 73-82, 2005.

Asghar HN, Zahir ZA, Arshad M and Khalig A. Plant growth regulating substances in the rhizosphere: microbial production and functions. *Adv. Agron.* **62:** 146–151, 2002.

Ashraf M and Foolad MR. Roles of *Glycine betaine* and proline in improving plant abiotic stress resistance. *Environ. Exp. Bot.* **59:** 206–216, 2007.

Aspray TJ, Frey-Klett P, Jones JE, Whipps JM, Garbaye J and Bending GD. Mycorrhization helper bacteria: a case of specificity for altering ectomycorrhiza architecture but not ectomycorrhiza formation. *Mycorrhiza,* **16:** 533–541, 2006b

Aspray TJ, Jones EE, Whipps JM and Bending GD. Importance of mycorrhization helper bacteria cell density and metabolite localization for the *Pinus sylvestris-Lactarius rufus* symbiosis. *FEMS Microbiol. Ecol.*, **56:** 25–33, 2006a.

Aubert B. citrus greening disease, a serious limiting factor for Citriculture in Asian and Africa. Proc. Int. Soc. Citriculture. Vol. 2 pp.817-820, 1992.

Azcon-Aguilar C and Barea JM. Arbuscular mycorrhizas and biological control of soil-borne plant pathogens - An overview of the mechanisms involved. *Mycorrhiza* 6: 457-464, 1996.

Bais HP, Loyola Vargas VM, Flores HE and Vivanco JM. Root specific metabolism: the biology and biochemistry of underground organs. *In vitro Cell Dev Biol. Plant* **37:** 730–741, 2001.

Bais HP, Weir TL, Perry LG, Gilroy S and Vivanco JM. The Role of Root Exudates in Rhizosphere Interactions with Plants and Other Organisms. *Annu. Rev. Plant Biol.* **57:** 233-266, 2006.

Balestrini R and Bonfante P. The interface compartment in arbuscular mycorrhizae: a special type of plant cell wall? *Plant Biosyst*. **139:** 8–15, 2005.

Barea JM. Azcón-Aguilar C and Azcón R. Vesicular arbuscular mycorrhiza improve both symbiotic N_2 fixation and N uptake from soil as assessed with a ^{15}N technique under field conditions. *New Phytologist.* **106**:717-725, 1987.

Becard G, Douds DD and Pfeffer PE. Extensive *in vitro* hyphal growth of vesicular-arbuscular mychorrhizal fungi in the presence of CO2 and flavonols. *Appl. Environ. Microbiol.* **58:** 821–825, 1992.

Bending G. What are the mechanisms and specificity of mycorrhization helper bacteria? *New Phytol.*, **174:** 707–710, 2007.

Benhamou N and Nicole M. Cell biology of plant immunization against microbial infection: the potential of induced resistance in controlling plant diseases. *Plant Physiol Biochem* **37:** 703-719, 1999.

Bernier F and Berna A. Germins and germin-like proteins: Plant do-all proteins. But what do they do exactly? *Plant Physiol. Biochem*. **39:** 545-554, 2001.

Bertin C, Yang XH and Weston LA. The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil*. **256:** 67–83, 2003.

Besserer A, Puech-Pagès V, Kiefer P, Gomez-Roldan V and Jauneau A. Strigolactones Stimulate Arbuscular Mycorrhizal Fungi by Activating Mitochondria. PLoS Biol 4(7): e226. doi: 10.1371,2006.

Bethlenfalvay GJ and Linderman RG. Mycorrhizae in Sustainable Agriculture. St Paul, MN: *The American Phytopathological Society* Special Publication **54:** 124, 1992.

Bethlenfalvay J and Linderman RG. Mycorrhizae in Sustainable Agriculture. ASA Special Publication No. 54, (eds.) Madison, WI: ASA Special Publication No. 54, pp. 45-70, 1992.

Bhutia LP, Chakraborty BN and Chakraborty U. Management of charcoal stump rot disease using AMF and PGPR in temi tea estate, Sikkim. *J. Mycol. Plant. Pathol.* **42(1):** 1-12, 2012.

Bianciotto V and Bonfante P. Arbuscular mycorrhizal fungi: a specialized niche for rhizosphere and endocellular bacteria. Antonie van Leeuwenhoek, **81:** 365-371, 2002.

Bol JF, Linthorst HJM and Cornelissen BJC. Plant pathogenesis-related proteins induced by virus infection. *Annu. Rev. Phytopathol*. **28:** 113-138, 1990.

Boller T. and Mauch F. Colorimetric assay for chitinase. *Meth. Enzymol.* **161:** 430-435, 1998.

Boman BJ and Obreza TA. Fertigation nutrient sources and application considerations for citrus. Univ. of Fla. IFAS, Coop. Exten, 2002.

Bonfante-Fasolo P and Grippiolo R. Cytochemical and biochemical observations on the cell wall of the spore of *Glomus epigaeum*. *Protoplasm.* **123:** 140-151, 1984.

Bowen GD and Theodorou C. Interactions between bacteria and ectomycorrhizal fungi. *Soil Biology and Biochemistry,* **11:** 119–126, 1979.

Bowles DJ. Defense-related proteins in higher plants. *Annu. Rev. Biochem.* **59:** 873-907, 1990.

Brundrett M. Mycorrhizas in natural ecosystems. *Adv. Eco. Res.* **21:** 171-313, 1991.

Brundrett MC. Coevolution of roots and mycorrhizas of land plants. *New Phytol.* **154:** 275–304, 2002.

Bruns TD, White TJ and Talyor JW. Fungal molecular systematics. *Annu. Rev. Ecol. Sys.* **22:** 525-564, 1991.

Bryan GT, Daniels MJ and Osbourn AE. Comparison of fungi within the Gaeumannomyces-Phialophora complex by analysis of ribosomal DNA sequence. *Appl. Environ. Microbiol.* **61:** 681-689, 1995.

Buchel AS and Linthorst HJM. PR-1: A group of plant proteins induced upon pathogen infection. In: Pathogenesis-related proteins in plants. S.K. Datta and S. Muthukrishnan (eds.). CRC Press LLC, Boca Raton. **pp.** 21-47, 1999.

Bucher M. Functional biology of plant phosphate uptake at root and mycorrhizal interfaces. *New Phytol.* **173:** 11–26, 2007.

Budi SW, van Tuinen D, Martinotti G and Gianinazzi S. Isolation from the *Sorghum bicolor* mycorrhizosphere of a bacterium compatible with arbuscular mycorrhiza development and antagonistic towards soilborne fungal pathogens. *Appl. Environ. Microbiol.* **65:** 5148-5150, 1999.

Burr TJ and Caesar A. Beneficial plant bacteria. *Crit. Rev. Plant. Sci.* **2:**1–20, 1984.

Campillo ED and Lewis LN. Identification and kinetics of accumulation of proteins induced by ethylene in bean abscission zone. *Plant Physiol*. **98:** 955-961, 1992.

Caron M. Potential use of mycorhizae in control of soil-borne diseases. *Canadian Journal of Plant Pathology*. **11:** 177-179, 1989.

Chakraborty BN. Antigenic disparity. In: Singh, U.S., Heiss, W.M., Weber, D.J., (Eds.), Experimental and Conceptual Pathology India. Oxford and IBH publishing Company, 477-484, 1988.

Chakraborty BN and Saha A. Detection and cellular location of cross-reactive antigens shared by *Camellia sinensis* and *Bipolaris carbonum*. *Physiol. Mol. Plant. Pathol.* **44:** 403-416, 1994.

Chakraborty BN, Basu P, Das R, Saha, A and Chakraborty U. Detection of cross reactive antigen between *Pestalotiopsis theae* ant tea leaves and their cellular location. *Ann. Appl. Biol.* **207**: 11-21, 1995.

Chakraborty BN, Chakraborty U and Dey PL. Potential application of *Trichoderma* as biocontrol agents, their molecular characterization and diversity analysis. In: Plant diseases and their biological control (ed.) A. Singh, Aavishkar Publishers, Distributors, India, pp. 186-216, 2011.

Chakraborty BN, Chakraborty U, Barman BC, Bhutia LP and Ghosh PK. Induction of systemic resistance in tea plants against root rot pathogen upon field application of VAM, phosphate solubilizing fungus and bacterium. In: Rhizosphere Biotechology: Plant Growth Retrospect and Prospect. (Eds. Roy AK, Chakraborty BN, Mukadam DS and Rashmi) Scientific Publishers, Jodhpur, pp. 61-71, 2007.

Chakraborty BN, Chakraborty U, De U and Chakraborty AP. Biological control of sclerotial blight of tea using arbuscular mycorrizal fungus and plant growth promoting rhizobacterium. *Int. J. Tea. Sci.* **8(4):** 27-35, 2012.

Chakraborty BN, Chakraborty U, Saha A, Dey PL and Sunar K. Molecular characterization of *Trichoderma harzianum* isolated from soils of North Bengal based on rDNA markers and analysis of their PCR-RAPD profiles. *G. J. Biotech. Biochem.* **5:** 55-61, 2010.

Chakraborty BN, Chakraborty U, Sunar K and Dey PL. Harnessing beneficial microbial resources for crop improvement. In: Trends in Soil Microbial Ecology, pp. 175-201, 2014b.

Chakraborty BN, De UK and Allay S. Molecular detection of arbuscular mycorrhizal fungi and their application for plant health improvement. In: Biology of useful plants and microbes (Eds. Sen A), Narosa Publishing House Pvt. Ltd., pp. 13-31, 2014a.

Chakraborty U, Basnet M, Bhutia L and Chakraborty BN. Plant growth promoting activity of *Bacillus pumilus* and *Bacillus megaterium* from tea rhizosphere. In:*Proceedings of 2004 International Conference on O-Cha (tea) Culture and Science (ICOS),* Shizuoka, Japan, pp.102-105, 2004.

Chakraborty U, Chakraborty BN and Kapoor M. Changes in the level of peroxidase & Phenylalanine ammonia lyase in *Brassica napus* cultivars showing variable resistance to *Leptosphaeria maculans*. *Folia Microbiol.* **38:** 491-496, 1993.

Chakraborty U, Chakraborty BN, Allay S, De U and Chakraborty AP. Dual application of *Bacillus pumilus* and *Glomus mosseae* for improvement of health status of mandarin plants. *Acta. Hort.*, **892:** 215-229, 2011.

Chakraborty U, Chakraborty BN, Basnet M and Chakraborty AP. Evaluation of *Ochrobactrum anthropi* TRS-2 and its talc based formulation for enhancement of growth of tea plants and management of brown root rot disease. *J. Appl. Microbiol*., **107(2):** 625-634, 2009.

Chakraborty U, Pradhan D, Lama R, Pradhan B, De U and Chakraborty BN. Abiotic stress induced biochemical response in cereals and legumes and associated changes in arbuscular mycorrhizal population. In: *Microbial resources for crop improvement* (Eds. Chakraborty BN and Chakraborty U), Satish Serial Publishing House, New Delhi, pp.183-195, 2013.

Chandanie WA, Kubota M and Hyakumachi M. Interactions between the arbuscular mycorrhizal fungus *Glomus mosseae* and plant growth-promoting fungi and their significance for enhancing plant growth and suppressing damping-off of cucumber (*Cucumis sativus* L.). *Appl. Soil Ecology*. **41(3):** 336-341, 2009.

Chatterjee S, Sau GB, Sinha S and Mukherjee SK. Effect of co-inoculation of plant growth-promoting rhizobacteria on the growth of amaranth plants. *Arch. Agrn. Soil. Sci.* **58(12):** 1387-1397, 2012.

Chet I, Viterbo A, Brotman Y and Lousky T. Enhancement of plant disease resistance by biocontrol agent *Trichoderma*. Life Science. URL:http:// www.weizmann.ac.il/, 2006.

Clark RB. Arbuscular mycorrhizal adaptation, spore germination, root colonization, and host plant growth and mineral acquisition at low pH. *Plant and Soil*. **192(1):** 15-22, 1997.

Clausen J. Laboratory techniques in biochemistry and molecular biology. Vol 1, part-3, (ed. R. H. Burdon and P. H. Van Kinppenberg). pp: 64-65, 1988.

Colburn GC and Graham JH. Protection of citrus rootstocks against *Phytophthora* spp. with a hypovirulent isolate of *Phytophthora nicotianae. Phytopathology*. **97:** 958-963, 2007.

Cook RJ, Thomashow LS, Weller DM, Fujimoto D, Mazzola M, Bangera G and Kim D. Molecular mechanisms of defence by rhizobacteria against root disease. *Proc. Natl. Acad. Sci.* USA. **92:** 4197–4201, 1995.

Coughlan AP, Dalpe Y, Lapointe L and Piche Y. Soil pH induced changes in root colonization, diversity and reproduction of symbiotic Arbuscular Mycorrhizal fungi from healthy and declining maple forests. Can. J. Fores. Res. **30(10):** 1543-1554, 2000.

Dakora FD, Joseph CM and Phillips DA. Common bean root exudates contain elevated levels of daidzein and coumestrol in response to *Rhizobium* inoculation. *Mol. Plant-Microbe Interact*. **6:** 665–668, 1993.

Daniels BA and Skipper HD. Methods for the recovery and quantitative estimation of propagules from soil. In: *Methods and Principles of Mycorrhizal Research.* (Eds. Schenck NC), *The American Phytopathol. Soc.* pp. 29-36, 1982.

Darrah, P.R. The rhizosphere and plant nutrition: A quantitative approach. *Plant Soil*. **155/156:** 1–20, 1993.

Davis BJ. Disc electrophoresis II. Method and application of human serum proteins. *Annl. N. Y. Acad. Sci.***121**: 404-427, 1964.

Deene S, Garampalli R and Reddy CN. Influence of vesicular-arbuscular mycorrhiza (Glomus aggregatum) and Fusarium udum on plant vigour and wilt disease incidence of pigeon pea. *Phytomorphology*. **54:** 23-29, 2004.

Dehne HW. Interaction between vesicular-arbuscular mycorrhizal fungi and plant pathogens. *Phytopathology*. **72:** 1115-1119, 1982.

del Campillo E. and Lewis LN. Identification and Kinetics of Accumulation of Proteins Induced by Ethylene in Bean Abscission Zones. *Plant Physiol.* **98:** 955-961, 1992.

Dev N and Dawande AY. Biocontrol of soil borne pathogen *Rhizoctonia solani* using *Trichoderma* spp. and *Pseudomonas fluorescens*. *Asiatic J. Biotech. Res.* **1:** 39-44, 2010.

Deveau A, Palin B, Delaruelle C, Peter M Kohler A, Pierrat JC, Sarniguet A, Garbaye J Martin F and Frey-Klett P. The mycorrhiza helper *Pseudomonas fluorescens* BBc6R8 has a specific priming effect on the growth, morphology and gene expression of the ectomycorrhizal fungus *Laccaria bicolor* S238N. *New Phytol.* **175:** 743–755, 2007.

Dobbelaere S, Croonenberghs A, Thys A, Vande Broek A and Vanderleyden J. Photostimulatory effects of *Azospirillum brasilense* wild type and mutant strain altered in IAA production on wheat. *Pl. Soil*. **212:** 155-164, 1999.

Dodd JC. The role of arbuscular mycorrhizal fungi in agro-and natural ecossystems. *Outlook on Agriculture*. **29 (1):** 55-62, 2000.

Dohroo A and Sharma DR. Role of plant growth promoting rhizobacteria, arbuscular mycorrhizal fungi and their helper bacteria on growth parameters and root rot of apple. *World Journal of Science and Technology*, **2(12):** 35-38, 2012.

Dong X. NPR1, all things considered. *Curr Opin. Plant Biol*. **7:** 547-552, 2004.

Drew MC. Oxygen deficiency and root metabolism: injury and acclimation under hypoxia and anoxia. *Annu. Rev. Plant Physiol. Plant mol. Biol.* **48:** 223-250, 1997.

Dubey RC, Khare S, Kumar P, Dubey P and Maheshwari DK. Evaluation of diversity of *Bacilli* from chickpea rhizosphere by 16S ARDRA and assessment of their plant-growth-promoting attributes. *Arch. Phytopathol. Plant. Protect.,* 10.1080/03235408.2013.794528, 2013.

Duff SMG, Sarath G and Plaxton WC. The role of acid phosphatases in plant phosphorus metabolism. *Physiol. Plant.* **90:** 791–800, 1994.

Dugassa GD, Alton HV and Schonbeck F. Effects of arbuscular mycorrhiza (AM) on health of LINUM USITATISSIMUM L. infected by fungal pathogens. *Plant and Soil.* **185(2):** 173-182, 1996.

Duponnois R and Plenchette C. A mycorrhiza helper bacterium enhances ectomycorrhizal and endomycorrhizal symbiosis of Australian *Acacia* species. *Mycorrhiza.* **13:** 85–91, 2003.

Duponnois R, Garbaye J, Bouchard D and Churin JL. The fungus-specificity of mycorrhization helper bacteria (MHBs) used as an alternative to soil fumigation for ectomycorrhizal inoculation of bare-root Douglas-fir planting stocks with *Laccaria laccata*. *Plant Soil.* **157:** 257–262, 1993.

Duponnois R, Garbaye J. Mycorrhization helper bacteria associated with the Douglas fir–*Laccaria laccata* symbiosis: effects in aseptic and in glasshouse conditions. *Annales des Sciences Forestières.* **48:** 239–251, 1991.

Durn Estela L, Ploper LD, Ramallo JC, Grandi RAP, Giancoli gata CH and Azevedo JL. The foliar fungal endophytes of *Citrus limon* in Argentina. *Canadian Journal of Botany*. **83 (4):** 350, 2005.

Durrant WE and Dong X. Systemic acquired resistance. *Annu. Rev. Phytopathol.* **42:** 185–209. 2004.

Edreva A. Generation and scavenging of reactive oxygen species in chloroplasts: a submolecular approach. *Agr Ecosyst Environ.* **106:** 119-133, 2005.

Edreva A. Pathogenesis-related proteins: research in progress in the last 15 years. *Gen. Appl. Plant physiology*. **31(1-2):** 105-124, 2005.

Edreva A. Stress proteins of plants - PR(b)-proteins. *Sov. Plant Physiol*. **38:** 579-588, 1991.

Edreva AM. Induction of "pathogenesis - related" proteins in tobacco leaves by physiological (non-pathogenic) disorders. *J. Exp. Bot*. **41:** 701-703, 1990.

Edreva M. Plant stress proteins PRPs. *Fiziologia Rastenii*. **38:** 788–800, 1991.

El-Atrach F, Vierheilig H and Ocampo JA. Influence of nonhost plants on vesicular-arbuscular mycorrhizal infection of host plants and on spore germination. *Soil Biol. Biochem*. **21:** 161–163, 1989.

Elias KS and Safir GR. Hyphal elongation of *Glomus fasciculatus* in response to root exudates. *Appl. Environ. Microbiol*. **53:** 1928–1933, 1987.

El-Mohamedy RSR. Efficiency of different application methods of biocontrol agents and biocides in control of Fusarium root rot on some citrus rootstocks. *Arch.Phytopathol. Plant. Protect.* **42(9):** 819-828, 2009.

Estabrook EM and Yoder JI. Plant-plant communications: rhizosphere signaling between parasitic angiosperms and their hosts. *Plant Physiol*. **116:** 1–7, 1998.

Eyal Y, Meller Y, Lev Yadun S and Fluhr R. A basic-type PR-1 promotor directs ethylene responsiveness, vascular and abscission zones specific expression. *Plant J*. **4:** 25-234, 1993.

Fatima Z, Saleemi M, Zia M, Sultan T, Aslam M, Rehman RU and Chaudhary MF. Antifungal activity of plant growth-promoting rhizobacteria isolates against *Rhizoctonia solani* in wheat. *Afr. J. Biotechnol.* **8:**219–225, 2009.

Felsenstein J. Confidence limits on phylogenies: An approach using the bootstrap. *Evolution.* **39**:783-791, 1985.

Filion M, St-Arnaud M and Fortin JA. Direct interaction between the arbuscular mycorrhizal fungus *Glomus intraradices* and different rhizosphere microorganisms. *New Phytologist*. **141:** 525-533, 1999.

Filippi C, Bangnoli G, Citernesi S and Giovannetti M. Ultrastructural spatial distribution of bacteria associated with sporocarps of *Glomus mosseae*. *Symbiosis*. **24:** 1-12, 1998.

Fitter AH and Garbaye J. Interaction between mycorrhizal fungi and other soil organisms. In: *Management of Mycorrhizas in Agriculture, Horticulture and Forestry*. (Eds. Robson AD, Abbot LL and Malajckzuk N), Kluwer Academic Publishers, Netherlands. pp. 47-68, 1994.

Fraser RB. Evidence for the occurrence of the "pathogenesis-related proteins" in leaves of healthy tobacco plants during flowering. *Physiol. Plant Pathol*. **19:** 69-76, 1981.

Freitas SS and Vildoso CIA. Rizobactérias e promoção do crescimento e plantas cítricas. *R. Bras. Ci. Solo.* **28:** 987-994, 2004.

Frey-Klett P, Chavatte M, Clausse ML, Courrier S, Le-Roux C, Raaijmakers J, Martinotti M.G, Pierrat JC and Garbaye J. Ectomycorrhizal symbiosis affects functional diversity of rhizosphere fluorescent pseudomonads. *New Phytol.* **165:** 317–328, 2005.

Frey-Klett P, Garbaye J and Tarkka M. The mycorrhiza helper bacteria revisited. *New Phytol.* **176:** 22-36, 2007.

Friese CF and Allen MF. The spread of VA mycorrhizal fungal hyphae in the soil: Inoculum types and external hyphae architecture. *Mycological Research.* **92:**317-321, 1991.

Fry SC, Aldington S, Hetherington PR and Aitken J. Oligosaccharides as signals and substrates in the plant cell wall. *Plant Physiol.* **103:** 1–5, 1993.

Fujibe T, Watanabe K, Nakajima N, Ohashi Y, Mitsuhara I, Yamamoto KT and Takeuchi Y. Accumulation of pathogenesis-related proteins in tobacco leaves irradiated with UV-B. *J. Plant Res.* **113:** 387-394, 2000.

Gambrell RP and Patrick WH. Chemical and microbiological properties of anaerobic soils and sediments. In: *Plant Life in Anaerobic Environments*.375-423 (Hook, D.D. and Crawford, R.M.M., Eds.), *Ann Arbor Sci. Publ.*, Ann Arbor, MI, U.S.A, 1978.

Garbaye J and Bowen GD. Stimulation of mycorrhizal infection of *Pinus radiata* by some microorganisms associated with the mantle of ectomycorrhizas. *New Phytol.* **112:** 383-388, 1989.

Garbaye J. Helper bacteria: a new dimension to the mycorrhizal symbiosis. *New Phytol.* **128:** 197-210, 1994.

Garbeva P, Van JA and van Elsas JD. Microbial diversity in soil: selection microbial populations by plant and soil type and implications for disease suppressiveness. *Ann. Rev. Phytopathol.* **42:** 243-270, 2004.

Garcia-Sanchez F, Syvertsen, JP, Gimeno V, Botia P and Perez-Perez JG. Responses to flooding and drought stress by two citrus rootstock seedlings with different water-use efficiency. *Physiol Plant*. **130:** 532-542, 2007.

Gardner WK, Barber DA and Parbery DG. The acquisition of phosphorus by *Lupinus albus* L. III. The probabale mechanism by which phosphorus movement in the soil/root interface is enhanced. *Plant Soil*. **70:** 107–124, 1983.

Garmendia L, Goicoechea N and Aquirreolea J. Moderate drought influences the effect of arbuscular mycorrhizal fungi as biocontrol agents against Verticillium-induced wilt in pepper. Mycorrhizal. **15(5):** 345-56, 2005.

Gerdemann JW and Nicolson TH. Spores of mycorrhizal Endogone species extracted from soil by wet sieving and decanting. *Trans. B. Mycol. Soc.* **46:** 235-244, 1963.

Gerdemann JW. Vesicular arbuscular mycorrhizae. In: The Development and Function of Roots, (eds.). J.G. Torrey and D.T. Clarkson, Academic Press, New York. pp. 575-595, 1975.

Ghosh A, Das A, Pun KB, Kumar R and Meena R. Present status of Citrus tristeza virus infecting Citrus spp. in Darjeeling hills and its detection in different plant parts. *Phytoparasitica*. **42:** 381-386, 2014.

Gianinazzi-Pearson V, Branzanti B and Gianinazzi S. *In vitro* enhancement of spore germination and early hyphal growth of a vesicular-arbuscular mycorrhizal fungus by host root exudates and plant flavonoids. *Symbiosis*. **7:** 243–255, 1989.

Gibberd MR and Gibberd CPS. Effect of waterlogging and soil pH on the micro-distribution of naturalized annual legumes. *Aust. J. Agri. Res*. **48:** 223–229, 1997.

Gibberd MR, Gray JD, Cocks PS and Colmer TD. Waterlogging tolerance among a diverse range of *Trifolium* accessions is related *E.coli* pyrophosphatase lead to decreased vitality under oxygen deficiency. *Annals of Bot.* **96:** 717–726, 2001.

Gilbert GA, Knight JD, Allan DL and Vance CP. Acid phosphatase activity in phosphorus-deficient white lupin roots. *Plant Cell Environ*. **22:** 801–810, 1999.

Giovanetti M and Mosse B. An evaluation of techniques for measuring vesicular arbuscular mycorrhizal infection in roots. *New. Phytol.* **84:** 489-500, 1980.

Giri B and Mukerji KG. Mycorrhizal inoculant alleviates salinity stress in *Sesbania aegyptiaca* Pres and *S. grandiflora* Pres under field conditions: evidence for improved magnesium and decreased sodium uptake. *Mycorrhiza* (published online 23 Oct 2003)

Giri B, Kapoor R, Lata A, Mukerji KG. Preinoculation with arbuscular mycorrhizae helps *Acacia auriculiformis* in a degraded Indian wasteland soil. *Comm Soil Sci Plant Anal.* **35(1&2):** 193-204, 2003.

Glick B.R. The enhancement of plant growth by free living bacteria. *Can. J. Microbiol.* **41:** 109-117, 1995.

Glick BR, Patten CL, Holgin G and Penrose DM. Biochemical and genetic mechanisms used by plant growth promoting bacteria. Imperial College Press, London, pp.267, 1999.

Graham JH, Linderman RG and Menge JA. Development of external hyphae by different isolates of mycorrhizal *Glomus* spp. in relation to root colonization and growth of troyer citrange. *New Phytologist.* **91:**183-189, 1982.

Grant M and Lamb C. Systemic immunity. *Curr. Opin. Plant Biol.* **9:** 414-420, 2006.

Gray EJ and Smith DL. Intracellular and extracellular PGPR: commonalities and distinctions in the plant–bacterium signaling processes. *Soil. Biol. Biochem.* **37:** 395–412, 2005.

Gupta A, Gopal M and Tilak KV. Mechanism of plant growth promotion by rhizobacteria. *Ind. J. Exp. Biol.* **38:** 856–862, 2000.

Gurung A. ecology of vectors of Citrus tristwza virus and greening in Darjeeling district and its reations with the spread of citrus dieback.Ph.D. thesis (Unpublished), Bidhan Chandra Krishi Viwas vidyalaya, West Bengal, India, 1989.

Ham KS, Kauffmann S, Albersheim P and Darvill AG. A soybean pathogenesis-related protein with β-1,3-glucanase activity releases phytoalexin elicitor-active heatstable fragments from fungal wall. *Mol. Plant-Microbe Interact.* **4:** 545-552, 1991.

Hamdali H, Hafidi M, Virolle MJ and Ouhdouch Y. Rock phosphate solubilizing Actinomycetes: screening for plant growth promoting activities. *World. J. Microbiol. Biotechnol*. **24:** 2565–2575, 2008.

Hammerschmidt R and Kuc J. Induced resistance to disease in plants. Kluwer, Dordrecht, The Netherlands, 1995.

Hanfrey C, Fife M and Buchanan-Wollaston V. Leaf senescence in *Brassica napus*: expression of genes encoding pathogenesis-related proteins. *Plant Mol. Biol*. **30:** 597- 609, 1996.

Harley JL and Harley EL. A check-list of mycorrhiza in the British Flora. *New Phytol.* **105:** 1–102, 1987.

Harrier LA and Watson CA. The potential role of arbuscular mycorrhizal (AM) fungi in the bioprotection of plants against soil-borne pathogens in organic and/or other sustainable farming systems. *Pest Manag. Sci.* **60:** 149–157, 2004.

Harrison M. Biotrophic interfaces and nutrient transport in plant/fungal interfaces. *J. Exp. Bot.* **50:** 1013–1022, 1999.

Harrison MJ and Buuren MLV. A phosphate transporter from the mycorrhizal fungus *Glomus versiforme*. *Nature*. **378:** 626–629, 1995.

Harrison MJ, Dewbre GR and Liu J. A phosphate transporter from Medicago truncatula involved in the acquisition of phosphate released by arbuscular mycorrhizal fungi. *Plant Cell*. **14:** 2413–2429, 2002.

Harrison MJ. Molecular and cellular aspects of the arbuscular mycorrhizal symbiosis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **50:** 361–389, 1999.

Hartwig UA, Joseph CM and Phillips DA. Flavonoids released naturally from alfalfa seeds enhance growth of *Rhizobium meliloti*. *Plant Physiol*. **95:** 797–803, 1991.

Hawes MC, Brigham LA, Wen F, Woo HH and Zhu Y. Function of root border cells in plant health: Pioneers in the rhizosphere. *Annu. Rev. Phytopathol*. **36:** 311–327, 1998.

Hawes MC, Gunawardena U, Miyasaka S and Zhao X. The role of root border cells in plant defense. *Trends Plant Sci.* **5:** 128–133, 2000.

Hayat R, Ali S, Amara U, Khalid R and Ahmed I. Soil beneficial bacteria and their role in plant growth promotion: a review. *Ann. Microbiol*. **60:** 579–598, 2010.

Hibbett DS. Ribosomal RNA and fungal systematics. *Trans. Mycol. Soc.* **33:** 533-556, 1992.

Hildebrandt U, Janetta K and Bothe H. Towards growth of arbuscular mycorrhizal fungi independent of a plant host. *Appl. Environ. Microbiol*. **68:** 1919-1924, 2002.

Hooker JE, Jaizme-Vega M and Atkinson D. Biocontrol of plant pathogens using arbuscular mycorrhizal fungi. In: S. Gianinazzi and H. Schuepp (eds.). Impact of arbuscular mycorrhizas on sustainable agriculture and natural ecosystems. Basel: Birkhduser Verlag. pp. 191-200, 1994.

Horinouchi H, Muslim A and Hyakumachi M. Biocontrol of fusarium wilt of spinach by the plant growth promoting fungus *Fusarium equiseti* gf183. *Journal of Plant Pathology.* **92 (1):** 249-254, 2010.

Hwang SF, Chang KF and Chakravarty P. Effects of vesicular-arbuscular mycorrhizal fungi on the development of Verticillium and *Fusarium* wilts of alfalfa. *Plant Dis.* **76:** 239-243, 1992.

Ibarak Y. Evaluation of Photosynthetic Capacity in Micropropagated Plants by Image Analysis. In: S. Dutta Gupta and Y. Ibaraki (eds.). Plant Tissue Culture Engineering, Springer.15–29, 2006.

Ilan Chet, Ada Viterbo And Yariv Brotman. Plant Biocontrol by *Trichoderma* spp. 2006 (http://www.weizmann.ac.il/Biological_Chemistry/scientist/Chet/Chet.html)

Jalali BL and Jalali I. Mycorrhiza in plant disease control. In: D. K. Arora, B. Rai, K. G. Mukerji, and G. R. Knudsen (eds.). Handbook of Applied Mycology. Soil and Plants, Marcel Dekker, New York, Vol. 1 pp. 131-154, 1991.

Jaleel CA, Manivannan P, Lakshmanan GMA, Gomathinayagam M, Noctor G and Panneerselvam R. Alterations in morphological parameters and photosynthetic pigment responses of Catharanthus roseus under soil water deficits. *Colloids Surf. B: Biointerfaces*. **61:**298–303, 2008.

Jaleel CA, Manivannan P, Sankar B, Kishorekumar A, Gopi R, Somasundaram R and Panneerselvam R. *Pseudomonas fluorescens* enhances biomass yield and ajmalicine production in *Catharanthus roseus* under water deficit stress. *Colloids Surf. B: Biointerfaces* 60, 7-11, 2007.

Javot H, Penmetsa RV, Terzaghi N, Cook DR and Harrison MJ. A *Medicago truncatula* phosphate transporter indispensable for the arbuscular mycorrhizal symbiosis. *Proc. Natl. Acad. Sci.* USA **104:** 1720–1725, 2007.

Javot H, Pumplin N and Harrison MJ. Phosphate in the arbuscular mycorrhizal symbiosis: transport properties and regulatory roles. *Plant Cell Environ.* **30:** 310–322, 2007.

Jones DL and Darrah PR. Role of root derived organic acids in the mobilization of nutrients from the rhizosphere. *Plant Soil*. **166:** 247–257, 1994

Karagiannidis N, Bletsos F and Stavropoulos N. Effect of *Verticillium* wilt (*Verticillium dahliae* Kleb.) and mycorrhiza (*Glomus mosseae*) on root colonization, growth and nutrient uptake in tomato and eggplant seedlings. *Scientia Horticulturae*. **94(1-2):** 145-156, 2002.

Kauffmann S, Legrand M, Geoffroy P and Fritig B. Biological function of "pathogenesis- related" proteins: four PR proteins of tobacco have β-1,3-glucanase activity. *EMBO J.* **6:** 3209-3212, 1987.

Kean S, Soytong K and To-anun C. Application of biological fungicides to control citrus root rot under field condition in Cambodia. *J. Agricultural. Technology*. **6(2):** 219-230, 2010.

Keller S, Schneider K, Sussmuth RD. Structure elucidation of auxofuran, a metabolite involved in stimulating growth of fly agaric, produced by the mycorrhiza helper bacterium *Streptomyces* AcH 505. *Journal of Antibiotics (Tokyo)* **59:** 801–803, 2006.

Kessler A and Baldwin IT. Plant responses to insect herbivory: the emerging molecular analysis. *Annual Review of Plant Pathology.* **53:**299-328, 2002.

Khan AG, Kuek C, Chaudhry TM, Khoo CS and Hayes WJ. Plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation. *Chemosphere*. **41:**197–207, 2000.

Khan AG. Role of soil microbes in the rhizosphere of plants growing on trace metal contaminated soils in phytoremediation. *J. Trace. Elem. Med. Biol.* **18:** 355–364, 2005.

Kim SG, Khan Z, Jeon YH and Kim YH. Inhibitory effect of *Paenibacillus polymyxa* GBR-462 on *Phytophthora capsici* causing Phytophthora Blight in Chili Pepper. *J. Phytopathol*. **157:** 329–337, 2009.

Kitagawa T, Sakamoto Y, Furumi K and Ogura H. Novel enzyme immunoassays for specific detection of *Fusarium oxysporum* f.sp. *cucumerinum* and for general detection various *Fusarium* species. *Phytopathology*. **79:** 162-165, 1989.

Kloepper JW and Schroth MN. Plant growth promoting rhizobacteria on radishes. IV. *International Conference on Plant Pathogenic Bacteria*. Angers. France. **2:** 879-882, 1978.

Kloepper JW. Plant growth promoting rhizobacterial as biological control agents. In: Soil Microbial Ecology- Applications in Agricultural and Environmental Management (F. Metting, Jr., editor). pp. 255-274. Marcel Dekker, New York, 1993.

Knudsen D and Beegle D. Recommended phosphorous tests. In: Recommended chemical soil tests procedures for the north central region. WC Dahnke(ed) Bull North Dakota Agric Exp Stn. North Dakota, USA No. **499**: 122-15, 1988.

Kobayashi K, Laura G, Ana V and Jorge, y Cozzi. Suppressive effects of antagonistic bacteria and metabolites on a pathogenic *Rhizoctonia solani* strain. Increased production in a specific medium. *Rev. Invest. Agrop*. **29**: 63-76, 2000.

Kobra N, Jalil K and Youbert G. Effects of Three GLOMUS SPECIES as Biocontrol Agents Against Verticillium-Induced Wilt in Cotton. *Journal of Plant Protection Research.* **49(2):** 185-189, 2009.

Kombrink E, Ancillo G, Buchter R, Dietrich J, Hoegen E, Ponath Y, Schmelzer E, Stromberg A and Wegener S. The role of chitinases in plant defense and plant development. In: 6th International Workshop on PR-proteins. Spa, Belgium. Book of abstracts, pp. 11, 2001.

Koske RE. Evidence for a volatile attractant from plant roots affecting germ tubes of a VA mycorrhizal fungus. *Trans Brit Mycol Soc*. **79:** 305–310, 1982.

Kowalski S and Wojnowski L. Armillaria root rot dynamics in the experimental plantation with Scots pine (*Pinus sylvestris* L.) seedlings non-mycorrhized and mycorrhized with the fungi *Hebeloma crustuliniforme* and *Laccaria bicolor*. *Sylwan*. **153(1):**31-38, 2009.

Kragh KM, Hendriks T, Jong AJD, Schiavo FL, Bucherna N, Hojrup P, Mikkelsen JD and Vries SCD. Characterization of chitinases able to rescue somatic embryos of the temperature-sensitive carrot variant *ts11*. *Plant Mol. Biol*. **31:** 631-645, 1996.

Krishna KR and Bagyaraj DJ. Role of vesicular arbuscular mycorrhiza in the uptake of micronutrient by groundnut plants. *Curr. Res.* **20:** 173–175, 1991.

Kucuk C and Kivane M. Isolation of *Trichoderma* spp. and determination of their antifungal, biochemical and physiological features. *Turk J. Biol*. **27:** 247-253, 2003.

Laemmli UK. Cleavage of structural proteins during assembly of the head of bacteriophage T4. *Nature.* **227:** 680-685, 1970.

Lagrimini LM, Burkhart W, Moyer M and Rothstein S. Molecular cloning of complementary DNA encoding the lignin-forming peroxidase from tobacco: molecular analysis and tissue-specific expression. *Proc. Natl. Acad. Sci.* USA, **84:** 7542-7546, 1987.

Lama R and Chakraborty U. Polyethylene glycol induced water stress in maize seedlings and evaluation of antioxidant defense mechanisms. *NBU J. Plant Sci*. **6(1):** 35-40, 2012.

Lange L, Heide M and Olson LW. Serological detection of *Plasmodiophora brassicae* by dot immunobinding and visualization of the serological reaction by scanning electron microscopy. *Phytopathology*. **79**: 1066-1071, 1989.

Lawrence CB, Singh NP, Qiu J, Gardner RG and Tuzun S. Constitutive hydrolytic enzymes are associated with polygenic resistance of tomato to *Alternaria solani* and may function as an elicitor release mechanism. *Physiol. Mol. Plant Pathol.* **57:** 211-220, 2000.

Legrand M, Kauffmann S, Geoffroy P and Fritig B. Biological function of pathogenesis-related proteins: four tobacco pathogenesis-related proteins are chitinases. *Proc. Natl. Acad. Sci.* USA, **84:** 6750-6754, 1987.

Li WH and Graur D. Fundamentals of molecular evolution. *Sinauer Associates Inc.* Sunderland, Massachsetts, 1991.

Lieckfiledt E, Samules GJ, Helgard I, Nirenderg and Petrini O. A morphological and molecular perspectives of *T. viride* is it one or two species? *Appl. Environ. Microbiol.* **65(6):**2418-2428, 1999.

Linderman RG and Paulitz TC. Mycorrhizal-rhizobacterial interactions. In: D. Hornby (ed.). Biological control of soil-borne plant pathogens. Wallingford: CAB International, pp. 261-283, 1990.

Linderman RG. Effects of mycorrhizas on plant tolerance to diseases. In: Y. Kapulnik and D. D. Douds. (eds.). Arbuscular Mycorrhizas: Molecular Biology and Physiology. Kluwer Press, pp. 345-365, 2000.

Linderman RG. Role of VAM fungi in biocontrol. In: F. L. Pfleger and R. G. Linderman (eds.). Mycorrhizae and Plant Health., APS Press, St. Paul, MN, pp. 1-26, 1994.

Linderman RG. Mycorrhizal interactions with the rhizosphere microflora: the mycorrhizosphere effect. *Phytopathologist* **78:** 366–371, 1988.

Liu RJ. Effect of vesicular-arbuscular mycorrhizal fungi on verticillium wilt of cotton. *Mycorrhiza.* **5(4):** 293-297, 1995.

Lowe RH and Evans HJ. Carbon dioxide requirement for growth of legume nodule bacteria. *Soil Sci.* **94:** 351–356, 1962.

Lowry O H, Rosebrough NJ, Farr AL and Randall R J. Protein measurement with folin phenol reagent. *J. Biol. Chem.* **193:** 265-275, 1951.

Lu Z, Tombolini R, Woo S, Zeilinger S, Lorito M, Jansson JK. In vivo study of *Trichoderma*–pathogen–plant interactions, using constitutive and inducible green fluorescent protein reporter systems. *Appl. Environ. Microbiol.* **70:** 3073–3081, 2004.

Lucy M, Reed E and Glick BR. Applications of free living plant growth-promoting rhizobacteria. *Rev. Antonie. Van. Leeu.* **86:**1–25, 2004.

Lugtenberg BJJ, Dekkers L and Bloemberg GV. Molecular determinants of rhizosphere colonization by *Pseudomonas*. *Annual Review of Phytopathology.* **39:** 461-490, 2001.

Lumsden RD, Lewis JA and Fravel DR. Formulation and delivery of bio-control agents for use against soil-borne plant pathogens. In: Biorational Pest Control Agents Formulation and Delivery. Eds. Hall F.R., Bary J.W., American Chemical Society, Washington, DC, USA, pp. 166-182, 1995.

M'Piga P, Belanger RR, Paulitz TC and Benhamou N. Increased resistance to *Fusarium oxysporium* f.sp. *radicis- lycopersici* in tomato plants treated with the endophytic bacterium *Pseudomonas fluorescens* strain 6328. *Physiology and Molecular Plant Pathology.* **50:** 301-320, 1997.

Mahadevan A and Sridhar R. Methods in physiological plant pathology. 2nd Ed. Sivakami Publ. India, 1982.

Maldonado AM, Doerner P, Dixon RA, Lamb CJ, Cameron RK. A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. *Nature*, **419:** 399-403, 2002.

Mansoori M, Heydari A, Hassanzadeh N, Rezaee S and Laleh Naraghi. Evaluation of *Pseudomonas* and *Bacillus* bacterial antagonists for biological control of cotton *Verticillium* wilt disease. *J. Plant. Pathol. Rev.* **53(2):** DOI: 10.2478/0023, 2013.

Marschner H and Dell B. Nutrient uptake in mycorrhizal symbiosis. *Plant and Soil.* **159:** 89–102, 1994.

Marschner H. Mineral Nutrition of Higher Plants. 2nd Edn. Academic Press, London. 889, 1995.

Mart´ınez-Medina A, Pascual JA, Lloret E and Roldan A. Interactions between arbuscular mycorrhizal fungi and *Trichoderma harzianum* and their effects on Fusarium wilt in melon plants grown in seedling nurseries. *J. Sci. Food Agric.* **89:** 1843–1850, 2009.

Mathivanan N, Prabhavathy VR and Vijayanandraj VR. Application of talc formulations of *Pseudomonas fluorescens* Migula and *Trichoderma viride* Pers.ex S.F. Gray decrease the sheath blight disease and enhance the plant growth and yield in rice. *J. Phytopathol*. **153:** 697-701, 2005.

McDowell JM and Dangl JL. Signal transduction in the plant immune response.*Trends Biochem Sci.* **25(2):**79-82, 2000.

Mehlich A. Mehlich 3 soil test extractant: A modification of the Mehlich 2 extractant. *Commun. Soil. Sci. Plant. Anal.* **15:** 1409-1416, 1984.

Mihaela, E and Mioara, A. Microscopical examination of plant reaction in case of infection with *Trichoderma* and *Mycorrhizal* fungi. *Roum. Biotechnol. Lett.* **13(6):** 13-19, 2008.

Minaxi SJ. Disease suppression and crop improvement in moong beans (*Vigna radiata*) through *Pseudomonas* and *Burkholderia* strains isolated from semi arid region of Rajasthan. *BioControl.* **55(6):** 799-810, 2010.

Misra N, Gupta G and Jha PN. Assessment of mineral phosphate-solubilizing properties and molecular characterization of zinc-tolerant bacteria. *J. Basic. Microbiol*. **52:** 549-558, 2012.

Montealegre J, Valderrama L, Herrera R, Besoain X and Perez LM. Biocontrol capacity of wild and mutant *T. harzianum* (Rifai) strains on *R. solani*. 618: Effect of temperature and soil type during storage. *Electronic Journal of Biotechnology* **12(4):** 1-11, 2009.

Moorby J and Besford RT. Mineral nutrition and growth. In: A. Lauchli and R.L. Bieleski (eds.). Encyclopedia of Plant Physiology New Series Vol. 15 B: Iorganic Plant Nutrition. Springer-Verlag, Berlin, pp. 481–515, 1983.

Moricca S, Ragazzi A, Kasuga T and Mitchelson KR. Detection of *Fusarium oxysporum* f. sp. *vasinfectum* in cotton tissue by polymerase chain reaction. *Plant. Pathol.* **47:** 486-494, 1998.

Morsey EM, Abdel-Kawi KA and Khalil MNA. Efficiency of *Trichoderma viride* and *Bacillus subtilis* as Biocontrol Agents against *Fusarium solani* on Tomato Plants. *Egypt. J. Phytopathol.* **37(1):** 47-57, 2009

Morton JB and Benny GL. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glomineae and Gigasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. *Mycotaxon.* **37**:471-491, 1990.

Morton JB. Evolutionary relationships among arbuscular mycorrhizal fungi in the Endogonaceae. *Mycologia*. **82**:192-207, 1990.

Mosse, B. The establishment of vesicular-arbuscular mycorrhiza under aseptic conditions. *J. Gen. Microbiol.* **27:** 509-520, 1962.

Moukahmedov R, Hu X, Nazar RN and Robb J. Use of polymerase chain reaction-amplified ribosomal intergenic sequence for diagnosis of *Verticillium tricorpus*. *Phytopathology.* **84:** 256-259, 1994.

Mukherjee PK, Verma A and Latha J. PCR fingerprinting of some *Trichoderma* isolates from two Indian type culture collection- a need for re-identification of these economically importance fungi. *Science. Correspondence.* **83(4):** 372-374, 2002.

Mulaw TB, Kubicek CP and Druzhinina IS. The rhizosphere of *Coffea arabica* in its native highland forests of Ethiopia is associated with a distinguished diversity of *Trichoderma. Diversity.* 2, 527–549 10.1111/j.1462-2920.2008.01736.x, 2010.

Muthumeenakshi S, Mills PR, Brown AE and Seaby DA. Interspecific molecular variation among *T. harzianum* isolates colonizing mushroom compost in the British Isles. *Microbiol. Reading.* **140(4):**769-777, 1994.

Nagahashi G and Douds DD. Partial separation of root exudates components and their effects upon the growth of germinated spores of AM fungi. *Mycol. Res*. **104:** 1453–1464, 2000.

Narayanasamy P. Biological Disease Management Systems for Agricultural Crops. In: *Progress in Biological Control.* **16:**189-235, 2013.

Nardi S, Concheri G, Pizzeghello D, Sturaro A, Rella R and Parvoli G. Soil organic matter mobilization by root exudates. *Chemosphere*. **5:** 653–658, 2000.

Navazio L, Baldan B, Moscatiello R, Zuppini A, Woo SL, Mariani P, Lorito M. Calcium-mediated perception and defense responses activated in plant cells by metabolite mixtures secreted by the biocontrol fungus *Trichoderma atroviride*. *BMC Plant Biol.* **7:**41, 2007.

Nazar RN, Hu X, Schmidt J, Culham D and Robb J. Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of *Verticillium* wilt pathogens. *Physiol. Mol. Plant. Pathol.* **39:** 1-11, 1991.

Neda H, Halasz K, Posa T, Peter G, Hrotko K, Gaspar L and Lukacs N. Diversity of endophytic fungi isolated from cherry (*Prunus avium). Journal of Horticulture, Forestry and Biotechnology*. **15(2):** 1- 6, 2011.

Neeraj and Singh, K. Impact of AM fungi along with Pseudomonas fluorescens and mustard oil cake in controlling root-rot of French bean. *Mycorrhizal News.* **21(1):** 12-14, 2009.

Neha D and Dawande AY. Biocontrol of soil borne plant pathogen *Rhiozoctonia solani* using *Trichoderma* spp. and *Pseudomonas fluorescens*. *Asiatic J. Biotech. Res.* **01:** 39-44, 2010.

Nemec S. Characteristics of *Furasium soloni* infected pioneer roots on blight diseased ad healthy *Citrus*. Proc. Soil and Crop. Sci. Fla **43:**177-183, 1984.

Oba H, Tawaraya K and Wagatsuma T. Inhibition of presymbiotic hyphal growth of the arbuscular mycorrhizal fungus *Gigaspora margarita* by root exudates of *Lupinus* spp. *Soil Sci. Plant Nutr*. **48:** 117–120, 2002.

Oberson A, Friesen DK, Rao IM, Buhler S and Frossard E. Phosphorus transformations in an oxisol under contrasting land-use system: The role of the microbial biomass. *Plant. Soil*. **237:** 197-210, 2001.

Orak AB and Demir S. Effects of arbuscular mycorrhizal fungi and different phosphorus doses against cotton wilt caused *Verticillium dahlia* Kleb. *Plant Pathology Journal*. pp. 1-7, 2011.

Ospina MD, Royse DJ, Chen X and Romaine P. Molecular Phylogenetic analysis of biological control strains of *T. harzianum* and other biotypes of *Trichoderma* spp. associated with mushroom green mold. *Phytopathology*. **89(4):** 313-317, 1999.

Ouchterlony O. Immunodiffusion and immunoelectrophoresis. In: D.M. Weir (ed.). *Handbook of Experimental Immunology.* Blackwell Sci. Pub., Oxford & Edinburgh. pp.655, 1967.

Pan SQ, Ye XS and Kuc J. A technique for detection of chitinase,β-1,3 glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis or isoelectric focussing. *Phytopathology*. **81**: 970-974, 1991.

Pandey A, Soccol CR and Mitchell D. New developments in solid state fermentation: 1-Bioprocesses and products. *Process Biochem.* **35:** 1153-1169, 2000.

Pandey A, Soccol CR, Rodriguez-Leon JA and Nigam P. Factors that influence on solid state fermentation In: Pandy, A. (ed.) Solid state fermentation in biotechnology; fundamentals and applications. New Delhi Asiatech Publishers Inc. pp. 21-29, 2001.

Pandey AK and Upadhyay VB. Impact of refrigeration of eggs and pre-refrigeration period on the weight of shell of *Bombyx mori* L. *J Adv Zool.* **21(2):** 98-101, 2000.

Park CJ, JM A, Shin YC, Kim KJ, Lee BJ and Paek KH. Molecular characterization of pepper germin-like protein as the novel PR-16 family of pathogenesis-related proteins isolated during the resistance response to viral and bacterial infection. *Planta.* **219:** 797-806, 2004(a).

Park CJ, Kim KJ, Shin R, Park JM, Shin YC and Peak KH. Pathogenesis-related protein 10 isolated from hot pepper functions as a ribonuclease in an antiviral pathway. *Plant J*. **37:** 186-198, 2004(b).

Parveen M, Hasan MK, Takahashi J, Murata Y, Kitagawa E, Kodama O and Iwahashi H. Response of *Saccharomyces cerevisiae* to a monoterpene: evaluation of antifungal potential by DNA microarray analysis. *J Antimicrob Chemother.* **54(1):** 46-55, 2004.

Paulo L. *Penicillium digitatum*, *Penicillium italicum* (Green mould, Blue mould). In: Post harvest decay: Control strategies (Eds. Bautista-Banos S), Academic Press, Elsevier, pp. 45-88, 2014.

Peng DL, Shandong, Wang J, Chen C and Zhou M. Integrated biological and chemical control of rice sheath blight by *Bacillus subtilis* NJ-18 and jinggangmycin. DOI 10.1002 ps. 3551, 2013.

Penninsi E. The secret life of fungi. *Science.* **304:** 1620-1622, 2004.

Phillips DA, Joseph CM, Yang GP, Martinez-Romero E, Sanborn JR and Volpin H. Identification of lumichrome as a *Sinorhizobium* enhancer of alfalfa root respiration and shoot growth. *Proc. Natl. Acad. Sci.* USA **22:** 12275–12280, 1999.

Phillips JM and Hayman DS. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. *Trans. British. Mycol. Soc.* **55:** 157-160, 1970.

Pikovskaya RE. Solubilisation of phosphorous in soil in connection with vital activity of some microbial species. *Microbiologia*. **17**:362-370, 1948.

Pinior A, Wyss U, Piche Y and Vierheilig H. Plants colonized by AM fungi regulate further root colonization by AM fungi through altered root exudation. *Can. J. Bot.* **77:** 891–897, 1999.

Poole EJ, Bending GD, Whipps JM and Read DJ. Bacteria associated with *Pinus sylvestris* _ *Lactarius rufus* ectomycorrhizas and their effects on mycorrhiza formation *in vitro*. *New Phytol.* **151:**743-751, 2001.

Porter WM, Robson AD and Abbot LK. Factors controlling the distribution of vesicular-arbuscular mycorrhizal fungi in relation to soil pH. *Journal of Applied Ecology*. **24(2):** 663-672, 1987.

Pozo MJ, Cordier C, Dumas-Gaudot E, Gianinazzi S, Barea JM and Azcon-Aguilar C. Localized versus systemic effect of arbuscular mycorrhizal fungi on defence responses to *Phytophthora* infection in tomato plants. *Journal of Experimental Botany*, **53 (368):** 525-534, 2001.

Probanza A, Mateos JL, Garcia JAL, Ramos B, Felipe MR and Mafiero FJG. Effects of Inoculation with PGPR *Bacillus* and *Pisolithus tinctorius* on *Pinus pinea* L. growth, bacterial rhizosphere colonization, and mycorrhizal infection. *Microbial Ecol.* **41:** 140-148, 2001.

Pruthi HS and Mani MS. *Imp. Conc.Agriic. Res.Aci. Monoger*, **16**:42, 1945.

Purkayastha RP. Phyto-immunology an emerging discipline of plant science. *Everyman's. Sci.* **29(2):** 41-44, 1994.

Rajasab AH and Kallurmath RS. Rhizosphere mycoflora of Onion (*Allium cepa* L.) crop with special reference to *Aspergillus niger* V. Tieghem. *Journal of Mycology and Plant Pathology.* **30(3):** 339-342, 2000.

Rambelli A. The rhizosphere of mycorrhizae. In: MarksGC, KozlowskiTT, eds. *Ectomycorrhizae, their ecology and physiology*. New York, USA: Academic Press, 299–349, 1973.

Rausch C, Daram P, Brunner S, Jansa J, Laloi M, Leggewie G, Amrhein N and Bucher MA. phosphate transporter expressed in arbuscule-containing cells in potato. *Nature.* **414:** 462–470, 2001.

Recourt K, Schripsema J, Kijne JW, Van Brussel AAN and Lugtenberg BJJ. Inoculation of *Vicia sativa* subsp. *nigra* roots with *Rhizobium leguminosarum* biovar *viciae* results in release of *nod* gene activating flavanones and chalcones. *Plant Mol. Biol.* **16:** 841–852, 1991.

Reddy DM, Mohan BK, Nataraja S, Krishnappa M and Abhilash M. Isolation and molecular characterization of *Bacillus megaterium* isolated from different agro climatic zones of Karnataka and its effect on seed germination and plant growth of *Sesamum indicum*. *Res. J. Pharmaceu, Biolog.Chem.Sci.* **1(3):**614-625, 2010.

Reddy MM and Gasber E D. *Bot. Gaz.* pp. 132-158, 1973.

Redecker D, Morton JB and Bruns TD. Ancestral lineages of arbuscular mycorrhizal fungi (Glomales). *Mol. Phylogen. Evol*. **14:** 276–284, 2000.

Rehman A, Rehman A, Javed UN, Malik AU and Mehboob S. Toxin production by *Fusarium solani* from declining citrus plants and its management. *African Journal of Biotechnology*. **11(9):** 2199-2203, 2012.

Remy W, Taylor TN, Hass H, Kerp H. Four hundred-million-year-old vesicular arbuscular mycorrhizae. *Proc. Natl. Acad. Sci., USA* **91:**11841–11843, 1994.

Rillig MC and Mummey DL. *Tansley review: Mycorrhizas and soil structure*. *New Phytologist*, 2006.

Rillig MC. *Arbuscular mycorrhizae, glomalin, and soil aggregation. Can. J.Soil. Sci.* **84**:355-363, 2004.

Roberts WK and Selitrennikoff CP. Plant and bacterial chitinases differ in antifungal activity. *J. Gen. Microbiol.* **134:** 169-176, 1988.

Roesti D, Ineichen K, Braissant O, Redecker D, Wiemken A and Aragno M. Bacteria associated with spores of Arbuscular Mycorrhizal fungi *Glomus geosporum* and *Glomus constrictum. Appl. Environ. Microbiol.* **71:** 6673-6679, 2005.

Rovira AD. Plant root exudates. *Bot. Rev.* **35:** 35–57, 1969.

Run-Jin Liu. Effect of vesicular-arbuscular mycorrhizal fungi on verticillium wilt of cotton. *Mycorrhiza.* **5(4):** 293-297, 1995.

Saitou N. and Nei M. The neighbor-joining method: A new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution.* **4**:406-425, 1987.

Saldajeno MGB and Hyakumachi M. The plant growth-promoting fungus *FUSARIUM EQUISETI* and the arbuscular mycorrhizal fungus *GLOMUS MOSSEAE* stimulate plant growth and reduce severity of anthracnose and damping-off diseases in cucumber (*CUCUMIS SATIVUS*) seedlings. *Annals of Applied Biology*. **159 (1):** 28–40, 2011.

Sampo S, Massa N, Cantamessa S, D´Agostino U, Bosco D, Marzachi C and Berta G. Effects of two arbuscular mycorrhizal fungi on phytoplasma infection in the model plant *Chrysanthemum carinatum*. *Agricultural and food science*. **21:** 39-51, 2012.

Schaller A, Roy P and Amrhein N. Salicylic acid-independent induction of pathogenesis-related gene expression by fusicoccin. *Planta.* **210:** 599-606, 2000.

Schenck NC and Perz Y. Manual for the identification of VA Mycorrhizal Fungi. (Eds.3rd), Synergistic Publication, USA, 1990.

Schilling AG, Möller EM and Geiger HH. Polymerase chain reaction-based assays for specific detection of *Fusarium culmorum, F. graminearum,* and *F. avenaceum. Phytopathology.* **86:** 515-522, 1996.

Schreiner RP and Koide RT. Antifungal compounds from the roots of mycotrophic and non-mycotrophic plant species. *New Phytol.* **123:** 99–105, 1993.

Schu¨bler A, Schwarzott D and Walker C. A new fungal phylum, the Glomeromycota: phylogeny and evolution. *Mycological Research,* **105:**1413–1421, 2001.

Schuster A and Schmoll M. Biology and biotechnology of *Trichoderma. Appl. Microbiol. Biotechnol*. **87:** 787-799, 2010.

Schwyn B and Neiland JB. Universal chemical assay for the detection and determination of siderophores. *Anal. Biochem***. 160**: 47-56, 1987.

Selitrennikoff CP. Antifungal proteins. *Appl. Env. Microbiol*. **67:** 2883-2894, 2001.

Semikhatova OA. Maintenance Respiration and Plant Adaptation, Bot. Zh. (St. Petersburg) **85:**15–32, 2000.

Sharma P, Sharma M, Raja M and Shanmugam,V. Status of *Trichoderma* research in India: A review. *Indian Phytopath*. **67(1):** 1-19, 2014.

Shen D. Microbial diversity and application of microbial products for agricultural purposes in China. *Agric. Ecosyst. Environ*. **62:** 237–245, 1997.

Shetty KG, Hetrick BAD, Figge DAH and Schwab AP. Effects of mycorrhizae and other soil microbes on revegetation of heavy metal contaminated mine spoil. *Environ. Pollut.* **86:** 181–188, 1994.

Shilev S, López AF, Prieto MS and Puebla EDS. Induced protein profile changes in arsenate tolerant and sensitive *Pseudomonas fluorescens* strains. *J. Environ. Eng. Land. Manag.* **15(4):** 221-226, 2007.

Shivankar VJ, Rao CN and Shyam S. Integrated Pest management in *Citrus* national symposium on Citriculture: A Road Map, Indian Society of Citriculture, Nagpur, Maharashtra, 2006.

Shoresh M, Harman GE, Mastouri F. Induced systemic resistance and plant responses to fungal biocontrol agents. *Annu Rev. Phytopathol*. **48:** 21-43, 2010.

Sid Ahmed A, Ezziyyani M, Sanchez CP and Candela ME. Effect of chitin on biological control activity of *Bacillus spp*. and *Trichoderma harzianum* against root rot disease in pepper (*Capsicum annuum*) plants. *Eur. J. Plant. Pathol.* **109:** 633-637, 2003.

Siddiqui ZA and Mahmood I. Biological control of *Heterodera cajani* and *Fusarium udum* on pigeonpea by *Glomus mosseae*, *Trichoderma harzianum* and *Verticillium chlamydosporium*. *Isr. J. Plant. Sci*. **44:** 49–56, 1996.

Siddiqui ZA and Mahmood I. Role of plant symbionts in nematode management: A Review. *Bioresource Technol*. **54:** 217–226, 1995.

Siddiqui ZA, Mahmood I and Khan MW. VAM fungi as prospective biocontrol agents for plant parasitic nematodes. In: D.J. Bagyaraj, A. Verma, K.K. Khanna and, H.K. Kehri (eds.), Modern Approaches and Innovations in Soil Management. Meerut, India: Rastogi, pp. 47–58, 1999.

Silveira APD, Freitas SS, Silva LRC, Lombardi MLCO and Cardoso EJBN. Interações de micorrizas arbusculares e rizobactérias promotoras do crescimento em plantas de feijão. *R. Bras. Ci. Solo.* **19:** 205-211, 1995.

Singh HP and Singh B. Horticulture research, education and development in historical persepective – A way forward. In: Shodh Chintan (Eds. Srivastava AK, Singh B, Patel NL, Ahlawat TR and Singh A), ASM Foundation, New Delhi, pp.01-41, 2014.

Singh NK, Bracker CA, Hasegawa PM, Handa AK, Buckel S, Hermodson MA, Pfankoch E, Regnier FE and Bressan RA. Characterization of osmotin. A thaumatin like protein associated with osmotic adaptation in plant cells. *Plant Physiol*. **85:** 529- 536, 1987.

Singh PK, Singh M and Vyas D. Biocontrol of Fusarium wilt of chickpea using arbuscular mycorrhizal fungi and *Rhizobium leguminosorum* biovar. *Caryologia*, **63 (4):** 349-353, 2010.

Singh R, Soni SK and Kalra A. Synergy between *Glomus fasciculatum* and a beneficial *Pseudomonas* in reducing root diseases and improving yield and forskolin content in *Coleus forskohlii* Briq. under organic field conditions. *Mycorrhiza*. **23**: DOI 10.1007/s00572-012-0447-x, 2013.

Singh S, Rekha PD, Arun AB, Huang YM, Shen FT and Young CC. Wastewater from monosodium glutamate industry as a low cost fertilizer source for corn *(Zea mays* L.). *Biomass Bioenerg.* **35:** 4001-4007, 2011.

Smith FA and Smith SE. Structural diversity in vesicular-arbuscular mycorrhizal symbioses. *New. Phytologist*. **137:** 373-388, 1997.

Smith SE and Read DJ. *Mycorrhizal Symbiosis*. 2nd ed Academic Press, London. 605, 1997.

Sonia LS, Jeddi FB, Tisserant B, Debiane D, Rezgui S, Grandmougin-Ferjani A and Hadj Sahraoui AL. Role of arbuscular mycorrhizal symbiosis in root mineral uptake under $CaCO_3$ stress. *Mycorrhiza.* **22:** 337–345, 2013.

Steinkellner S, Hage-Ahmed K, García-Garrido JM, Illana A, Ocampo JA and Vierheilig H. A comparison of wild-type, old and modern tomato cultivars in the interaction with the arbuscular mycorrhizal fungus *Glomus mosseae* and the tomato pathogen *Fusarium oxysporum* f. sp. *lycopersici Mycorrhiza*. Published online, 2011

Stitcher L, Mauch-Mani B and Metraux JP. Systemic acquired resistance. Annual Review of Phytopathology. **35:** 235–70, 1997.

Sukhada M, Manjula R and Rawal RD. Evaluation of arbuscular mycorrhiza and other biocontrol agents against *Phytophthora parasitica* var. *nicotianae* infecting papaya (*Carica papaya* cv. Surya) and enumeration of pathogen population using immunotechniques. *Biological Control*. **58(1):** 22-29, 2011.

Sunar K, Chakraborty U and Chakraborty BN. Exploitation of native microflora of Darjeeling hills for sclerotial rot management and growth promotion in pulses. *Indian Phytopath*. **67(1):** 59-69, 2014.

Tamas L, Huttova J and Igov Z. Accumulation of stress-proteins in intercellular spaces of barley leaves induced by biotic and abiotic factors. *Biol. Plant*. **39:** 387-394, 1997.

Tamura K, Nei M and Kumar S. Prospects for inferring very large phylogenies by using the neighbor-joining method. *Proceedings of the National Academy of Sciences* (*USA*) **101**:11030-11035, 2004.

Tamura K, Stecher G, Peterson D, Filipski A and Kumar S. MEGA6: Molecular Evolutionary Genetics Analysis version 6.0. *Molecular Biology and Evolution.* **30:** 2725-2729, 2013.

Tarafdar A, Biswas KK and Ghosh PD. Molecular characterization of the *Citrus tristeza* virus (CTV) genome. In: Biology of useful plants and microbes (Eds Sen A), Narosa Publishing House Pvt. Ltd, pp. 1-11, 2014.

Tawaraya K, Hashimoto K and Wagatsuma T. Effect of root exudate fractions from P-deficient and P-sufficient onion plants on root colonisation by the arbuscular mycorrhizal fungus *Gigaspora margarita*. *Mycorrhiza*. **8:** 67–70, 1998.

Tawaraya K, Watanabe S, Yoshida E and Wagatsuma T. Effect of onion (*Allium cepa*) root exudates on the hyphal growth of *Gigaspora margarita*. *Mycorrhiza*. **6:** 57–59, 1996.

Thapa A. Study of nematodes problem associated with Citrus dieback in Darjeeling idistrict of West Bengal. Ph.D. thesis, Bidhan Chandra Krishi Viswavidyalaya,West Bengal, 1991.

Tilak KVBR and Reddy BS. *Bacillus cereus* and *B. circulans*- novel inoculants for crops. *Curr. Sci.* **90:** 642-644, 2006.

Tisdall JM and Oades JM. Organic matter and water-stable aggregates in soils. *J.Soil.Sci.* **33**:141-163, 1982.

Toro M, Azcn R and Barea JM. Improvement of arbuscular mycorrhizal development by inoculation with phosphate-solubilizing rhizobacteria to improve rock phosphate bioavailability (32P) and nutrient cycling. *Appl. Environ. Microbiol.* **63:** 4408– 4412, 1997.

Trappe JM. Synoptic keys ti the genera and species of zygomycetous Mycorrhizal fungi. *Phytopathology.* **72:** 1102-1108, 1982.

Treseder KK. The extent of mycorrhizal colonization of roots and its influence on plant growth and phosphorus content. *Plant and soil.* **371:** 1-13, 2013.

Truman W, Bennett MH, Kubigsteltig I, Turnbull C and Grant M. *Arabidopsis* systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. *Proc. Natl. Acad. Sci.* USA. **104:** 1075-1080, 2007.

Tsai SM and Phillips DA. Flavonoids released naturally from alfalfa promote development of symbiotic *Glomus* spores *in vitro*. *Appl. Environ. Microbiol.* **57:** 1485–1488, 1991.

Turan M, Gulluce M and Sahin F. Effects of plant growth promoting rhizobacteria on yield, growth, and some physiological characteristics of wheat and barley plants. *Commu. Soil. Sci. Plant. Anal.* **43:**1658–1673, 2012.

Uren NC and Reisenauer HM. The role of root exudates in nutrient acquisition. *Adv. Plant Nutr*. **3:** 79–114, 1988.

Urrea EM, Paul S, Khodaverdi V, Seifert J, von Bergen M, Kretzschmar U and Adrian L. Identification and Characterization of a Re-Citrate Synthase in *Dehalococcoides* Strain CBDB1. *Journal of Bacteriology.* **193:** 5171-5178, 2011.

Vallad GE and Goodman RM. Systemic Acquired Resistance and Induced Systemic Resistance in Conventional Agriculture. *Cropsci.* **44(6):** 1920-1934, 2004.

Van Loon LC and Van Strien EA. The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol*. **55:** 85-97, 1999.

Van Loon LC, Bakker PAHM and Peiterse CMJ. Systemic resistance induced by rhizosphere bacteria. *Ann. Rev. Phytopathol.* **36:** 453-483, 1998.

Van Loon LC, Pierpoint WS, Boller T and Conejero V. Recommendations for naming plant pathogenesis-related proteins. *Plant Mol. Biol. Reporter.* **12:** 245-264, 1994.

Van Loon LC. Occurrence and properties of plant pathogenesis-related proteins. In: S.K. Datta and S. Muthukrishnan (eds.). Pathogenesis-related proteins in plants. CRC Press LLC, Boca Raton, pp. 1-19, 1999.

Van Loon LC. The families of pathogenesis-related proteins. In: 6th International Workshop on PR-proteins. May 20-24, 2001, Spa, Belgium. Book of abstracts, pp. 9, 2001.

Veerabhadraswamy AL and Garampall RH. Effect of Arbuscular Mycorrhizal Fungi in the Management of Black Bundle Disease of Maize caused by *Cephalosporium acremonium*. *Science Research Reporter*. **1(2):** 96 – 100, 2011.

Venkateswarlu R, Reddi KM, Reddy ENP and Sudhakar P. Molecular characterization of *Trichoderma* spp. used against *Fusarium* wilt with PCR based RAPD and ITS-PCR. *J. Mycol. Plant. Pathol.* **38(3):**569-563, 2008.

Vessey JK. Plant growth promoting rhizobacteria as biofertilizers. *Plant Soil.* **255:** 571–586, 2003.

Vierheilig H and Piche Y. Signalling in arbuscular mycorrhiza: facts and hypotheses. In: B. Buslig , J. Manthey (eds.). Flavonoids in cell function. Kluwer Academic/Plenum, New York, pp. 23–39, 2002.

Vierheilig H, Lerat S and Pich Y. Systemic inhibition of arbuscular mycorrhiza development by root exudates of cucumber plants colonized by *Glomus mosseae. Mycorrhiza*. **13:** 167–170, 2003.

Vierheilig H, Schweigerb P and Brundrett M. An overview of methods for the detection and observation of arbuscular mycorrhizal fungi in roots. *Physiologia Plantarum* 125: 393–404, 2005.

Vierheilig H. Regulatory mechanisms during the plant-arbuscular mycorrhizal fungus interaction. *Can. J. Bot.* **82:** 1166–1176, 2004.

Vigers AJ, Roberts WK and Selitrennikoff CP. A new family of plant antifungal proteins. *Mol. Plant-Microbe Interact*. **4:** 315-323, 1991.

Vinale F, Ghisalberti EL, Sivasithamparam K, Marra R, Ritieni A, Ferracane R, Woo S, Lorito M. Factors affecting the production of *Trichoderma harzianum* secondary metabolites during the interaction with different plant pathogens. *Lett. Appl. Microbiol*. **48:**705–711, 2009.

Vinale F, Marra R, Scala F, Ghisalberti EL, Lorito M, Sivasithamparam K. Major secondary metabolites produced by two commercial *Trichoderma* strains active against different phytopathogens. *Lett. Appl. Microbiol*. **43:**143–148, 2006.

Vlot AC, Klessig DF and Park SW. Systemic acquired resistance: the elusive signal(s). *Current Opinion in Plant Biology*. **11:** 436–442, 2008.

Voesenek LACJ, Rijnders JHGM, Peeters AJM, Van de Steeg HMV and De Kroon H. Plant hormones regulate fast shoot elongation under water: from genes to communities. *Ecology.* **85:** 16–27, 2004.

Vogeli-Lange R, Frundt C, Hart CM, Beffa R, Nagy F and Meins F Jr. Evidence for a role of β 1,3-glucanase in dicot seed germination. *Plant J.* **5:** 273-278, 1994.

Wakeham AJ and White JG. Serological detection in soil of Plasmodophora brassicae resting spores. *Physiol. Mol. Plant. Pathol*. **48**: 289-303, 1996.

Walker C. Systematics and taxonomy of the Arbuscular endomycorrhizal fungi (Glomales), a possible way forward. *Agronomie.* **12:** 887-897, 1992.

Walley FL and Germida JJ. Failure to decontaminate *Glomus clarum* NT4 spores is due to spore wall-associated bacteria. *Mycorrhiza.* **6:** 43-49, 1996.

Walling LL. The myriad plant responses to herbivores. *Journal of Plant Growth Regulation.* **19:** 195–216, 2000.

Walsh GA, Murphy RA, Killen GF and Headon Power DR RF. Technical note: detection and quantification of supplemental fungal β-glucanase activity in animal feed. *J. Animal. Sci.* **73**: 1074-1076, 1995.

Wang P, Zhang JJ, Shu B and Xia RX. Arbuscular mycorrhizal fungi associated with citrus orchards under different types of soil management, southern China. *Plant. Soil. Environ.* **58 (7):** 302–308, 2014.

Warcup JH. The soil-plate method for isolation of fungi from soil. *Nature.* **166(4211)**:117–118, 1950.

Weir TL, Park SW and Vivanco JM. Biochemical and physiological mechanisms mediated by allelochemicals. *Curr. Opin. Plant Biol.* **7:** 472–79, 2004.

Whitelaw MA. Growth promotion of plants inoculated with phosphate solubilizing fungi. *Adv. Agron.* **69:** 99-151, 2000.

Wilkinson DG, Bailes JA, Champion JE and Mcmahon AP. A molecular analysis of mouse development from 8 to 10 days post coitum detects changes only in embryonic globin expression. *Developments.* 493-500, 1987.

Wisneiwska M and Golinowski W. Immunolocalization of ∞-Expansion protein (NtEXPA5) in tobacco roots in the presence of the arbuscular mycorrhizal fungus

Glomus mosseae Nicol. & Gerd. *Acta Biologica Cracoviensia* Series Botanica. **53/2:**113-123, 2011.

Woo SL, Scala F, Ruocco M, Lorito M. The molecular biology of the interactions between *Trichoderma* spp., phytopathogenic fungi, and plants. *Phytopathology*. **96:**181–185, 2006.

Wright SF and Upadhyaya A. A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. *Plant and Soil.* **198**:97-107, 1998.

Wu SC, Cao ZH, Li ZG, Cheung KC and Wong MH. Effects of biofertilizer containing N-fixer, P and K solubilizers and AM fungi on maize growth: a greenhouse trial. *Geoderma* **125:** 155–166, 2005.

Xavier LCJ and Germida JL. Bacteria associated with *Glomus clarum* spores influence mycorrhizal activity. *Soil Biol. Biochem*. **35:** 471-478, 2003.

Xia Y, Suzuki H, Borevitz J, Blount J, Guo Z, Patel K, Dixon RA and Lamb C. An extracellular aspartic protease functions in *Arabidopsis* disease resistance signalling. *EMBO J.* **23:** 980-988, 2004.

Yaseen T and D'Onghia AM. *Fuarium* spp. associated to citrus dry root rot: An emerging issue for Mediterranean citriculture. *Acta Hort*. **940:** 647-655, 2012.

Yedida I, Benhamou N and Chet I. Induction of defense responses in cucumber plants (*Cucumis sativus*) by the biocontrol agent *Trichoderma harzianum*. *Appl. Environ MIcrobiol*. **65(3):** 1061-1070, 1999.

Yedida I, Benhamou N, Kapulnik Y and Chet I. Induction and accumulation of PR proteins activity during early stages of root colonization by the mycoparasite *Trichoderma harzianum* strain T-230. *Plant Physiol. BIochem*. **38:** 863-873, 2000.

Zak B. Role of mycorrhizae in root disease. *Annual Review of Phytopathology* **2:** 377-392, 1964.

Zhang X, Dai Y, Xiong Y, DeFraia C, Li J, Dong X and Mou Z. Overexpression of *Arabidopsis* MAP kinase kinase 7 leads to activation of plant basal and systemic acquired resistance. *Plant J*. **52:** 1066-1079, 2007.

Zhu Y, Pierson LS III and Hawes MC. Release of nodulation gene inducing chemicals from root border cells. *Plant Physiol*. **115:** 1691–1698, 1998.

APPENDIX A: List of thesis related publications

In Journals

Allay S and Chakraborty BN. Activation of defense response of mandarin plants against *Fusarium* root rot disease using *Glomus mosseae* and *Trichoderma hamatum*. *Journal of Mycology and Plant Pathology*. **40(4):** 499-511, 2010.

Chakraborty U, Chakraborty BN, Allay S, De U and Chakraborty AP. Dual Application of *Bacillus pumilus* and *Glomus mosseae* for Improvement of Health Status of Mandarin Plants. *Acta Horticulturae*, **892:** 215-229, 2011.

Allay S, De UK and Chakraborty BN. Root colonization of mandarin plants grown in orchards of Darjeeling hills and plains with Arbuscular Mycorrhizal Fungi. *NBU Journal of Plant Science.* **6** (1) : 41-47, 2012.

Allay S, Lama R, Chakraborty U and Chakraborty BN. Antioxidative responses of mandarin plants to water stress. *NBU Journal of Plant Science.* **7(1):** 21-28, 2013.

Allay S and Chakraborty BN. Induction of Resistance in *Citrus reticulata* against *Fusarium solani* by Dual Application of AMF and *Trichoderma asperellum*. *International Journal of Bio-resource & Stress Management*. **4(4):** 588-592, 2013.

Publications in edited volumes

Chakraborty BN, Chakraborty U, Allay S, Khati S, De U and Dey PL. Rhizosphere mycoflora of *Citrus reticulata* and their diversity analysis. In: Microbial resources for crop improvement, eds. B.N. Chakraborty and U. Chakraborty, Satish serial publishing house, India, pp. 157-172, 2013.

Chakraborty BN, De UK and Allay S. Molecular detection of arbuscular mycorrhizal fungi and their application for plant health improvement. In. Biology of useful plant and microbes, ed. A. Sen, Narosa publishing house, New Delhi, India, pp. 13-31,2014.

Chakraborty BN, Chakraborty U, Rai K, Allay S and De U. *Citrus* decline in Darjeeling Hills and potential microorganisms for its management. In: Approaches and trends in plant disease management (eds.) S.K. Gupta and M. Sharma, Scientific Publishers (India), pp. 158-166, 2014.

APPENDIX B: List of Abbreviations

APS- Ammonium per sulphate
BLAST- Basic local alignment search tool
BSA- Bovine serum albumin
BSS-2- Biclonal seed stock- 2
$Ca_3(PO_4)_2$ - Tri-calcium phosphate
$CaHPO_4$ - Calcium phosphate
CAS- Chrome azurol S
CAT- Catalase
CDA- Chitinase detection agar
cfu- Colony forming unit
CHT- Chitinase
$CuSO_4$ - Copper sulphate
DAC-ELISA- Direct antigen coating- Enzyme linked immune-sorbent assay
DAPG- 2,4-Diacetylphloroglucinol
dATP- Deoxy adenosine tri-phosphate
dCTP- Deoxy cytosine tri-phosphate
DEAE cellulose- diethyl aminoethyl cellulose
dGTP- Deoxy guanosine tri-phosphate
DMAB- Di methyl amino benzaldehyde
DNA- Deoxyribonucleic acid
dNTPs- Deoxy nucleotide tri-phosphates
DNSA- Dinitro salicylic acid
dTTP- Deoxy thymidine tri- phosphate
EDTA- Ethylene diamine tetra acetic acid
ELISA- Enzyme linked immune- sorbent assay
ER- Root endosphere
$FeCl_3$- Ferric chloride
FITC- Fluorescein isothiocyanate
g- gram
Gfp- Green fluorescent protein
GlcNAc- N-acetyl glucosamine
H_2O_2 - Hydrogen peroxide
H_2So_4- Sulphuric acid
HCl- Hydrochloric acid
HCN- Hydrocyanic acid
HDTMA- Hexa-decytrimethyl ammonium bromide
HPLC- High performance liquid chromatography
IAA- Indole acetic acid
ISR- Induced systemic resistance
KCl- Potassium chloride
KH_2PO_4 – Potassium dihydrogen phosphate
MEGA 4- Molecular Evolutionary Genetics Analysis 4
mg- Mili gram

$MgCl_2$- Magnesium chloride
ml- Mili litre
Na_2CO_3- Sodium carbonate
Na_2HPO_4- Di sodium hydrogen phosphate
Na_2MoO_4 - Sodium molybdate
NaCl- Sodium chloride
NaN_3- Sodium azide
$NaNO_2$- Sodium nitrite
NaOH- Sodium Hydroxide
NB- Nutrient Broth
NBT/BCIP substrate- Nitro blue tetrazolium/ (5-bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate substrate
NCBI- National Center for Biotechnology Information
NCM- Nitrocellulose membrane
NH_4Cl- Ammonium chloride
PAL- Phenylalanine ammonia lyase
PBS-Tween- Phosphate buffer saline- Tween
PCA- Phenazine-1-carboxylic acid
PCI- Water saturated phenol: Chloroform: Isoamyl alcohol
PCR- Polymerase chain reaction
PCR–RFLP- Polymerase chain reaction- Restriction fragment length polymorphism
PGPR- Plant Growth Promoting Rhizobacteria
PGPTs- Plant growth promoting traits
pNPP- p- nitrophenyl phosphate
POX- Peroxidase
PPO- Polyphenol oxidase
PR proteins- Pathogenesis related proteins
PRN- Pyrrolnitrin
PSB- Phosphate solubilising bacteria
PVK- Pikovskaya's agar
PVLG – Poly vinyl Lacto Glycerate
PVP- Poly vinyl- pyrollidone
RNA- Ribonucleic acid
RP- Rock phosphate
RS- Rhizosphere soil
SA- Salicylic acid
SAR- Systemic acquired resistance
SDS- Sodium dodecyl sulphate
SDS-PAGE- Sodium dodecyl sulphate- Poly-acrylamide gel electrophoresis
SKM- Skim milk agar
T-17- Teenali-17
TAE buffer- Tris Acetic Acid and EDTA buffer
TE buffer- Tris-EDTA buffer
TEMED- N,N,N',N'-Tetramethylethylenediamine

Tris Hcl- Tris hydrochloric acid
TV- Toklai variety
UP- Upasi variety
UPGMA- Unweighted Pair Group Method with Arithmetic Mean
yr- Year
β-1,3-GLU- β-1,3 glucanase
µl- Micro litre

APPENDIX C: List of Chemicals

Ammonium chloride
Ammonium per sulphate
Bovine serum albumin
Calcium phosphate
Carboxy methyl cellulose
Chrome azurol S
Colloidal chitin
Copper sulphate
Deoxy nucleotide tri-phosphates
Di methyl amino benzaldehyde
Di sodium hydrogen phosphate
Diethyl aminoethyl cellulose
Dinitro salicylic acid
Ethylene diamine tetra acetic acid
Ferric chloride
Fluorescein isothiocyanate
Helicase (3%)
Hexa-decytrimethyl ammonium bromide
Hydrochloric acid
Hydrocyanic acid
Hydrogen peroxide
Indole acetic acid
LR White resin
Magnesium chloride
N,N,N',N'-Tetramethylethylenediamine
N-acetyl glucosamine
Nitro blue tetrazolium/ (5-bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate substrate
O-dianisidine (5 mg/ml methanol)
p- nitrophenyl phosphate
Phosphate buffer saline- Tween
Poly vinyl- pyrollidone
Potassium chloride
Potassium dihydrogen phosphate
Sodium azide
Sodium carbonate
Sodium chloride
Sodium dodecyl sulphate
Sodium Hydroxide
Sodium molybdate
Sodium nitrite
Sulphuric acid
Tri-calcium phosphate

Tris Acetic Acid and EDTA buffer
Tris hydrochloric acid
Tris-EDTA buffer

Water saturated phenol: Chloroform: Isoamyl alcohol
0.05(M) sodium phosphate buffer (pH 6.8)
0.1M sodium phosphate buffer pH (7.4)
0.1(M) sodium acetate buffer (pH 5.0)
0.2M Na-phosphate buffer (pH 5.4)
0.3mM borate buffer (pH 8.0)
1 M K-PO4 buffer (pH 7.1)
1-amino-cyclopropane-1-carboxylic acid hydrochloride
1M Na-acetate buffer (pH 4)
Sodium borate buffer (pH 8.8)
2 mM β- mercaptoethanol
1 M Na-borate buffer (pH 9.8)
2% L-phenylalanine
2,4-Diacetylphloroglucinol
4 mM H_2O_2.
4% laminarin
2.5% Glutaraldehyde
30%-90% absolute alcohol
1% aqueous toluidine blue solution
2% uranyl acetate
0.2% lead acetate

Smt. Guman Devi Verma Memorial Best Woman Scientist Award

Activation of Defense Response of Mandarin Plants Against *Fusarium* Root Rot Disease Using *Glomus mosseae* and *Trichoderma hamatum*

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Abstract

Mandarin (*Citrus reticulata*) is one of the ancient commercial crop that is cultivated in Darjeeling and Sikkim hills. Citrus decline in nursery grown saplings of *Citrus reticulata* caused by *Fusarium solani* is increasingly posing as a major threat to the farmers. Polyclonal antibodies (PAbs) were raised against mycelial antigens of *F. solani*, IgG were purified and further packaged into immunological formats such as PTA-ELISA, dot immunobinding assay, western blot and indirect immunofluorescence for quick and accurate detection of pathogen from soil and mandarin root tissue. Besides, for PCR based molecular detection of root pathogen (*F. solani, F. oxysporum* and *F. graminearum*) as well as biocontrol fungi (*Trichoderma hamatum* and *T. asperellum*) isolated from mandarin rhizosphere, genomic DNA were prepared, purified and PCR amplification of 18S rDNA was done using ITS region specific primer pair with good product yield and minimum non-specific amplification. The product size was approximately 550 bp for *Fusarium* and 600 bp for *Trichoderma* with the size variation across the isolates. RAPD profile was obtained using four random decamers from Operon technology kit OPA1, OPA-4, A-5 and A-11 for *Fusarium* and A11, OPD6 and A04 specific primers for *Trichoderma*. Screening of arbuscular mycorrhizal fungi from rhizosphere of mandarin grown in orchards yielded *Glomus mosseae*, *G. fasciculatum*, *G. intraradices*, *G. versiforme*, *Gigaspora margarita*, *G. rosea*, *G. gigantea, Acaulospora spinosa*, *A. bireticulata* and *Scutellospora* sp. Among these *G. mosseae* was found to be dominant and maximum percent root colonization was evident. It was selected for mass multiplication in sorghum plants. Application of *G. mosseae* in mandarin saplings exhibited marked increase in growth of plants. Application of *G. mosseae* and *T. hamatum* singly or jointly suppressed root rot of mandarin caused by *F. solani*. Defense enzymes, chitinase, glucanase and peroxidase showed enhanced activities during disease suppression and was confirmed by immunological assays. PAGE analyses of peroxizomes revealed one induced isoform each following *T. hamatum* treatment and *G. mosseae* inoculated roots.

Keywords: *Citrus reticulata*, *Fusarium solani*, *Trichoderma hamatum*, *Glomus mosseae* immunodiagnosis, rDNA, PCR-RAPD

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Citrus reticulata is the most extensively grown citrus species in India. It is commonly known as mandarin. 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 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 (Mukhopadhyay and Thapa 2001). Darjeeling mandarin is an indigenous economic crop of Darjeeling hills situated between 27°13' and 26°27' and between 88°53' and 87°59' (Chettri et al 2006). Darjeeling oranges have a distinct flavour and quality and hence they have a distinct appreciation in the market. The hills of Darjeeling grows mandarin from the time immemorial and its ethno botanical records are reflected in the local folklore of highlanders (Thapa 2007). 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 tonnes and an average productivity of 8.11 tonnes/ha. The mandarin cultivation in Darjeeling has shown a massive decline due to various pathological, entomological and nutritional stresses (Mukhopadhyay

et al 1996). *Fusarium solani, F. oxysporum* and *Macrophomina phaseolina,* infects roots of both nursery grown and field grown mandarin plants. Outbreaks are common in fields where mandarin is grown under water stress. The economic condition of the villagers, lack of proper infrastructures and facilities and unscientific management practices add to the decline of the crop. Rhizosphere soil of plants contains a wide variety of microorganisms- bacteria, fungi and actinomycetes which are both beneficial and harmful. Among the beneficial microorganisms, two groups are very important agriculturally – the arbuscular mycorrhizal fungi (AMF) and biocontrol agents (BCA). Since microorganisms do not live in isolation in the soil, their effects on plant growth, nutrition and protection against diseases are due to their interactions.

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 dual activity of AMF and BCA has provided ample evidence as bioprotector as well as biofertilizer and they play a significant role in sustainable agriculture. 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 pathogen (*Fusarium*) and also for biocontrol agent (*Trichoderma*) 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 selective detection of *Fusarium* species associated with root damage of *C. reticulata* and for *Trichoderma* species to develop the management strategies of root diseases of mandarin plant. In the present study, a mycorrhizal fungus- *Glomus mosseae* and a plant growth promoting fungus – *Trichoderma hamatum* were selected and tested for their effects on growth promotion and root rot disease suppression of *Citrus reticulata*. Mechanism of the observed effects by the dual application of the microorganisms were determined both biochemically and immunologically.

Materials and Methods

Plant material. Mandarin (*Citrus reticulata*) seedlings (8 mo old) were obtained from Nirmaldass Orchard, Gurung Brothers Nursery, Baramangwa Busty, Darjeeling. The selected seedlings were maintained in plastic pots (30 cm dia) and watered regularly for proper growth in a glasshouse and were used for experimental purpose.

Isolation of AMF. AMF associated with mandarin roots were isolated from rhizosphere of *C. reticulata* by wet sieving and decanting technique (Gerdemann and Nicolson 1963). Approximately 1kg of soil was suspended in 4 l water and heavier particles were allowed to settle down. The upper aqueous phase was taken and decanted through sieves of decreasing size (BS 60, BS 80, BS 100, BS 150, BS170 and BS 200). Spores were collected by fine brushes and further purification was done by sucrose gradient centrifugation method (Daniels and Skipper 1982). In this method, spores and minimal amount of organic particles were further purified by suspending and sieving in 40% sucrose solution and centrifuging at 2000 rpm (approximate 370 x g) for 1 min. The supernatant (with spores) was passed through a sieve of 400 mesh and rinsed with distilled water to remove sucrose residue. Clean AMF spores were separated using the help of a simple microscope (20x), stained with Melzar's reagent, microscopical observations were made and photographs were taken. The spores were stored in Ringer's Solution (8.6 g NaCl, 0.3 g KCl, 0.33 g $CaCl_2$ in 1 l of boiled distilled water) at 4 C or in sterile water for further use.

Isolation of *Trichoderma* sp. from mandarin rhizosphere. *Trichoderma* sp. was isolated from rhizosphere of *C. reticulata* in *Trichoderma* selective media (TSM) (Elad et al 1980) 0.2 g $MgSO_4(7H_2O)$, 0.9 g KH_2PO_4, 0.14 g KCl, 1 g NH_4NO_3, 3.0 g anhydrous glucose, 0.15 g Rose Bengal, 20 g agar, 950 ml distilled water]. *T. hamatum* and *T. asperellum* were identified through National Center of Fungal Taxonomy, IARI, New Delhi and used for present investigation.

Fungal pathogen. *Fusarium solani* (Acc. No. 3719) and *F. oxysporum* (Acc. No. 581) causing root rot of mandarin was obtained from Culture collection of Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi. *F. graminearum, Macrophomina phaseolina*, *Curvularia lunata* and *Sphaerostilbe repens* were obtained from culture collection of Immuno-Phytopathology Laboratory, Department of Botany, NBU.

Inoculation technique. Pathogen (*F. solani*) was grown in sand-maize meal medium (maize meal: sand: water-1:9:1 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 100 g of previously prepared inoculum of *F. solani* to the rhizosphere soil.

Disease assessment. Disease assessment was performed following the method of Chakraborty et al (2006) after 15, 30 and 45 d of inoculation. Disease intensity was assessed as root rot index on a scale of 0-6, depending on both underground and above ground symptoms as: 0=no symptoms; 1=small roots turn brownish and start rotting; 2=leaves start withering and 20-40% of roots turn brown; 3=leaves withered and 50% of roots affected; 4=shoot tips also start withering and 60-70% roots affected; 5=shoots withered with defoliation of lower withered leaves, 80% roots affected; 6=whole plants die, with upper withered leaves still remaining attached; roots fully rotted.

In vitro studies. *F. solani* was paired with *T. hamatum* on solid medium as described by Chakraborty and Chakraborty (1989).

Inocula preparation and application of biocontrol fungi

AMF. Spores of *G. 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 of 7-10 d old seedlings of sorghum plants grown in black plastic pots (30 cm) having autoclaved soil to discard the presence of other fungal propagules. After 45 d 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/100g could be considered as potent inocula for application.

BCA. Inoculum of *T. hamatum* was prepared by inoculating wheat bran (sterilized) with 5 mm disc of the fungus and incubating at 28 ºC 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. hamatum* was mixed to give a conc of 10^5 cfu/g of soil as described by Chakraborty et al (2003).

Determination of growth of mandarin seedlings. Growth promotion was studied in terms of increase in height, number of leaves and biomass in potted plants. Plants were grown under natural conditions of light and temp (25±2 C). The numbers of leaves and branches were also determined visually. Leaves were dried and the dry biomass of leaves was determined at 1 mo interval up to three months of application. In each treatment, avg of 20 replicate plants were taken and analyzed.

Extraction and quantification of soil phosphate. Soil sample (1g) was air dried and suspended in 25 ml of the extracting solution (0.025N H_2SO_4, 0.05N HCl) to which activated charcoal (0.01g) was also added, shaken well for 30 min on a rotary shaker and filtered through Whatman No. 2 filter paper (Mehlich 1984). Quantitative estimation of phosphate was done following ammonium molybdate-ascorbic acid method as described by Knudsen and Beegle (1988).

Assay of enzyme activities. Leaves of mandarin seedlings grown in treated or control potted soil were used for all biochemical analyses. Leaves were collected for assay 72 h after inoculation. β-1,3-glucanase (E.C. 3.2.1.39) was extracted and assayed from leaf samples following the method of Pan et al (1991). The amount of glucose liberated was determined spectrophotometrically using a standard curve. Activity was expressed as µg glucose released /min/g tissue. Chitinase (E.C. 3.2.1.14.) was extracted and assayed following the method of Boller and Mauch (1988). The amount of GlcNAc released was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as µg GlcNAc released /min/ g fresh wt. tissue. Peroxidase (E.C. 1.11.1.7.) was extracted and estimated following the method described by Chakraborty et al (1993). O-dianisidine was used as substrate and activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2. Specific activity expressed as the increase in Δ A 465/g tissue/min.

Preparation of antigen. Antigens were prepared from mycelia of *F. solani* as well as from healthy and *F. solani* infected root tissues 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 *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 *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 (*F. solani*, *F. oxysporum*, *Macrophomina phaseolina*), non pathogens (*Curvularia lunata*, *Sphaerostilbe repens*), healthy and artificially inoculated (with *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 *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 *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 *F. solani* and goat antisera specific to rabbit globulins conjugated with FITC were used for indirect immunofluorescence study to detect pathogen in root tissues and in soil as per 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 and F. graminearum* and isolates of *Trichoderma* (*T. hamatum* and *T. asperellum*) were done. Fungal mycelia (5 d old) was crushed with liquid nitrogen and incubated with lysis buffer containing 250 mM Tris-HCl (pH 8.0), 50 mM EDTA (pH 8), 100 mM NaCl and 2% SDS, for 1 h 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 0.5M 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 TE buffer pH 8.

Qualitative and quantitative estimation of DNA. Total genomic DNA extracted from isolates of *Fusarium* and *Trichoderma* was resuspended in 100 µl 1 X TE buffer, treated with RNAse (60µg) 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. The DNA from all isolates produced clear sharp bands, indicating good quality of DNA.

ITS-PCR amplification. All isolates of *Fusarium* and *Trichoderma* were taken up for ITS-PCR amplification. Genomic DNA was amplified by mixing the template DNA (50 µg), 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 µ1) 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.

Table 1. Nucleotide sequence used for ITS PCR

Seq Name	Primer Seq 5'-3'	Mer	TM	% GC
Fusarium sp				
Fcg17F	TCGATATACCGT GCGATTTCC	21	65	47%
Fcg17R	TACAGACACCGT CAGGGGG	19	66	63%
Trichoderma sp.				
T/ITS 1	TCTGTAGGTGAA CCTGCGG	19	63.9	57%
T/ITS4	TCCTCCGCTTATT GATATGC	20	61.5	45%

RAPD -PCR. Four selective primers - OPA1, OPA-4, A-5 and A-11 for *Fusarium,* and three A11, OPD6 and A04 specific primers for *Trichoderma* were selected for RAPD-PCR. 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 µ1) 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. 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 Biostatics, 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 four different locations of mandarin orchards were evaluated for resistance to *F. solani* separately. Disease assessment was based on percentage loss in dry mass of inoculated roots as well as on the color intensity of infected roots, 15 d after inoculation in relation to control.

Table 2. Screening for resistance of mandarin (*Citrus reticulata)* against *Fusarium solani*

Root samples (Location)	Loss in dry mass of roots (% compared to control)	Color intensity of infected roots
Bijanbari	42.5	Blackish brown
Kurseong	39.7	Deep brown
Mirik	82.0	Black
Kalimpong		
Block I	87.5	Black
Block II	79.6	Black

In relation to control, data from three separate trials of 50 plants each

Two locations, Mirik and Kalimpong where disease was prevalent showed the highest populations when isolation was made. Trend was also same in the artificial inoculation test (Table 2).

Population of different species of AM fungi isolated from the rhizosphere of mandarin from different regions was determined. Sites selected were hilly regions of Kalimpong, Mirik, Bijanbari and Kurseong of Darjeeling hill. Table 3, Fig.1 (a-h).

Table 3 Population of AM fungi in the rhizosphere of mandarin plants

Genus and species	AM spores (%)			
	Kalim Pong	Mirik	Bijanbari	Kurseong
Glomus				
G. aggregatum	54	23	48	42
G.mosseae	87	62	18	56
G. fasciculatum	45	30	38	32
G. viscosum	-	-	-	12
G. macrocarpum	05	08	08	10
G. microaggregatum	13	12	10	18
G. versiforme	-	-	-	06
Gigaspora				
G. decipiens	60	57	48	03
G. gigantea	75	58	62	14
G. margarita	65	42	50	34
G. rosea	25	20	-	08
Acaulospora				
A. bireticulata	15	18	16	24
A. spinosa	08	12	10	22
A. denticulata	10	12	10	10
A. tuberculata	02	-	01	04
Scutellospora				
S. rubra	06	08	06	12
S. calospora	04	02	04	05

Table 4 Soil phosphate content in rhizosphere of mandarin plants following root colonization with *G. mosseae* and *G. fasciculatum*

Treatment	Soil phosphate (µg/g tissue)
Control	49.37±1.18
G. mosseae	33.12±0.57
G.fasciculatum	31.25±3.60
G. mosseae + *G.fasciculatum*	30.75±0.33
CD *(P=0.05)*	3.103
CV *(%)*	6.25

Among the AM fungi, *Glomus mosseae* could be determined as the most predominant, followed by other genera such as *Gigaspora*, *Acaulospora* and *Scutellospora*. Percentage of AM spores determined from different regions showed high levels of different *Glomus* sp., *Acaulospora* and *Scutellospora* in Kalimpong and Mirik orchards. On the basis of consistent association and maximum colonization with mandarin roots, *G. mosseae* was selected for further multiplication in sorghum seedlings, and root colonization was confirmed after three weeks (Fig.1, i-k). Scanning electron microscopic observation of *G. mosseae* was made (Fig.1, l). 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 4). PAbs raised against *F. solani* were tested with homologous and heterologous antigens of mandarin roots. Strong precipitin reactions occurred in homologous reactions in immunodiffusion test (Fig. 2 C). Among the root antigens of mandarin plants of six different locations tested against PAbs of *F. solani*, strong and positive reactions were noticed in root antigens of four specific locations (Table 5).

Table 5. Detection of cross reactive antigens among *C. reticulata* and *F. solani* using agar gel double diffusion

Root antigens of *C. reticulata*	Pab *F. solani*
Rangli Rangliot	Absent
Bijanbari	Absent
Sukhia Pokhari	Present
Kurseong	Weak
Mirik	Present
Kalimpong Block I	Present
Kalimpong BlockII	Present
Gorubathan	Weak

Dot immunobinding assays confirm the effectiveness of raising antibodies against *F. solani*. 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 ug/l. Doubling dilutions of *F. solani* ranging from 1:125 to 1:4000 were initially tested. ELISA values decreased with the dilution. IgG dilution of 1:125 was selected for further assay. Dilutions of antigen conc in two-fold series ranging from 25 to 1600 ug/l were tested against two antiserum dilutions (1:125 and 1:250). ELISA values increased with a concomitant increase of antigens levels. Concentrations as low as 25 µg/l could be easily detected by ELISA at both antisera dilutions. PTA-ELISA could readily detect reaction between root antigen and PAb of pathogens (*F. solani*). Antigens extracted from healthy and artificially inoculated with *F. solani* were tested against PAbs of the pathogens separately. 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 2 mg/l (Table 6). Kitagawa et al (1989) successfully used a competitive ELISA technique to develop an assay to identify *F. oxysporum* f.sp. *cucumerinum* among other *Fusarium* sp.

Table 6. PTA-ELISA values showing reaction of PAbs of *Fusarium solani with* antigens of healthy and inoculated mandarin roots

Citrus saplings Locality	Root Antigen concentration (40 mg/L) Healthy	Inoculated[a] *F. solani*
Rangli Rangliot	0.812	1.182
Bijanbari	0.890	1.139
Sukhia Pokhari	0.715	1.345
Kurseong	0.972	1.265
Mirik	0.664	1.876
Kalimpong Block I	0.907	1.766
Kalimpong Block II	0.787	1.980
Gorubathan	0.938	1.765

Absorbance at 405 nm; PAb of *F. solani* (1:125 dilution); [a] 3 days after inoculation

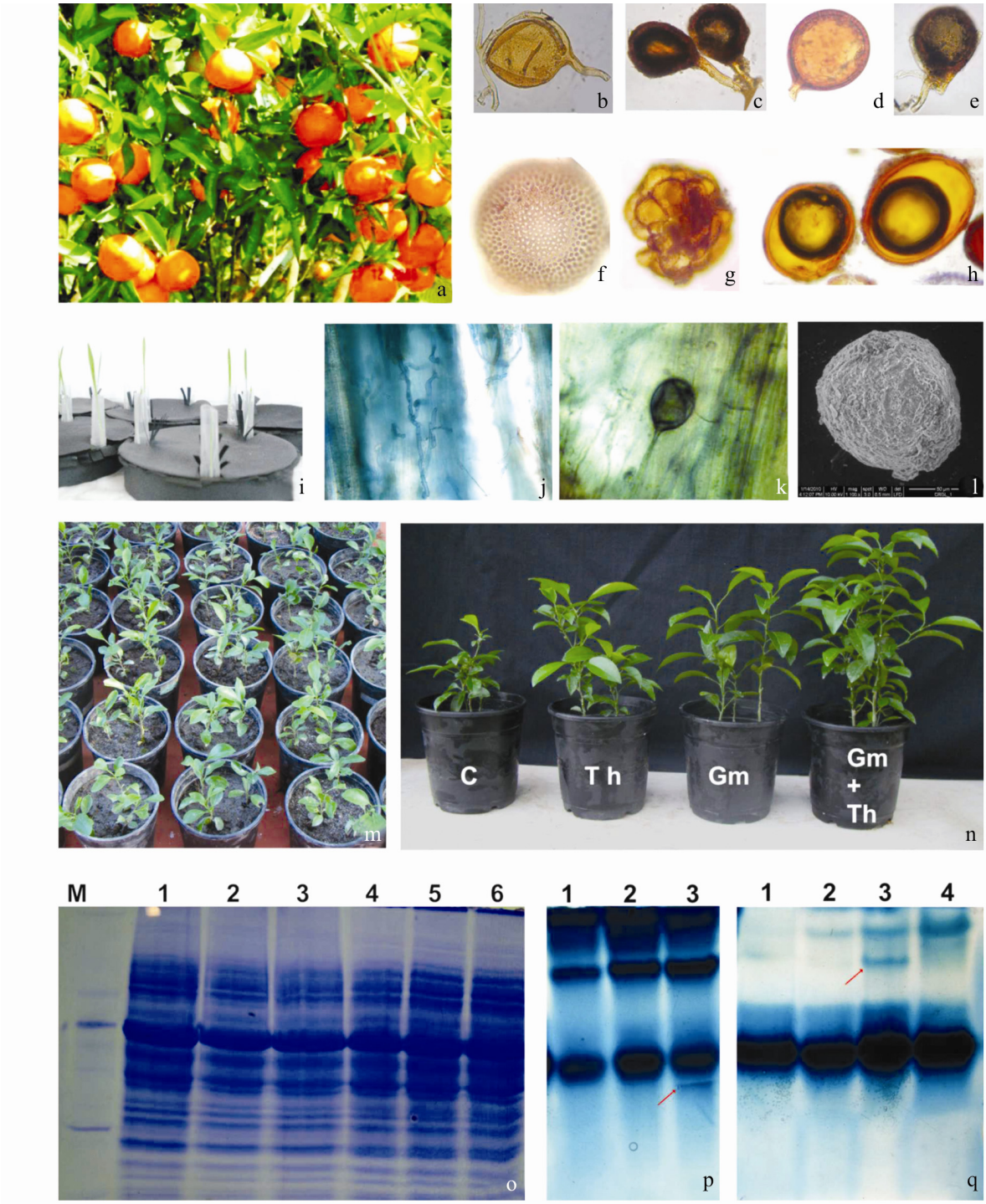

Figure 1. a = *Citrus reticulata*; b = spore of *Glomus mosseae*; c = *G. fasciculatum*; d = *G. badium*; e = *G. fasciculatum*; f = *Acaulospora spinosa*; g = *Glomus* sporocarp; h = *Scutellospora rubra*; i = Sorghum seedlings inoculated with *G. mosseae*; j-k = root colonization with *G. mosseae*; l = SEM of *G. mosseae*; m = mandarin seedlings; n = plants inoculated with *G. mosseae* and *T. hamatum* singly or jointly; o = SDS-PAGE of leaf proteins of mandarin plants following inoculation with *Glomus* and *Trichoderma*; p = PAGE of peroxidase of mandarin roots (lanes 1 = healthy; 2 = *T. hamatum* inoculated; 3 = *G. mosseae* inoculated); q = PAGE of peroxidase of mandarin leaves (lanes 1 = healthy; 2 = *T. hamatum* inoculated; 3 = *G. mosseae* and *T. hamatum* inoculated; 4 = *G. mosseae* inoculated)

Mycelial antigen of *F. solani* was analysed on SDS-PAGE (Fig. 2 a) and then using PAb of *F. solani*, western blot analyses were done (Fig. 2 b). 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 were confirmed as apple green fluorescence in young hyphal tip as well as in conidia of the pathogen (Fig. 2 d).

Amplification of target DNA through PCR with sequence specific primers are potentially more sensitive and rapid than microbiological techniques, as a number of constrains 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; Moukhamedove 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 18S small subunit (SSU), 5.8S, and 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* and *Trichoderma* spp. ITS region of rDNA was amplified using genus specific T/ITS1 and T/ITS4(for *Trichoderma*) and Fcg17F and Fcg17R (for *Fusarium*) primers. Amplified products of 550bp size was produced by *Fusarium* (Fig.2 e), while 600bp size was evident in case of *Trichoderma* (Fig. 2 h). The primer pairs Fcg17F and Fcg17R were highly specific for *Fusarium* genus. These results are also in accordance with several workers who observed the amplified rDNA fragment of approximately 600 bp by ITS-PCR in *Trichoderma* (Ospina et al 1999: Muthumeenakshi et al 1994; Lieckfiledt et al 1999; Mukherjee et al 2002, Venkateswarlu et al 2008, Chakraborty et al 2010). The genetic relatedness among isolates of *F. solani, F. oxysporum,* and *F. graminearum* among isolates of *Trichoderma hamatum* and *T. asperellum* were analyzed by random primers to generate reproducible polymorphisms. All amplified products with the primers had shown polymorphic and distinguishable banding patterns which indicated the genetic diversity of all isolates. A total of 54 reproducible and scorable polymorphic bands ranging from approximately 2000bp to 6000bp were generated in *F. solani, F oxysporum* and *F. graminearum* (Table 7, Fig. 2 f & g) and 34 reproducible and scorable polymorphic bands ranging from approximately 100bp to 2000bp were generated with primers among the isolates of *T. hamatum* and *T. asperellum* (Table 8, Fig. 2 I & j). In the RAPD profiles showed that primer OPD-6 scored highest bands which ranged between 200 to 6000 bp in all case of *T. asperellum* and *T. hamatum* isolates and A-5 showed maximum polymorphism among the isolates of *F. solani, F. oxysporum* and *F. graminearum* and random RAPD primer OPD-6 showed 87.5% polymorphism in the case of *T. asperellum* and *T. hamatum.*

Relationships among the isolates were evaluated by cluster analysis of the data based on the similarity matrix. The dendogram was generated by un weighted pair-group methods with arithmetic mean (UPGMA) using NTSYSpc software. Based on the results obtained all the four isolates can be grouped into two main clusters.

Table 7 Analysis of the polymorphism obtained with RAPD markers in *F. solani*, *F. graminearum, F oxysporum* and 4 *Trichoderma* isolates

Isolates	Seq Name	Total no RAPD bands	Approximate band size (bp). Min	Max.	Monomorphic bands	Polymorphic bands	Polymorphic (%)
Fusarium	OPA1	11	200	6000	0	11	100
Fusarium	OPA-4	17	200	6000	0	17	100
Fusarium	A-5	15	100	6000	0	15	100
Fusarium	A-11	11	100	6000	0	11	100
Trichoderma	A-11	06	100	2000	06	00	0
Trichoderma	OPD-6	08	100	2000	01	07	87.5
Trichoderma	AA-04	07	100	1000	04	03	42.85

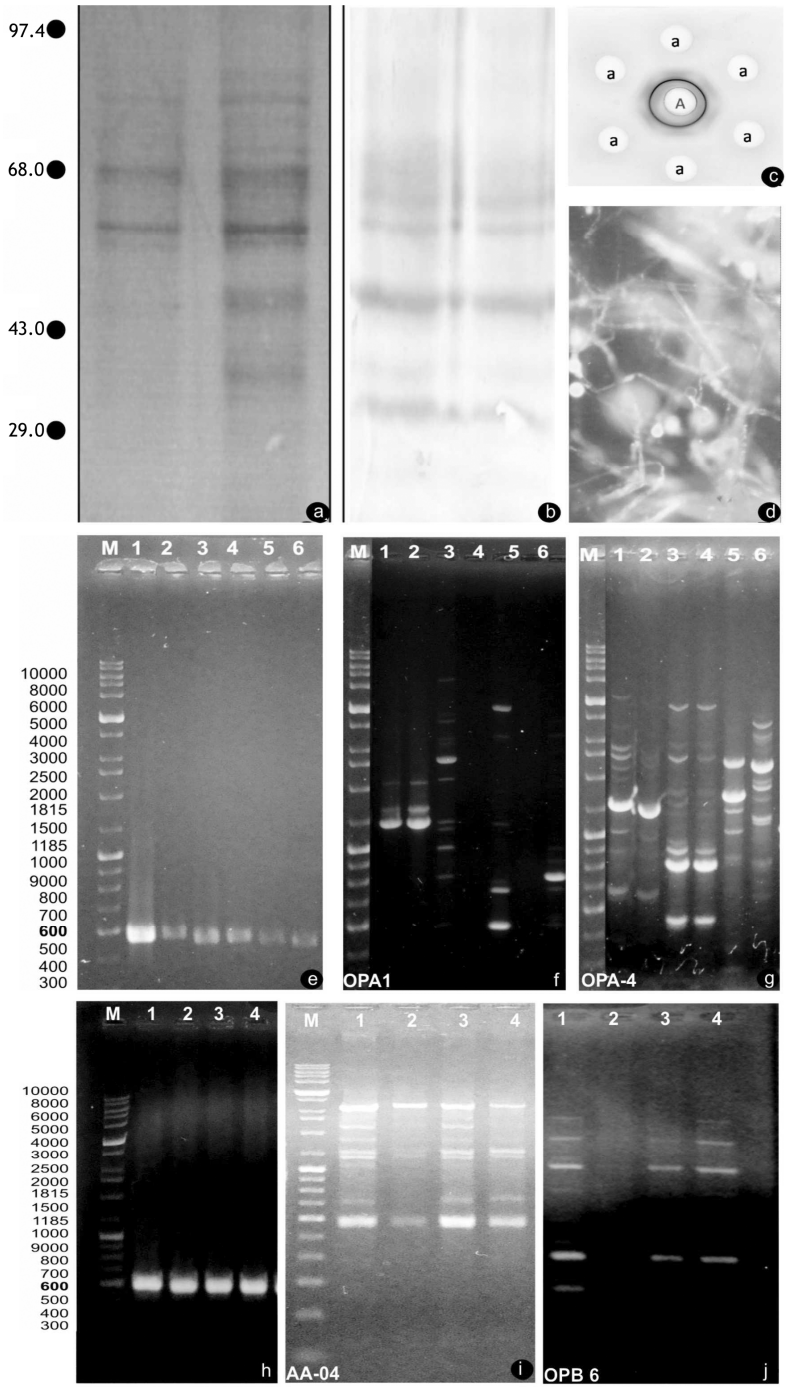

Figure 2. SDS PAGE of protein profile (a), Western Blot (b) and immunodiffusion (c) of antigens of *F solani* (peripheral wells) treated with homologous PAb(central well). d = immunofluorescence of hyphae labeled with FITCconjugate; e = agarose gel (2%) electrophoresis of ITS region, lane M-high range DNA ladder, 1-2 for *F. solani*, 3-4 for *F graminearum,* 5-6 for *F oxysporum*; RAPD analysed with primer OPAl (f) and with primer OPA-4 (g); h = asgarose gel of ITS region, lanes 1-2. *Trichoderma asperellum* and 3-4 *T hamatum*; i-j = agarose gel (2%) electrophoresis of RAPD, lanes 1-4 for *T asperellum* and *T hamalum* with primer AA-04 and OPB 6, respectively.

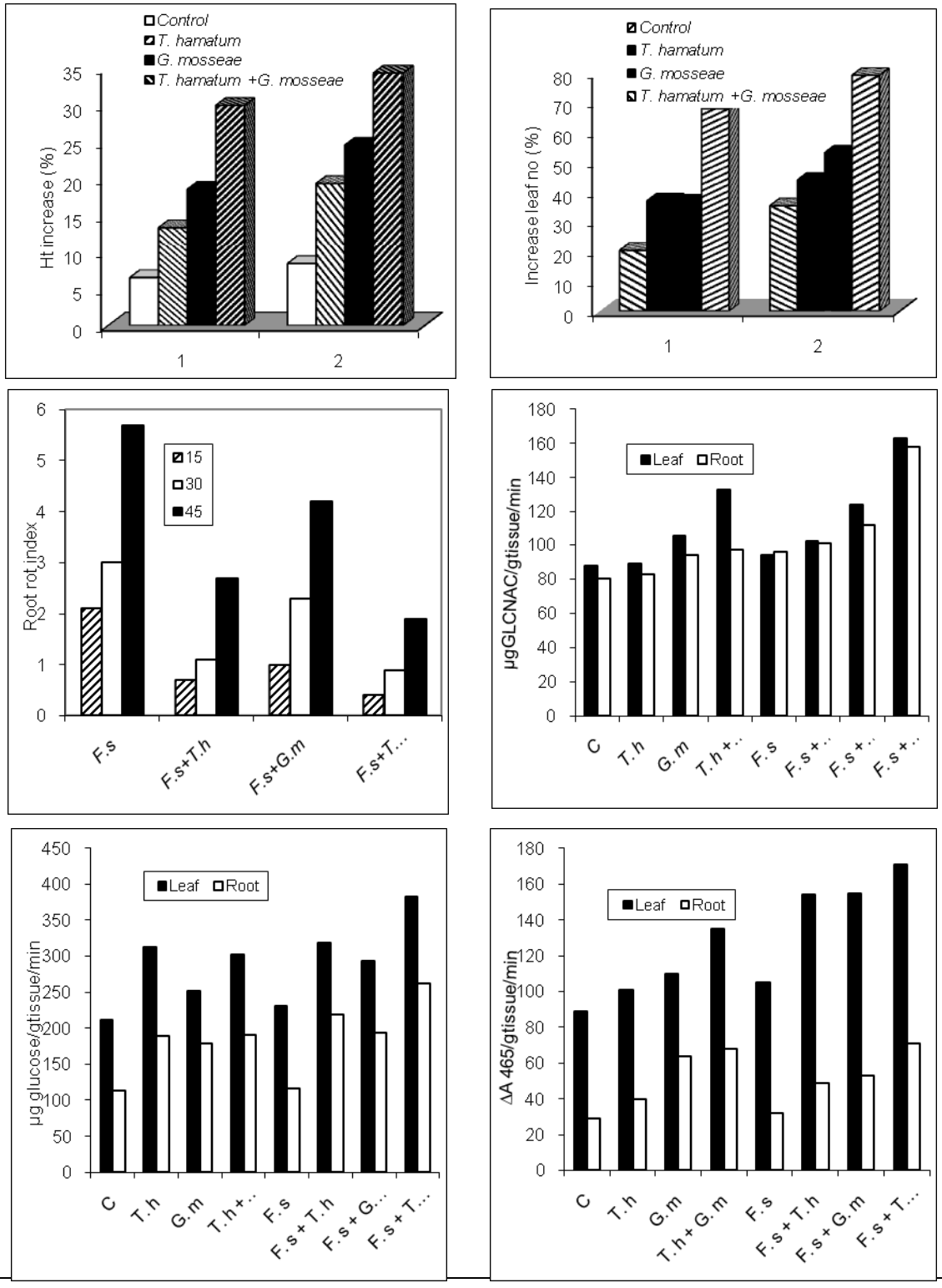

Figure 3. Effect of application of *T. hamatam* and *G. mosseae* on growth of citrus seedlings: increases in ht and leaves (in 2 months) (top); on root rot caused by *F. solani* and chitinase (middle); β-1, 3 glucanase and peroxidase activities

Table 8. ELISA and Dot-Blot values of reactions between PAbs of defense enzymes and enzyme extracts from treated mandarin plants

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	Light pink	0.049	Light pink
F.solani inoculated	0.036	Light pink	0.052	Light pink
F.solani+ *G.mosseae* treated	0.520	Deep purplish	0.368	Dark pink
F.solani + *T. hamatum* treated	0.768	Dark pink	0.445	Dark pink
F.solani + *G.mosseae* + *T. hamatum*	0.982	Deep purplish	0.865	Deep purplish

One cluster represents *T. hamatum* and other *T. asperellum*. In the case of the RAPD profile of *F. solani, F. graminearum* and *F. oxysporum* grouped into three main clusters. The clusters consist of *F. solani, F. graminearum* and *F. oxysporum* respectively. RAPD banding patterns reveled that the isolates of *F. solani, F. graminearum* and *F. oxysporum* were genetically different and showed polymorphism among each other.

The present work was aimed at developing a management strategy to control root rot and wilt complex of mandarin plants by biological means. Antibiosis to *F. solani* by biocontrol agent (*T. hamatum*) was evaluated in vitro and in vivo. The application of *T. hamatum* to the soil as a biocontrol agent, in glasshouse, not only resulted in reduced disease severity but also enhanced plant growth. 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). 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 numbers of leaves (Fig. 3). Joint inoculation with both the microorganisms (G. *mosseae* and *T. hamatum*) gave most significant results (Fig. 1, m & n). Defense responses of mandarin plants were demonstrated during early stages of root colonization by *T. hamatum* and *G. mosseae.* Marked reduction in disease development was evident following dual inoculations of G. *mosseae* and *T. hamatum.* The observed root rot reduction following separate and dual application of *G. mosseae* and *T. hamatum* may be correlated with increased accumulation of defense enzymes such as chitinase, β-1, 3- glucanase and peroxidase. 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* (Fig 1, p, lane 3). However, new isoforms were noticed in mandarin leaves following inoculation of roots with *G. mosseae* and *T. hamatum,* singly or jointly (Fig. 1, q). Interestingly dual application of *G. mosseae* and *T. hamatum* induced additional isozyme (Fig. 1, q, lane 3). 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 as evident in immunological assays (Fig.1, O &Table 9). 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)

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.

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References

Boller T and Mauch F. 1988. Colorimetric assay for chitinase. *Meth Enzymol*. 161: 430-435.

Bruns TD, White TJ and Talyor JW. 1991. Fungal molecular systematics. *Annu Rev Ecol Sys.* 22: 525-564.

Bryan GT, Daniels MJ and Osbourn AE. 1995. Comparison of fungi within the Gaeumannomyces-Phialophora complex by analysis of ribosomal DNA sequence. *Appl Environ Microbio.* 61: 681-689.

Chakraborty BN. 1988. Antigenic disparity. In Experimental and Conceptual Pathology ed Singh, U S, Heiss, W M and Weber D J. pp. 477-484. India: Oxford and IBH publishing Company**.**

Chakraborty BN and Chakraborty U. 2003. Immunodetection of Plant Pathogenic Fungi. In Frontiers of Fungal Diversity in India ed. Rao GP, Manoharachari C, Bhat DJ, Rajak RC and Lakhanpal TN pp. 23-41. India: International Book Distributing Company.

Chakraborty BN, Basu P, Das R, Saha A and Chakraborty U. 1995. Detection of cross reactive antigens between *Pestalotiopsis theae* and tea leaves and their cellular location. *Ann Appl Biol.* 127, 11–21.

Chakraborty BN, Chakraborty U, Saha A, Dey PL and Sunar K 2010. Molecular characterization of *Trichoderma harzianum* isolated from soils of North Bengal based on rDNA markers and analysis of their PCR-RAPD profiles. *Global Journal of Biotechnlogy & Biochemistry* 5: 55-61.

Chakraborty BN and Purkayastha RP 1983. Serological relationship between *Macrophomina phaseolina* and soybean cultivars. *Physiol Plant Pathol* 23, 197–205.

Chakraborty BN and Sharma M 2007. Serological detection and immunogold localization of cross-reactive antigens shared by *Camellia sinensis* and *Exobasidium vexans Journal of Applied Microbiology* 103: 1669-1680.

Chakraborty U and Chakraborty BN. 1989. Interaction of *Rhizobium leguminosarum* and *Fusarium solani* f. sp. *pisi* on pea affecting disease development and phytoalexin production. *Can. J. Bot.* 67: 1698-1701.

Chakraborty U, Chakraborty BN, Basnet M 2006. Plant growth promotion and induction of resistance in *Camellia sinensis* by *Bacillus megaterium*. *J Basic Microbiol* 46: 186-195.

Chakraborty U, Chakraborty BN, Kapoor M 1993. Changes in the levels of peroxidase and phenyl alanine ammonia lyase in *Brassica napus* cultivars showing variable resistance to *Leptosphaeria maculans*. *Folia Microbiol* 38: 491-496.

Chakraborty U, Sarkar B and Chakraborty BN 2003.Protection of soybean root rot by *Bradyrhizobium japonicum* and *Trichoderma harzianum*, associated changes in enzyme activities and phytoalexins production. J Myol Pl Pathol 33: 21-25.

Chakraborty BN, Chakraborty U, Barman BC, Bhutia LP and Ghosh PK 2007. Induction of systemic resistance in tea plants against root rot pathogen upon field application of VAM, phosphate solubilizing fungus and bacterium. In: Rhizosphere Biotechology: Plant Growth Retrospect and Prospect. (Eds A K Roy, B N Chakraborty, D S Mukadam and Rashmi) Scientific Publishers, Jodhpur, 61-71.

Chet, I. (1987) *Trichoderma*- application, mode of action and potential as a biocontrol agent of soilbome plant pathogenic fungi, ln: Innovative approaches to plant disease control. *Edition John Wiley and Sons*, New York. 137-160.

Chettri GB, Gurung TR and Roder W 2006. A Review on Seed Sector Development. Department of Agriculture, Thimphu.

Clausen, J 1988. Immunochemical techniques for the identification and estimation of macromolecules. In: RH Burden and PH Van Knippenberg (Eds.) Laboratory Techniques in Biochemistry and Molecular Biology. Vol-1 Part III p. 464.

Daniels BA and Skipper HD 1982. Methods for the recovery and quantitative estimation of propagules from soil. p. 29-35. In: N Schenck(eds.), Methods and Principles of mycorrhizal Research Amer. Phytopathol. Soc., St. Paul, Minnesota.

DeVay JE and Adler HE 1976. Antigens common to host and parasites. *Annu Rev Microbial* 30, 147-168.

Egberongbe HO, Akintokun AK, Babalola OO and Bankole MO 2010**.** The effect of *Glomus mosseae* and *Trichoderma harzianum* on proximate analysis of soybean (Glycine *max* (L.) Merrill.) seed grown in sterilized and unsterilized soil. *J Agricul Ext Rur Develop*. 2(4): 54-58.

Elad, Y., Chet, I. and Karan, J. (1980) *Trichoderma harzianum*: A biocontrol agent effective against *Sclerorium rotfsii* and *Rhizoctonia solani*. *Phytopathology* 70: l19-l2l

Gawande SJ, Chimote VP, Shukla A and Gadewar AV 2006. Serological and Molecular diagnosis of plant pathogens. In plant Protection in New Millenium ed. Gadewar AV and Singh BP pp. 563-588.

Gerdemann JW and Nicolson TH 1963. Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. *Trans Br Mycol Soc* 46: 235-244.

Harman GE (2000) Myth and dogmas of biocontrol changes in perceptions derived from research on *Trichoderma harzianum***.** T-22**.** *J. Plt Dis***.** 84: 377-393**.**

Hibbett DS 1992. Ribosomal RNA and fungal systematics. *Trans Mycol Soc* 33: 533-556.

Kitagawa T, Sakamoto Y, Furumi K and Ogura H 1989. Novel enzyme immunoassays for specific detection of *Fusarium oxysporum* f.sp. *cucumerinum* and for general detection various *Fusarium* species. *Phytopathology* 79: 162-165.

Knudsen D, Beegle D 1988. Recommended phosphorous tests.122-125.In: WC Dahnke (ed), Recommended chemical soil tests procedures for the north central region. Bull North Dakota Agric Exp Stn. North Dakota, USA No. 499.

Laemmli UK 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T4, *Nature* (Lond.) 227-680-685.

Lange L, Heide M and Olson LW 1989. Serological detection of *Plasmodiophora brassiceae* by dot immunobinding and visualization of the serological by scanning electron microscopy. *Phytopathology* 79: 1066-1071.

Lieckfiledt E, Samules GJ, Helgard I, Nirenderg and Petrini O 1999. A morphological and molecular perspectives of *T. viride* is it one or two species? *Appl Environ Microbiol* 65(6):2418-2428.

Mehlich A 1984. Mehlich 3 Soil test extractant: A modification of the Mehlich 2 extractant. Comm. *Soil Sc Plant Anal* 15: 1409-1416.

Moricca S, Ragazzi A, Kasuga T and Mitchelson KR 1998. Detection of *Fusarium oxysporum* f. sp. *vasinfectum* in cotton tissue by polymerase chain reaction. *Plant Pathol* 47: 486-494.

Moukahmedov R, Hu X, Nazar RN and Robb J 1994. Use of polymerase chain reaction-amplified ribosomal intergenic sequence for diagnosis of *Verticillium tricorpus*. *Phytopathology* 84: 256-259.

Mukherjee PK, Verma A and Latha J 2002. PCR fingerprinting of some *Trichoderma* isolates from two Indian type culture collection- a need for re-identification of these economically importance fungi. *Science Correspondence* 83(4): 372-374.

Mukhopadhyay S and Thapa K 2001. Citrus: Legacy and biodiversity in Eastern Himalayas.

Mukhopadhyay S, Jaishree R, Sharma BC, Gurung A and Sengupta RK 1996. Production of Disease free Darjeeling orange plants by shoot tip grafting technique. *Proc Int Soc Citriculture* 2: 855-857.

Muthumeenakshi S, Mills PR, Brown AE and Seaby DA 1994. Interspecific molecular variation among *T. harzianum* isolates colonizing mushroom compost in the British Isles. *Microbiol Reading* 140(4):769-777.

Nazar RN, Hu X, Schmidt J, Culham D, Robb J 1991. Potential use of PCR-amplified ribosomal intergenic sequences in the detection and differentiation of *Verticillium* wilt pathogens. *Physiol Mol Plant Pathol* 39: 1-11.

Ospina MD, Royse DJ, Chen X and Romaine P 1999. Molecular Phylogenetic analysis of biological control strains of *T. harzianum* and other biotypes of Trichoderma spp. Associated with mushroom green mold. *Phytopathology* 89(4): 313-317.

Ouchterlony O 1967. Immunodiffusion and immuno-electrophoresis. In: DM Weir (ed.). Handbook of Experimental Immunology. *Blackwell Sci. Publ*., Oxford and Edinburgh: p655.

Ousley MA, Lynch M and Whipps JM (1994) Potential of Trichoderma as consistent plant growth stimulation. *Biol. Fertilizer Soils* 17: 85-90

Pan SQ, Ye XS, Kue J 1991. A technique for detection of chitinase, β- 1,3- glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis and isoelectric focusing. *Phytopathology* 81:970-974.

Rohlf FJ 1993. NTSYSpc: Numerical Taxonomy and Multivariate analysis system. Ver 2.11 W. Exteer Software: Setauket, N Y. Science and Culture 67(1-2): 42-46.

Schilling AG, Möller EM, Geiger HH 1996. Polymerase chain reaction-based assays for specific detection of *Fusarium culmorum*, *F. graminearum*, and *F. avenaceum*. *Phytopathology* 86: 515-522.

Sundaresan P, Raja UN and Gunasekaran P 1993. Induction and accumulation of phytoalexins in cowpea roots infected with a mycorrhizal fungus *Glomus fasciculatum* and their resistance to *Fusarium* wilt disease. *J Biosciences* 18: 291-301.

Thapa KK 2007. Citrus: An indigenous crop of Darjeeling hills. *Asian Agri-History* 11(2): 133-137.

Venkateswarlu R, Reddi KM, Reddy ENP and Sudhakar P 2008. Molecular characterization of *Trichoderma* spp. used against *Fusarium* wilt with PCR based RAPD and ITS-PCR. *Journal of Mycology and Plant Pathology* 38(3):569-563.

Wakeham AJ and White JG 1996. Serological detection in soil of *Plasmodiophora brassicae* resting spores. *Physiol Mol Pl Pathol* 48: 289-303.

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Root colonization of mandarin plants grown in orchards of Darjeeling hills and plains with Arbuscular Mycorrhizal Fungi and their effects on plant growth

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Abstract
Citrus reticulata is an ancient commercial crop being cultivated in Darjeeling-Sikkim hills. Many diseases are prevalent in mandarin plants, out of them, bacterial & fungal diseases are mostly dangerous. Arbuscular Mycorrhizal Fungi were screened from rhizosphere of mandarin plants from the four different regions using wet sieving and decanting method. Microscopical observation revealed the presence of different genus of AM fungi present in the root as hyphae, spores and sporocarp. *Glomus mosseae, G. fasciculatum, G. aggregatum, G. badium, G. constrictum, G. versiforme, Gigaspora gigantea, G. margarita, Acaulospora capsicula, A. bireticulata, Sclerocystis* and *Scutellospora rubra* were found to be dominant in all the soil samples of mandarin. Species of *Glomus* were found to be high in both hilly and foothill regions. *Glomus mosseae* and *G. fasciculatum* were selected for mass multiplication in maize plant in pots. Histopathological study of root showed the presence of vesicles and arbuscules. AMF infection and total number of spores per 100 gram of soil were recorded. Scanning Electron Microscopy (SEM) of AMF spores of mandarin revealed clear morphology, spore wall characters and hyphal attachment of spores. Total phosphate content of the soil, soil analysis and enzyme activities in roots and leaves of mandarin plant from the different regions were studied. Three major defense enzymes peroxidase, chitinase and β-1,3- glucanase showed enhanced activities and the total phosphate content also decreased in soil with respect to control. Present study evaluates the effect of AMF in plant growth and phosphate solubilization.

Keywords: *Citrus reticulata*, *Glomus mosseae, G. fasciculatum*

Mandarin orange (*Citrus reticulata* Blanco) the loose jacket orange which is a principal cash crop of India is cultivated to an alleviation of 1500 mm in Darjeeling – Sikkim hills. It belongs to the family Rutaceae under the order Sapindales. Mandarin is grown in the tropical / sub -tropical regions 35° N to 35° S of equator. There are four natural and one hybrid cultivars of mandarin in India grown in five different belts as a dominant cash crop. The total production is reckoned at about 760 thousand tonnes per year. Loose jacketed orange such as Nagpur Santra, Coorg orange, Kamala orange of Manipur, Khasi orange of Assam, Sikkim orange of Darjeeling and Kinnow of Punjab belong to the category of Mandarins in India.

Mandarin is widely consumed as fresh fruit and also used for producing canned segments, juice- concentrate, squash, beverages, jams as well as marmalades. The peel of Mandarin is the source of essential oils which are used in the cosmetic and pharmaceutical industries (Frazier and Westhote, 1978). It can also serve as a basic material for the production of cattle feed, candies and alcohol.

However, mandarin orange is susceptible to various pest and diseases which results to decline in production and productivity. The intensity sometimes is so severe that thousands of hectare cultivated areas are declined every year which is commonly referred as citrus decline or citrus dieback. The commons citrus diseases are growing, tristeza, cranker, foot/root rot, wilting etc. Root rot is an alarming problem of Darjeeling mandarin and one of its important causal organism is *Fusarium* spp.

AMF are an important group of soil–borne microorganisms that contribute sustainability to the establishment, productivity and longevity of natural or man-made ecosystems by the virtue of forming a symbiotic association with most terrestrial plants by forming an extensive network of external hyphae functioning as plant rootlets by spreading into a vast area underground and absorb nitrogen, phosphorus, potassium, calcium, sulfur, ferric, manganese, copper and zinc from the soil and then translocate these nutrients to the plants with those roots they are associated (Gerdemann, 1975). The symbiotic associations of AMF with most terrestrial plans are well documented but there are only few reports of symbiotic association between mandarin plant and AMF. The extrametrical fungal hyphae can extend several centimeters into the soil and absorb large amounts of nutrients for the host root (Khan *et al*., 2000).

These extraradical hyphal networks and their hyphae help in improving the texture of the soil as they contain and release glomalin, which is a putative glycoprotein,

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assayed from soil. Glomalin is a Glomalin-related soil protein (GRSP) that is correlated with aggregate water stability (Wright and Upadhyaya 1998, Rillig 2004; Rillig and Mummey 2006). Improved soil structure increases water infiltration and can reduce soil erosion (Tisdall and Oades 1982).Efforts are being undertaken to develop a bio formulations which can minimize the disease occurrence.

Considering the importance of association of AMF with mandarin orange the present investigation was made to assess the AMF population from three different locations of Darjeeling hills (Kalimpong, Mirik, Bijanbari). For the assessment of AMF population in plains, rhizosphere soil was collected from mandarin plants being grown in experimental garden of Immuno-Phytopathology Laboratory, Department of Botany, NBU.

Materials and method

Isolation of AMF spores

Arbuscular mycorrhizal fungal spores were screened from those soil samples of Mandarin rhizosphere by the wet sieving and decanting method (Gerdeman & Nicholson, 1963). Soil samples (100gm each of the representative root zone) were collected, suspended in water (1 l) in order to obtain a uniform suspension. Soil clusters are carefully dispersed in the water and is kept for 10 minutes to settle down the heavy particles. Aqueous suspension was passed through a set of sieves of different pore size (200, 170, 150, 80, 50µm) arranged one below the other. The spores were picked by the help of fine bristles / brushes and transferred to grooved slides or vials and observed under dissecting microscope. Few spores were stained with Melzar's reagent and studied under sterio-microscope. Healthy spores are separated by fine brush and are stored in autoclaved glass vials either in sterile distilled water or Ringer's Solution (8.6gm NaCl, 0.3gm KCl, 0.33gm $CaCl_2$ in one liter of boiled distilled water) at 4ºC for further study and observation. It is evident from various studies that each plant has multiple AM fungi population. The soils of the collected samples were further analyzed to know the chemical composition of the soil, viz. moisture content, pH, amount of carbon, N_2 etc.

Plant material

Citrus reticulata seedlings were obtained from orchards of Kalimpong, Mirik, Bijanbari and experimental field of Immuno-phytopathology Laboratory of North Bengal University, Siliguri. They were maintained in 12" earthen pots with sterilized soil.

Identification of AMF spores

Spore samples were separated according to their morphology, size, colour, shape, wall thickness, wall layers, and other accessory structures like hyphal attachment etc. for the purpose of identification. The spores were identified up to species level with the help of standard keys (Walker 1981; Schneck and Perez 1987). Spores were critically examined with special reference to variation in vesicles (size, shape, wall thickness, wall layers, position and abundance), hyphal branching patterns, the diameter, structure (especially near entry points) and the staining intensity of hyphae.

Spore count

Rhizosphere soil (100g) was taken and suspended in 250 ml water. Wet sieving and decanting method was used for isolation of spores. Total number of spores were then counted and spore percentage of different genera was obtained.

Histo-pathological analysis

The root specimen were taken from field and washed with tap water. The root were cut into pieces, after washing treated with 10%KOH added, kept in water bath for 1h, then 1% HCL was added to neutralize the alkalinity. The root pieces were then washed with water (after 30 min) and staining was done by simmering the roots in cotton blue: lactophenol(1:4) for 3-4min with mild heating. Degree of contrast between fungal tissues and back ground plant cells was obtained according to the duration of storage of tissues. 1% HCl was added to acidify the tissues, as most histological stains are acidic. A little amendment in this process is noteworthy because it has been noticed that extraradical spore bearing hyphae and other extraradical fungal tissues with root segments are destroyed or dissolved when it is boiled in hot water bath at 90˚C twice with 2% KOH followed by 0.05 cotton blue and lacto glycerol for staining the internal structures of AMF inside the root segments i.e. arbuscules, vesicles, auxiliary cells etc. The total staining process can be done without heating but keeping the root fragments in 1-2% KOH for 24-48 hours in a Petri dish and another 12 to 18 hours in cotton blue and lactoglycerol with minimum movements of the samples yields remarkable result. In this method the spore bearing hyphal structures, auxiliary cells etc. are clearly visible and percent colonization can be determined with better accuracy. After preparing the roots the hyphal structures were viewed under dissecting stereomicroscope under 20X and 40X magnification. Percent root colonization was estimated by using slide method as described by Giovannetti and Mosse (1980).

Mass multiplication of AM spores

Glomus mosseae and *G. fasciculatum* were selected from among the mass of other AM fungi with the help of fine tweezers under dissecting microscope. The spores were washed several times with distilled water and Chloramin T to remove adhered debris. They were then inoculated in roots of 7-10 days old maize seedlings which were grown in petri plates. After inoculation they were transferred to black plastic pots (12 inch) having autoclaved soil to discard the presence of other fungal propagules. After 45 days the presence of spores of *G. mosseae* and *G. fasciculatum* were confirmed.

Artificial inoculation of mandarin roots

Healthy spores of *G. mosseae* and *G. fasciculatum* were collected from the maize plants and rinsed with sterile distilled water. Filter paper were cut into small circles of 5 mm diameter and about 5-6 spores were transferred to the filter paper. The paper was then adhered to the roots

Table 1: Physico-chemical factors and AM infection (%) in rhizosphere soil samples of mandarin

Factors	Kalim-pong	Mirik	Bijan-bari	Foot-hills
Soil type:	Clay	Sandy clay	Clay	Clay
Sand(%):	48	54	42	46
Silt(%):	10	04	16	14
Clay(%):	42	42	42	40
pH:	6.01	4.81	4.31	5.07
Moisture(%):	21.64	19.27	11.62	20.95
P_2O_5 ppm:	23.94	34.82	20.67	31.35
K_2O ppm:	31.34	197.67	84.89	48.97
Organic C(%):	0.48	1.11	1.31	0.75
Nitrogen(%):	0.05	0.11	0.14	0.08
Colonization (%):	98	79	86	84

of 1 month old seedlings of mandarin plants with the help of tweezers.

Extraction and quantification of soil phosphate

Soil sample (1g) was air dried and suspended in 25 ml of the extracting solution (0.025N H_2SO_4, 0.05N HCl) to which activated charcoal (0.01g) was also added, shaken well for 30 min on a rotary shaker and filtered through Whatman No. 2 filter paper (Mehlich 1984). Ammonium molybdate-ascorbic acid method was followed for quantitative estimation of phosphate as described by Knudsen and Beegle (1988).

Assay of enzyme activities

Leaves and roots of mandarin seedlings grown in treated or control potted soil collected from different regions were used for all biochemical analyses. Samples were collected for assay 1 month after inoculation.

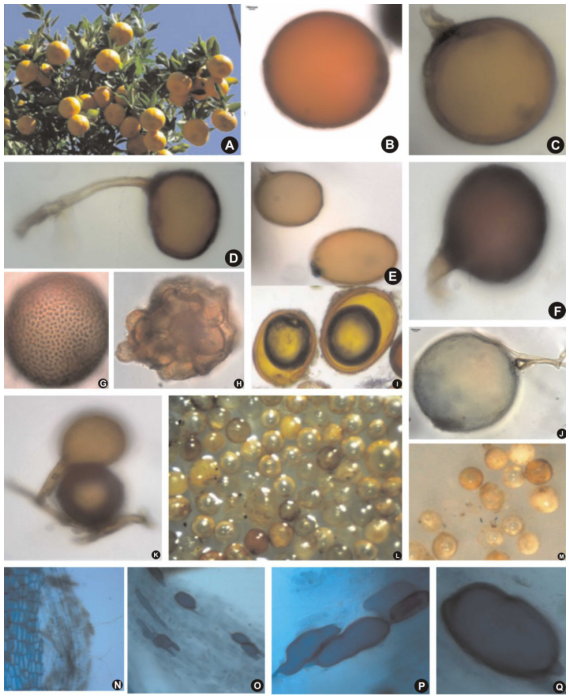

Figure 1 A: Fruit bearing mandarin plant; B: *Acaulospora capsicula*, C: *Giomus mosseae*, D: *G. mosseae* with long hyphal attachment, E: *G. badium*, F: *G. constrictum*, G: *Acaulospora bireticulata*, H: Sporocarp of *Glomus*, I: *Scutellospora rubra*, J: *Gigaspora margarica*, K: *Glomus fascicuiata*, L: Mass multiplied spores of *Gigaspora giganta*, M: Enlarged view of the same, N: Mycorrhizal hyphae in root tissue, O-Q: Vesicle, flattened vesicles and single enlarged view of vesicle

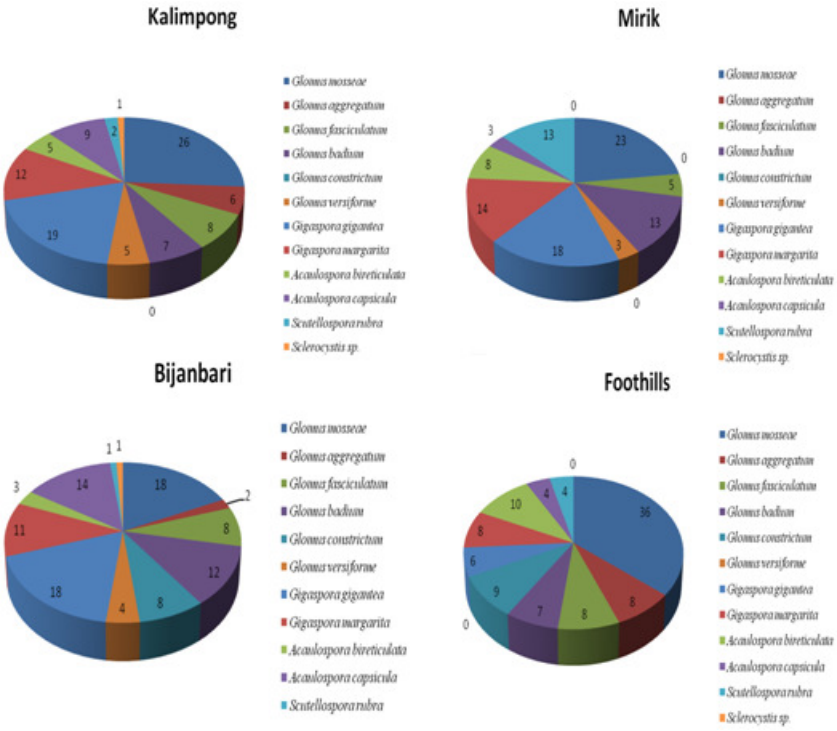

Figure 2: Percentage population of dominant AMF spores in mandarin soil

Table 2: Microscopic characters of AMF Spores associated with mandarin roots

Genus & species	Colour	Shape	Spore layer	Spore size (µm)	Other descriptions
Glomus fasciculatum	Pale yellow to bright brown	Globose to subglobose	3	70-120	Spore layer continuous
Glomus mosseae	Brown to orange -brown	Globose to subglobose	3	200	Hyphae are double layered
Glomus aggregatum	Pale yellow	Globose to oval	1-2	200-1800 x 200-1400	Sporocarps formed in loose clusters
Glomus badium	Reddish brown to dark brown to black	Globose, subglobose to ovoid	3	51-90 x 75-120	Subtending hypha of each spore is usually very short
Glomus constrictum	Brownish orange to dark brown	Globose to subglobose, sometimes ovoid	2	110-130 x 150-160	Subtending hyphae straight or curved, usually markedly constricted at the spore base
Glomus versiforme	Orange to red brown	Globose to subglobose, sometimes ovoid	2	60-160	Sporocarps are irregular, they arise from a basal pad of pale grayish yellow, loose mycelium with a few interspersed spores
Gigaspora gigantea	Greenish yellow	Globose to subglobose	2	250-270 x 265-370	Formed terminally or laterally on a bulbous sporogenous cell
Gigaspora margarita	Yellowish white to sunflower yellow	Globose to subglobose	2	300-340 x 360-380	Spores produced singly in the soil, blastically at the tip of a bulbous sporogenous cell
Acaulospora capsicula	Orange red to capsicum red	Globose to subglobose	3	220-310 x 290-440	Sporiferous saccule pale yellow to brownish yellow which usually falls off when spores mature
Acaulospora bireticulata	Brownish	Globose	3	280-410	Surface ornamentation is prominent. Spores are borne laterally from the neck of a sporiferous saccule.
Scutellospora rubra	Dark orange-brown to red-brown	Globose to subglobose	3	140-220	Germinal walls are formed completely separate from the spore wall
Sclerocystis	Brown to blackish brown	Globose to subglobose		300-600 x 400-700	Chlamydospores arranged side by side in a single layer radially arranged on a central plexus of hyphae

Table 3: Soil phosphate (µg/g tissue) content in rhizosphere of mandarin plants after application of microorganisms

	Kalimpong	Mirik	Bijanbari	Foothills
Control	47.19± 0.625	49.13 ± 0.0144	48.39 ± 0.387	47.12 ± 0.071
Glomus mosseae	33.67 ± 0.287	32.45 ± 0.262	31.99 ±0.41	33.06 ±0.461
G. fasciculatum	31.75 ± 0.2165	31.64 ± 0.086	32.40 ± 0.318	32.03 ± 0.14
G. mosseae + *G. fasciculatum*	30.38 ± 0.198	30.23 ± 0.296	29.85 ± 0.13	29.58 ± 0.29±

Peroxidase (POX, EC1.11.1.7.). Extraction and assay of peroxidase was done following the method described by Chakraborty *et al* (1993). O-dianisidine was used as substrate and activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2. Specific activity expressed as the increase in Δ A 465/g tissue/ min.

Chitinase (CHT, EC 3.2.1.14). Chitinase was extracted and assayed following the method of Boller and Mauch (1988). The amount of GlcNAc released was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as µg GlcNAc released /min/ g fresh wt. tissue.

β- 1,3- glucanase (β-GLU, EC 3.2.1.38). β-1,3-glucanase was extracted and assayed from the samples following the method of Pan *et al* (1991). The amount of glucose liberated was determined spectrophotometrically using a standard curve. Activity was expressed as µg glucose released /min/g tissue.

Table 4: Activities of β-1, 3 glucanase in leaves and roots of mandarin following application of *G. mosseae* and *G. fasciculatum*

Glucanase	Kalimpong		Mirik		Bijanbari		Foothills	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Control	212	113	200	105	198	123	215	118
Glomus mosseae	313	189	323	156	309	176	320	168
Glomus fasciculatum	252	179	263	163	261	175	250	173
G. mosseae + *G.fasciculatum*	302	191	346	170	304	169	321	198

Table 5: Peroxidase activity in leaves and roots of mandarin following application of *G. mosseae* and *G. fasciculatum*

Peroxidase	Kalimpong		Mirik		Bijanbari		Foothills	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Control	89	29	78	30	88	36	76	32
Glomus mosseae	110	64	102	61	120	71	116	72
G. fasciculatum	101	40	105	45	106	42	98	50
G. mosseae + *G.fasciculatum*	135	68	129	70	131	72	140	73

Results and discussion

The benefits and wide host range of AM fungi has led to it being used as a bioinoculant to improve plant nutrition and growth. This study focussed on the use of AM fungi, its application and the quantification of the increased defence related enzymes responsible for disease resistance and subsequently improving plant health status. Arbuscular Mycorrhizal fungi from the selected places of hills (Kalimpong, Mirik, Bijanbari) and foothills were screened from the rhizosphere of mandarin plant. On observation it was found that *Glomus mosseae* dominated the AM population in all the soil samples followed by *G. fasciculatum.* Results are presented in Fig 2. Percentage of AM spores determined from different regions showed maximum of different *Glomus* sp., followed by *Gigaspora* sp., *Acaulospora* and *Scutellospora.* Histopathological study revealed the presence of vesicles and arbuscules in the root segments determining the fact that infection of the AM spores has taken place (Fig 1). Organisms of AMF have a bimodal pattern of differentiation (Morton, 1990). The vegetative thallus consists of arbuscules, intraradical vesicles (shared only by species in the suborder Glomineae), extraradical auxiliary cells (shared only by species in the suborder Gigasporineae), and intraradical and extraradical hyphae (Smith and Read, 1997; Morton and Benny, 1990). Arbuscules are finely branched structures in close contact with the cell plasma membrane, functioning in exchange of nutrients between host and fungal cells (Smith and Read, 1997). Hyphae are important in nutrient acquisition and as propagules to initiate new root colonization (Graham et al., 1982; Friese and Allen, 1991). Vesicles are globose structures arising from swelling of the hyphae and filled with glycogen granules and lipids and are considered to be storage structures (Bonfante-Fasolo, 1984; Brundrett, 1991).The different types of spores which were observed in the rhizosphere of mandarin soil have been identified and described as shown in Table 2. Spore colour, shape and size are the most prominent factors in their identification. The hyphae of each genus also differs in their morphology and number of wall layers.

Scanning Electron Microscopy (SEM) (Fig 3) of the spores revealed the spore wall morphology. *Glomus mosseae* has a rough outer surface. The outer layer is sloughed. Spores of *G. fasciculatum* are found in aggregates of 2-6. The surface is rough and shape is subglobose. The wall of *Gigaspora gigantea* is smooth walled with the typical bulbous suspensor. The polygonal reticulum on the spore wall surface is characteristic of *Acaulospora reticulata*.

Total phosphate content of soil had decreased due to application indicating that the plant could uptake phosphorus which had been solubilized by AMF (Table 3). Dual application of *G. mosseae* and *G. fasciculatum* was more effective in solubilising the insoluble phosphate present in the soil than when applied singly. This study aimed at improving the understanding of AM fungal interactions in the rhizosphere using a field trial which was to an extent successful. 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. hamatum*) gave most significant results (Allay and Chakraborty, 2010). *B. pumilus* along with *G. mosseae* could improve seedling growth in terms of height and leaf number and also helped in solubilising phosphate, suggesting a synergistic effect (Chakraborty *et al,* 2011). Fresh shoot

Table 6: Chitinase activity in leaves and roots of mandarin following application of *G. mosseae* and *G.fasciculatum*

Chitinase	Kalimpong		Mirik		Bijanbari		Foothills	
	Leaf	Root	Leaf	Root	Leaf	Root	Leaf	Root
Control	88	80	86	75	89	77	92	81
Glomus mosseae	106	94	105	93	98	99	110	101
G. fasciculatum	89	83	93	91	101	82	102	85
G. mosseae + *G.fasciculatum*	133	97	124	95	126	98	145	100

biomass was also found to be increased when tomato plants were treated with *Glomus mosseae, Acaulospora laevis* and *Trichoderma harzianum* (Tanwar *et al,* 2010).

The pH of soil varied from 4.5 to 6. The pH of Kalimpong soil is around 6.0 which is ideal for citrus plants (Table 1). Not much difference was seen in the availability of carbon and nitrogen respectively in the 4 different regions. The moisture content of Kalimpong soil is also the highest. Citrus trees prefer draining sandy loam soil hence the amount of sand in all the soil was high.

Activities of 3 defense enzymes- chitinase, 1,3 - β glucanase and peroxidase were assayed in leaves and roots of mandarin seedlings subjected to various treatments- i.e., *G. mosseae*, *G. fasciculatum* and *G. mosseae* + *G. fasciculatum*. Activities of all 3 enzymes, in both leaves and roots, were significantly enhanced due to the various treatments (Table 4, 5 & 6). The most significant was in dual application of AMF in all the cases. In an earlier study, when mandarin seedlings were pre-treated either with *B. pumilus* or *G. mosseae* prior to challenge inoculation with the pathogen (*Fusarium oxysporum*), activities of all three defense enzymes increased significantly (Chakraborty *et al,* 2011).

The overall results of the present study have shown that *G. mosseae* and *G. fasciculatum* can promote growth of mandarin plants. However, *G. mosseae* was found to be the best colonizer as well as responsible for induced accumulation of defense enzymes in the host plant.

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References

Allay, S. and Chakraborty, B.N. 2010. Activation of defense response of mandarin plants against *Fusarium* root rot disease using *Glomus mosseae* and *Trichoderma hamatum*. *Journal of mycology and plant pathology* Vol. 40(4) 2010. 499-511.

Boller ,T., and Mauch, F. 1988. Colorimetric assay for chitinase. Meth Enzymol. 161: 430-435.

Bonfante-Fasolo, P. 1984. Anatomy and morphology of VA mycorrhizae. Pp. 5-33. In: *VA Mycorrhiza.* C.Ll. Powell and D.J. Bagyaraj (Eds.). CRC Press, Boca Raton, Florida.

Brundrett, M.C. 1991. Mycorrhizas in natural ecosystems. *Advances in Ecological Research* 21:171-213.

Chakraborty, U., Chakraborty, B.N., Kapoor, M. 1993. Changes in the levels of peroxidase and phenyl alanine ammonia lyase in *Brassica napus* cultivars showing variable resistance to *Leptosphaeria maculans*. Folia Microbiol. 38: 491-496.

Chakraborty, U., Chakraborty, B.N., Allay, S., De U. and Chakraborty A.P. 2011. Dual Application of *Bacillus pumilus* and *Glomus mosseae* for Improvement of Health Status of Mandarin Plants *Acta horticulture* p. 215-229

Daniels, B.A. and Skipper, H.D. 1982. Methods for the recovery and quantitative estimation of propagules from soil. pp. 20-45 In: Methods and principles of mycorrhizal research, ed. by N. C. Schenck, *The American Phytopathological Society*, St. Paul.

Daniels, B.A. & Skipper, H.A. 1982. Methods for the recovery and quantitative estimation of propagules from soil. In Methods and Principles of Mycorrhizal Research. Ed. N C Schenk, *American Phytopathological Society*, St. Paul, Minn., pp 29-35.

Friese, C.F., and M.F. Allen. 1991. The spread of VA mycorrhizal fungal hyphae in the soil: Inoculum types and external hyphae architecture. *Mycological Research* 92:317-321.

Gange, A.C., and H.M. West. 1994. Interactions between arbuscular Mycorrhizal fungi and foliar-feeding insects in Plantago lanceolata L. *New Phytologist* 128:79-87.

Gerdemann J.W. and Nicolson T.H. 1963. Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. *Trans Br Mycol Soc* 46: 235-244.

Gerdemann, J.W. 1965. Vesicular-arbuscular mycorrhizae formed on maize and tuliptree by *Endogone fasciculata. Mycologia* 57: 562-575

Gerdemann, J.W. and Trappe, J.M. 1974. The Endogonaceae in the Pacific Northwest 5: 1-76.

Gerdemann, J.W . 1975. Vesicular arbuscular mycorrhizae. In: The Development and Function of Roots, (eds.). J.G. Torrey and D.T. Clarkson, Academic Press, New York. Pp. 575-595.

Graham, J.H., Linderman, R.G., and Menge, J.A. 1982. Development of external hyphae by different isolates of mycorrhizal *Glomus* spp. in relation to root colonization and growth of troyer citrange. *New Phytologist* 91:183-189.

Gupta, R., and Kumar, P. 2000. Mycorrhizal plants in response to adverse environmental conditions. p. 67-84. In K. G. Mukerji, B. P. Chamola and J. Singh (eds.) *Mycorrhizal Biology*. Kluwer Academic/Plenum Publishing, New York

Hall, I.R. and Abbott, L.K. 1984. Some Endogonaceae from southwestern Australia. *Trans. Br. Mycol. Soc.* 83: 203-208.

Ibijbijen, J., Urquaiga, S, Ismali, M, Alve, J.R. and Boddey, R.M. 1996. Effect of arbuscular mycorrhizal fungi on growth, mineral nutrition, and nitrogen fixation of three varieties of common bean (*Phaseolus vulgaris*). *New Phytologist* 134:353-360.

Jakobsen, I. 1999. Transport of phosphorus and carbon in arbuscular mycorrhizas. p.535- 542. In A. Varma and B. Hock (eds.) Mycorrhiza: Structure, Function, *Molecular Biology*, 2nd ed. Springer, Berlin.

Khan, A.G., Kuek, C., Chaudhry, T.M., Khoo, C.S., Hayes, W.J., 2000. Plants, mycorrhizae and phytochelators in heavy metal contaminated land remediation. Chemosphere 41, 197– 207.

Knudsen, D., Beegle, D. 1988. Recommended phosphorous tests.122-125.In: W.C. Dahnke (ed), Recommended chemical soil tests procedures for the north central region. Bull North Dakota Agric Exp Stn. North Dakota, USA No. 499.

Lu, X.H., and R.T. Koide. 1994. The effect of mycorrhizal infection on components of plant-growth and reproduction. *New Phytologist* 128:211-218.

Morton, J. B. 1990. Evolutionary relationships among arbuscular mycorrhizal fungi in the Endogonaceae. *Mycologia* 82:192-207.

Morton, J.B., and Benny, G.L. 1990. Revised classification of arbuscular mycorrhizal fungi (Zygomycetes): a new order, Glomales, two new suborders, Glomineae and Gigasporineae, and two new families, Acaulosporaceae and Gigasporaceae, with an emendation of Glomaceae. *Mycotaxon* 37:471-491.

Frazier, W.C and Westhofe, D.C 1978. Contamination, preservation and spoilage of vegetables and fruits. *Food Microbiology*. New York. McGraw Hill Book Company, 3rd edition 12:194-214.

Pan, S.Q., Ye, X.S. and Kue, J. 1991. A technique for detection of chitinase, β- 1,3- glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis and isoelectric focusing. Phytopathology. 81:970-974.

Rillig, M.C. 2004. *Arbuscular mycorrhizae, glomalin, and soil aggregation*. Canadian Journal of Soil Science. 84:355-363.

Rillig, M.C. and Mummey, D.L. 2006. *Tansley review: Mycorrhizas and soil structure*. New Phytologist (in press).

Rothwell, F. M. and Trappe, J. M. 1979. *Acaulospora bireticulata* sp. nov. *Mycotaxon* 8:471-475.

Schenck, N.C. and Perez, Y. 1987. *Manual for the Identification of VA Mycorrhizal Fungi.* Second Edition. International Culture Collection of VA Mycorrhizal Fungi (INVAM), University of Florida, Gainesville, Florida

Smith, S.E., and Read, D.J. 1997. *Mycorrhizal Symbiosis*. 2nd ed Academic Press, London. 605 pp.

Tanwar, A., Kumar, A., Mangla, C and Aggarwal A. 2010. Effect of AM Fungi and *Trichoderma harzianum* on Growth Response of *Lycopersicon esculentum. Journal of mycology and plant pathology* 40:219-223.

Tisdall, J.M. and Oades, J.M. 1982. Organic matter and water-stable aggregates in soils. *Journal of soil Science*. 33:141-163.

Walker. 1981. *Acaulospora spinosa* sp. nov. with a key to the species of *Acaulospora*. *Mycotaxon* 12: 512-521.

Walker, C. and Koske, R. E. 1987. Taxonomic concepts in the Endogonaceae. IV. *Glomus fasciculatum* redescribed. *Mycotaxon* 30: 253-262.

Walker, C., Mize, C.W. and McNabb, H.S. 1982. Populations of endogonaceous fungi at two locations in Central Iowa. *Canadian Journal of Botany* 60, 2518-2529.

Wright, S.F. and Upadhyaya, A. 1998. A survey of soils for aggregate stability and glomalin, a glycoprotein produced by hyphae of arbuscular mycorrhizal fungi. *Plant and soil*. 198:97-107.

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Induction of Resistance in *Citrus reticulata* against *Fusarium solani* by Dual Application of AMF and *Trichoderma asperellum*

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Abstract

Decline of health status of nursery grown saplings of *Citrus reticulata* due to root rot caused by *Fusarium solani* is increasingly posing a major threat to the farmers of Darjeeling hill. One of the potential biocontrol fungus-*Trichoderma asperellum* isolated from mandarin rhizosphere, showing *in vitro* antagonistic reaction against the root rot pathogen-*F. solani* as well as showing plant growth promotion has been selected for application following root inoculation with *Glomus fasciculatum* and *Gigaspora gigantea,* dominant arbuscular mycorrhizal fungi (AMF) associated with mandarin roots. 18S rDNA sequence based molecular detection of *F. solani* and *T. asperellum* was done. PAbs separately raised against *F. solani*, *T. asperellum*, *G. fasciculatum* and *Gi. gigantea*, were purified and packaged into serological formats such as PTA-ELISA, DIBA, western blot and immunofluorescence. Successful root colonization with *G. fasciculatum* and *Gi. gigantea* was confirmed by their cellular localization in mandarin root tissues following FITC labeled immunofluorescence assay. Enhanced growth of the saplings in AMF inoculated plants was evident as compared with untreated healthy plants. Application of *G. fasciculatum*, Gi. *gigantea* and *T. asperellum* singly or jointly suppressed root rot of mandarin. Induction of major defense enzymes such as chitinase, β, 1-3 glucanase and peroxidase by treatment with AMF and *T. asperellum* was evident. One induced isoform of peroxizymes following AMF inoculation as well as application of *T. asperellum* was confirmed in PAGE analyses. Concomitant increase in defense enzymes following inoculation with *F. solani* was correlated with the induction of resistance in mandarin plants using bioinoculants.

1. Introduction

Fusarium solani is one the most notorious pathogen causing root rot in Darjeeling mandarin. It is prevalent in almost all orchards of Darjeeling hills and is one of the major reasons of heavy fruit loss due to decline of health of nursery grown plants. The beneficial effects of Arbuscular mycorrhizal fungi and biocontrol fungi either singly or dual application in root rhizosphere of plants have been proved (Davis et al., 1978; Allay and Chakraborty, 2010; El-Mohamedy et al., 2012). Our present investigation was to test the effects of *G. fasciculatum, Gi. gigantea* and *Trichoderma asperellum* on suppression of root rot disease and induction of resistance on *Citrus reticulata*.

2. Materials and Methods

2.1. Plant material

Mandarin (*Citrus reticulata*) seedlings (1 yr old) were obtained from Nirmaldass Orchard, Gurung Brothers Nursery, Baramangwa Busty, Darjeeling.

2.2. Isolation of AMF

AMF were isolated from rhizosphere of *C. reticulata* by wet sieving and decanting technique (Gerdemann and Nicolson 1963). Clean AMF spores were separated using the help of a simple microscope (20×), mounted on Polyvinyl-Lacto-Glycerol (PVLG), microscopical observations were made and photographs were taken. The spores were stored in Ringer's Solution at 4ºC or in sterile distilled water for further use.

2.3. Isolation of trichoderma sp. from mandarin rhizosphere

Trichoderma sp. was isolated from rhizosphere of *C. reticulata* in *Trichoderma* selective media (TSM) (Elad et al., 1980) *T. asperellum* were identified through National Center of Fungal Taxonomy, IARI, New Delhi and used for present investigation.

2.4. Fungal pathogen

Fusarium solani (Acc. No. 3719) causing root rot of mandarin was obtained from Culture collection of Division of Plant Pathology, Indian Agricultural Research Institute, New Delhi.

2.5. Inoculation technique

F. solani was grown in sand-maize meal medium (maize meal: sand: water-1:9:1 w:w:v) in autoclavable plastic bags (sterilized at 20 lbs. pressure for 20 min) for a period of three weeks at 28ºC until the mycelia completely covered the substrate. Nursery grown mandarin seedlings were inoculated by adding 100 g of the prepared inoculum of *F. solani* to the rhizosphere soil.

2.6. Disease assessment

Disease assessment was performed following the method of Chakraborty et al. (2006) after 15, 30 and 45 d of inoculation.

2.7. In vitro studies

F. solani was paired with *T. asperellum* on solid medium as described by Chakraborty and Chakraborty (1989).

2.8. Inocula preparation and application of biocontrol fungi

2.8.1. AMF

Spores of *Glomus fasciculatum* and *Gigaspora gigantea* were separated by fine tweezers and needles under dissecting microscope. After washing with distilled water several times to remove the adhered debris they were separately inoculated in the roots of 7-10 d old seedlings of maize plants grown in black plastic pots (30 cm) having autoclaved soil. After 45 days, inocula were prepared by chopping roots of maize plants where extra radical spores of *G. fasciculatum* and *G. gigantea* were present. Approximately>175 spores 100 g^{-1} could be considered as potent inocula for application.

2.8.2. BCA

Inoculum of *T. asperellum* was prepared by inoculating wheat bran (sterilized) with 5 mm disc of the fungus and incubating at 28ºC for 10 days as described by Chakraborty et al. (2003).

2.9. Determination of growth of mandarin seedlings

Growth promotion was studied in terms of increase in height, number of leaves and biomass in potted plants. In each treatment, average of 20 replicate plants were taken and analyzed.

2.10. Extraction and quantification of soil phosphate

Soil sample (1g) was extracted as described by Mehlich, 1984 and quantitative estimation of phosphate was done as described by Knudsen and Beegle (1988).

2.11. Assay of enzyme activities

Leaves and roots of mandarin seedlings were collected for assay 72 h after inoculation. β-1,3-glucanase (E.C.3.2.1.39) was extracted and assayed from leaf samples following the method of Pan et al. (1991). Chitinase (E.C.3.2.1.14.) was extracted and assayed following the method of Boller and Mauch (1988). Peroxidase (E.C.1.11.1.7.) was extracted and estimated following the method described by Chakraborty et al. (1993).

2.12. Preparation of antigen

Antigens were prepared from mycelia of *F. solani* as well as from healthy and *F. solani* infected root tissues of mandarin plants following the methods as described by Chakraborty and Purkayastha (1983). They were stored at -20ºC and used as mycelial and root antigens.

2.13. Production and purification of polyclonal antibody

New Zealand white male rabbits were used to raise polyclonal antibodies against mycelial antigens of *F. solani* following the method of Chakraborty and Purkayastha (1983). IgGs were purified by DEAE-Sephadex column chromatography.

2.14. Immunodiffusion

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

2.15. PTA-ELISA

Plate trapped antigen-enzyme linked immunosorbent assay (PTA-ELISA) was performed essentially as described by Chakraborty and Sharma (2007).

2.16. Dot immunobinding assay

Mycelial antigens prepared from root pathogens of mandarin (*F. solani* and *F. oxysporum*), healthy and artificially inoculated (with *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 *F. solani*.

2.17. 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 *F. solani*. 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.

2.18. Immunofluorescence

PAbs of AMF, *F. solani,* and goat antisera specific to rabbit globulins conjugated with FITC were used for indirect immunofluorescence study 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.

2.19. Preparation of genomic DNA.

Extraction of genomic DNA from 5 days old mycelia of *F. solani* and *T. asperellum* were done as described by Ma et al. (2001). The quality and quantity of the DNA sample was analyzed spectophtometrically.

2.20. ITS-PCR amplification

The isolates of *Fusarium* and *Trichoderma* were taken up for ITS-PCR amplification.

3. Results and Discussion

AMF from rhizosphere soil of mandarin were initially screened. High population of *Glomus fasciculatum* and *Gigaspora gigantea* were observed and hence were selected for inoculation. Total phosphate content of soil was determined after application of *G. fasciculatum,* and *G. gigantea* singly or jointly. Soil phosphate content decreased after application of AMF indicating that the plant could uptake phosphorus which had been solubilized by AMF (Table 1).

Dot immunobinding assays confirmed the effectiveness of raising antibodies against *F. solani*. Previous studies have also suggested that common antigens may be indicators of plant host-parasite compatibility (Chakraborty, 1988). Absorbance values in PTA-ELISA were significantly higher for infected root extracts than for healthy controls up to a concentration of 2 mg L.

Mycelial antigen of *F. solani* was analysed on SDS-PAGE and then western blot analyses were done using homologous PAb (Table 2). Antibody labeling with fluorescein isothiocyanate (FITC) is a powerful technique used 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 and in spores and hyphae of AMF. ITS region of rDNA was amplified using genus specific T/ITS1 & T/ITS4 (for *Trichoderma*) and Fcg 17 F & Fcg 17 FR (for *Fusarium*) primers. Amplified products of size in the range of 550-700 bp was produced by the all primers. The primer pairs Fcg 17 F and Fcg 17 R were found to be highly specific for *Fusarium* genus. Molecular techniques based on the polymerase chain reaction (PCR) have been used as a tool in genetic mapping, molecular taxonomy, evolutionary studies, and diagnosis of several fungal species (Williams et al., 1990, Clulow et al., 1991, Welsh et al., 1991 and McDonald, 1997). *Trichoderma* produced a single amplified product ranging from 600-620 bp. In order to identify one of our isolates of *T. asperellum* (RHS/M512) obtained from mandarin rhizosphere, 18S rRNA gene sequence which has been submitted to GenBank databases (Acc. No. HQ 265418) was compared and confirmed with other ten *Trichoderma* 18S rRNA gene sequences from NCBI database. Antibiosis to *F. solani* by biocontrol agent (*T. asperellum*) was evaluated *in vitro* and *in vivo*. The application of *T. asperellum* and AMF

Table 1: Soil phosphate content in rhizosphere of mandarin plants following root colonization with *G. fasciculatum* and *Gi. Gigantea*

Treatment	Soil phosphate (ug g^{-1} tissue)
Control	48.43
G. fasciculatum	35.11
Gi. gigantea	31.46
G. fasciculatum+Gi. gigantea	30.06

Table 2: ELISA and Dot-Blot values of reactions between PAbs of defense enzymes and enzyme extracts from treated mandarin plants

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	Light pink	0.049	Light pink
F. solani	0.036	Light pink	0.052	Light pink
F. solani+G. fasciculatum	0.499	Deep purplish	0.423	Dark pink
F. solani+Gi. gigantea	0.521	Deep purplish	0.368	Dark pink
F. solani+T. asperellum	0.487	Deep purplish	0.369	Dark pink
F. solani+G. fasciculatum+Gi. gigantea	0.768	Dark pink	0.445	Deep purplish
F. solani+G. fasciculatum+T. asperellum	0.745	Dark pink	0.525	Dark pink
F. solani+Gi. gigantea+T. asperellum	0.875	Dark pink	0.763	Deep purplish
F. solani+G. fasciculatum+Gi. gigantea+T. asperellum	0.980	Deep purplish	0.856	Dark pink

*Enzyme extracts from leaves of plants treated as mentioned

(*G. fasciculatum* and *Gi. gigantea*) to the soil as biocontrol agents, resulted in reduced disease severity and enhanced plant growth. Application of *G. fasciculatum, Gi. gigantea* and *T. asperellum* in the rhizosphere of *Citrus* plants gave most significant results in the growth of seedlings and numbers of leaves. Marked reduction in disease development was evident following triple inoculations of *G. fasciculatum, Gi. gigantea* and *T. asperellum*. Increased accumulation of defense enzymes

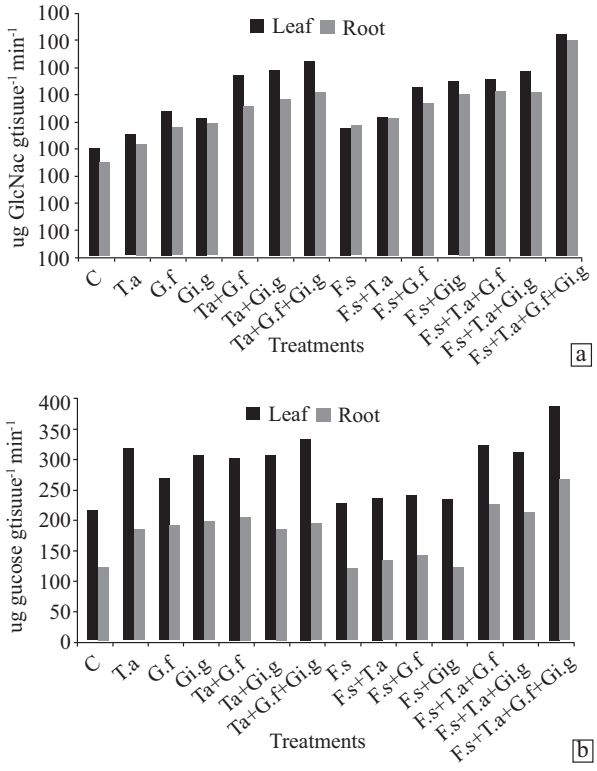

Figure 1: Chitinase (a) and Glucanase (b) activities following application of AMF and BCA

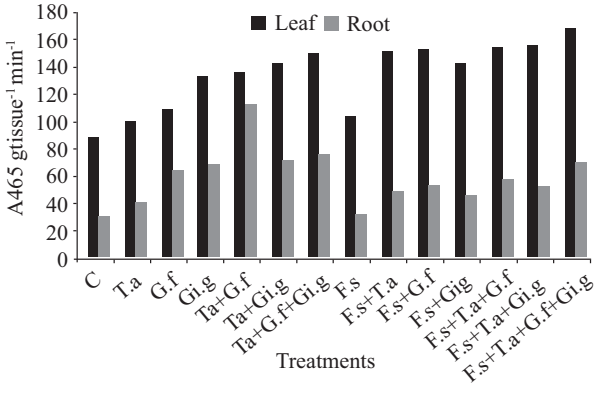

Figure 2: Peroxidase activities following application of AMF and BCA

such as chitinase (Figure 1a), β-1,3-glucanase (Figure 1b) and peroxidase (Figure 2) demonstrated defense responses of mandarin plants during early stages of root colonization.

Isoforms were observed on staining of peroxidase after native PAGE. A few of them were constitutively present in healthy roots and leaves. New isoforms were noticed in mandarin leaves following inoculation of roots with *G. fasciculatum, Gi. gigantea* and *T. asperellum,* singly or jointly. Increased activity of chitinase, β-1, 3-glucanase and peroxidase were also determined in mandarin plants following treatments with *G. mosseae* and *T. hamatum* (Allay and Chakraborty, 2010)

4. Conclusion

The present work mainly focused on developing a biological management strategy to control root rot and wilt complex of mandarin plants caused by *F. solani*. Results of present study indicate that *G. fasciculatum, Gi. gigantea* and *T. asperellum* could promote growth and suppress root rot of mandarin. Combined application of both AMF and BCA gave better results.

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6. References

Allay, S., Chakraborty, B.N., 2010. Activation of defense response of mandarin plants against *Fusarium* root rot disease using *Glomus mosseae* and *Trichoderma hamatum*. Journal of Mycology and Plant Pathology 40(4), 499-511.

Boller, T., Mauch F., 1988. Colorimetric assay for chitinase. Methods in Enzymology. 161, 430-435.

Chakraborty, B.N., Purkayastha, R.P., 1983. Serological relationship between *Macrophomina phaseolina* and soybean cultivars. Physiology and Plant Pathology 23, 197-205.

Chakraborty, B.N., Sharma, M., 2007. Serological detection and immunogold localization of cross-reactive antigens shared by *Camellia sinensis* and *Exobasidium vexans.* Journal of Applied Microbiology 103, 1669-1680.

Chakraborty, B.N., Basu, P., Das, R., Saha, A., Chakraborty, U., 1995. Detection of cross reactive antigens between *Pestalotiopsis theae* and tea leaves and their cellular location. Annals of Applied Biology 127, 11-21.

Chakraborty, B.N., 1988. Antigenic disparity. In: Singh, U.S., Heiss, W.M., Weber, D.J., (Eds.), Experimental and Conceptual Pathology India. Oxford and IBH publishing Company, 477-484.

Chakraborty, U., Chakraborty, B.N., 1989. Interaction of *Rhizobium leguminosarum* and *Fusarium solani* f. sp. *pisi* on pea affecting disease development and phytoalexin production. Canadian Journal of Botany 67, 1698-1701.

Chakraborty, U., Chakraborty, B.N., Basnet, M., 2006. Plant growth promotion and induction of resistance in *Camellia sinensis* by *Bacillus megaterium*. Journal of Basic Microbiology 46, 186-195.

Chakraborty, U., Chakraborty, B.N., Kapoor, M., 1993. Changes in the levels of peroxidase and phenyl alanine ammonia lyase in *Brassica napus* cultivars showing variable resistance to *Leptosphaeria maculans*. Folia Microbiologica 38, 491-496.

Chakraborty, U., Sarkar, B., Chakraborty, B.N., 2003.Protection of soybean root rot by *Bradyrhizobium japonicum* and *Trichoderma harzianum*, associated changes in enzyme activities and phytoalexins production. Journal of Mycology and Plant Pathology 33, 21-25.

Clulow, S.A., Lewis, B.C., Matthews, P., 1991. A pathotype classification for *Mycosphaerella pinodes.* Journal of Phytopathology 131, 322-332.

Davis, R.M., Menge, J.A., Zentmyer, G.A., 1978. Influence of vesicular-arbuscular mycorrhizae on Phytophthora root rot of three crop plants. Phytopathology 68, 1614-1617.

Elad, Y., Chet, I., Karan, J., 1980 *Trichoderma harzianum*: A biocontrol agent effective against *Sclerorium rotfsii* and *Rhizoctonia solani*. Phytopathology 70, l19-l2l.

El-Mohamedy, R.S.R., Morsey, A.A.1, Diab M.M.1, Abd- El-Kareem, F.1., Eman, S.F., 2012. Management of dry root rot disease of mandarin (*Citrus reticulate* Blanco) through biocomposted agricultural wastes. Journal of Agricultural Technology 8(3), 969-981.

Gerdemann, J.W., Nicolson, T.H., 1963. Spores of mycorrhizal *Endogone* species extracted from soil by wet sieving and decanting. Transactions of the British Mycological Society 46, 235-244.

Knudsen, D., Beegle, D., 1988. Recommended phosphorous tests. In: Dahnke, W.C. (Ed.), Recommended chemical soil tests procedures for the north central region. Bull North Dakota Agricultural Experimental Station, North Dakota, USA No. 499. 122-125

Laemmli, U.K., 1970. Cleavage of structural proteins during the assembly of head of bacteriophage T_4. Nature, 227-680-685.

Ma, Z., Boehn, E.W.A., Luo, Y., and Michailides, T., 2001. Population structure of *Botryosphaeria dothidea* from pistachio and other hosts in California. Phytopathology 91, 665-672.

McDonald, B.A., 1997. The population genetic of fungi: tools and techniques. Phytopathology 87, 448- 453.

Mehlich, A., 1984. Mehlich 3 Soil test extractant: A modification of the Mehlich 2 extractant. Communications in Soil Science and Plant Analysis 15, 1409-1416.

Ouchterlony, O., 1967. Immunodiffusion and immuno-electrophoresis. In: Weir, D.M. (Ed.), Handbook of Experimental Immunology. Blackwell Science Publication, Oxford and Edinburgh, 655.

Pan, S.Q., Ye, X.S., Kue, J., 1991. A technique for detection of chitinase, β-1,3-glucanase and protein patterns after a single separation using polyacrylamide gel electrophoresis and isoelectric focusing. Phytopathology 81, 970-974.

Welsh, J., Petersen, C., Mc Clelland, M., 1991. Polymorphisms generated by arbitrary primed PCR in mouse: application to strain identification and genomic mapping. Nucleic Acids Research 19, 303-306.

Williams, J.G.K., Kubelik, A.R., Livak, K.J., Rafalski, J.A., Tingey, S.V., 1990. DNA Polymorphisms amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Research, 18, 6531-6535.