Screening of Arbuscular Mycorrhizal Fungi and Plant Growth Promoting Rhizobacteria from rhizosphere of plantation crops and their evaluation for induction of resistance in tea plants against fungal Pathogen

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DECLARATION

I declare that the thesis entitled "**Screening of Arbuscular Mycorrhizal Fungi and Plant Growth Promoting Rhizobacteria from rhizosphere of plantation crops and their evaluation for induction of resistance in tea plants against fungal Pathogen.**" 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 Utanka Kumar De has prepared the thesis entitled "**Screening of Arbuscular Mycorrhizal Fungi and Plant Growth Promoting Rhizobacteria from rhizosphere of plantation crops and their evaluation for induction of resistance in tea plants against fungal Pathogen"**, for the award of Ph.D degree of the University of North Bengal, under my guidance. He has carried out the work at the Department of Botany, University of North Bengal.

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ABSTRACT

The associations that occur between plant roots and soil microorganisms have been known for many decades. Investigation on the relationship between roots and microbiota are essential to achieve innovations in agriculture and biotechnology. AMF and PGPR have gained worldwide importance and acceptance for agricultural benefits. This is due to the emerging demand for dependence diminishing of synthetic chemical products, to the growing necessity of sustainable agriculture within a holistic vision of development and to focus on environmental protection. Scientific researches involve multidisciplinary approaches to understand adaptation of PGPR and AMF, their effects on plant physiology and growth, induced systemic resistance, biocontrol of plant pathogens, biofertilization, and potential green alternative for plant productivity, viability of co-inoculating, plant microorganism interactions, and mechanisms of root colonization.

The present study examined the diversity of Arbuscular Mycorrhizal Fungi (AMF), colonisation nature and histopathological study of five different crop plants (tea, rubber, coffee, areca and cinnamon) along with isolation of Plant Growth Promoting Rhizobacteria (PGPR) from these rhizosphere and application of AM fungi singly and combined with potent PGPR to enhance the defence enzymes responsible for disease resistance against root pathogen *S. rolfsii* that is responsible for sclerotium blight disease in tea root in nursery conditions.

Tea [*Camellia sinensis* (L.) O. Kuntze, Family: Theaceae], Rubber [*Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg. Family: Euphorbiaceae], Areca (*Areca catechu* L. Family: Arecaceae), Coffee (*Coffea arabica* L. Family: Rubiaceae) and Cinnamon (*Cinnamomum zeylanicum* Blume, family Lauraceae) were selected for AMF isolation. Among all the genera, the genus *Glomus* was predominant followed by *Acaulospora*, *Gigaspora*, *Scutellospora*, *Sclerocystis. Glomus aggregatum*, *G. constrictum*, *G. mosseae, G. intraradices, G. fasciculatum* are most abundant species of the genus *Glomus* in all the plants. 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 tea varieties. The arbuscules characters that

were found during the investigation are full of diversities. Scanning electron microscopic observation was made of three most abundant genera, i.e. *Glomus, Gigaspora* and *Acaulospora*.

Germination of AMF spores *in vitro* was studied to screen their ability to grow in an artificial medium supplemented with root extract. Depending on the abundance two AMF spores were selected, i.e. *Glomus mosseae* and *G. fasciculatum* were taken up for all the *in vitro* and *in vivo* studies.

On the other hand a total of twenty two (22) bacterial isolates were obtained from the rhizosphere soil of different plantation crops. Among them 10 bacterial isolates were obtained from Tea, 7 from rubber and 5 from Cinnamomum. Isolated bacteria were studied under microscope after suitable staining and characterized based on morphological and biochemical studies following Bergey's manual of Systematic Bacteriology. Overall 15 bacterial isolates were gram positive, rod shaped whereas 7 isolates were gram negative. Among all the bacterial isolates two bacterial isolates designated as RHS/T-382 and TRS-6 obtained from the rhizosphere of tea showed positive tests for all the PGPR tests like phosphate solubilization, protease, chitinase IAA and siderophore production. On the basis of *in vitro* studies both the bacterial isolates RHS/T-382 and TRS-6 were designated as potential PGPR isolates and were taken up for all the *in vitro* and *in vivo* studies. Scaning electron microscopic studies of both the bacterial isolates showed that they were characteristically rod shaped and the size of the bacterial cells ranged from 4-7 µm.

The main objectives of the present study was to determine the efficacy of these bioformulations using AMF and PGPR singly and in combinations on plant growth promotion and biocontrol of sclerotial blight diseases of tea caused by *Sclerotium rolfsii* along with determination of cell defense responses in tea plants associated with induction of resistance toward *S. rolfsii* (RHS/T-381**)** 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. Different varieties of tea plant, which were maintained in the Germplasm

bank at Department of Botany, North Bengal University, were used for the experimental purposes.

The bacterial isolates RHS/T-382 and TRS-6 obtained from tea rhizosphere were designated as potential PGPR and both the isolates were confirmed with the help of 16S rDNA sequences. The BLAST query of the 16S r DNA sequence of the isolates against GeneBank database confirmed the identity of the isolate RHS/T-382 as *Bacillus pumilus* and TRS-6 as *B. amyloliquefaciens***.**

The sequences have been deposited in NCBI, GeneBank database under the accession no. JQ765580 for *B. pumilus* and JN983127 for *B. amyloliquefaciens*.

A multiple sequence alignment of ITS gene sequences of *S. rolfsii* was also conducted. Phylogenitic analysis of *S. rolfsii* was carried out with the Ex-type strain sequences obtained from NCBI Genbank Database which showed maximum homology with the isolate RHS/T-381.

Screening for resistance of tea varieties against *S. rolfsii* was carried out in sick plot developed specifically for this pathogen. Varietal resistance test of tea against *Sclerotium rolfsii* was carried out in ten (10) tea cultivars including five Toklai varieties (TV-25, TV-26, TV-9, TV-20, TV-18), two Teen Ali varieties (T-17 and T-78), two Upasi varieties (UP-3 and UP-26) and one Assam variety (AV-2). Three year old plant roots were inoculated with *S. rolfsii* and disease assessment was done on the basis of visual observation of symptoms and disease index.

The antagonistic effect of the both the isolates *B. pumilus* RHS/T-382 and *B. amyloliquefaciens*-TRS6 which showed positive results against tea root pathogens *viz*, *Sclerotium rolfsii, Fomes lamaoensis*, *Poria hypobrunnea* and *Spherostilbe repens*.

Glomus mosseae and *G. fasciculatum* were mass multiplied in sorghum and maize plants and was used singly and in combination with PGPR 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 PGPR and AMF however the results reveals that the growth of tea saplings grown under same environmental and physical conditions were enhanced to a greater extent when both *B. pumilus* and *G. mosseae* were applied jointly.

Statistical analysis (ANOVA) revealed that the decrease in soil P and increase in root and leaf P- content were significantly enhanced when both *G. mosseae* and *B. pumilus* were applied jointly. Similar results also obtained with *G. fasciculatum* and B. *amyloliquefaciens*.

Experiments were conducted to assess the effect of single as well as combined application on biochemical components of tea leaves. Activities of some of the enzymes which are involved in phenol metabolism as well as in defense-i.e., peroxidase, phenylalanine ammonia lyase, chitinase, β-1,3 glucanase were also determined. Polyphenols are major constituents of tea leaves and hence phenol contents were determined. Multifold increase in activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in roots was observed on application of AMF and PGPR to soil followed by inoculation with *S. rolfsii* which was significantly higher in those plants which were jointly inoculated with AMF and PGPR.

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 tissues. Fro this leaf section of the most responsive tea variety, TV-20 was taken. Immunolocalization of chitinase in treated as well as control tea leaves were observed by immunofluorescence study. *G. mosseae and B. pumilus* treated along with control tea leaves were treated with chitinase antibody and FITC.

In a different mode of investigation three mycorrhiza helper bacteria were isolated from *Glomus mosseae whose* 16 S rDNA sequence analysis was conducted and it was revealed as *Bacillus flexus* which also induced increased hyphal growth of *G. mosseae in vitro*.

Effects of AMF and PGPR in management of sclerotial blight disease of tea were tested in pot as well as nursery conditions. In the first set of trial *Glomus mosseae* and *Bacillus pumilus* were tested for their effect in inhibiting sclerotial blight of tea saplings in nursery conditions when applied singly and in combination. Under nursery condition, *G. mosseae* and *B. pumilus* alone could effectively reduce disease incidence. However combined inoculation with *G. mosseae* ad B. pumilus showed 20% disease incidence recorded after 45 days of pathogen challenge.

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expression within the leaf tissues. Fro this leaf section of the most responsive tea variety, TV-20 was taken. Immunolocalization of chitinase in treated as well as control tea leaves were observed by immunofluorescence study. *G. mosseae and B. pumilus* treated along with control tea leaves were treated with chitinase antibody and FITC labeled. Strong apple green fluorescence were evident in treated leaves.

Tea roots following colonization with AMF alone or in combination with PGPR could induced resistance against the pathogen. This was evident in the previous experiments when the treated tea leaves showed bright apple green fluorescence in mesophyll tissues when these were labelled with FITC and reacted with PAb of chitinase. The present investigation was designed to locate the pathogen in the rhizosphere as well as presence of AMF in rhizoplane as well as their cellular localization in root tissues using PAbs of the pathogen (*S.rolfsii*) and AMF (*G. mosseae*).

PREFACE

The associations that occur between plant roots and soil microorganisms have been known for many decades. Investigation on the relationship between roots and microbiota are essential to achieve innovations in agriculture and biotechnology. AMF and PGPR have gained worldwide importance and acceptance for agricultural benefits. Scientific researches involve multidisciplinary approaches to understand adaptation of PGPR and AMF, their effects on plant physiology and growth, induced systemic resistance, biocontrol of plant pathogens, biofertilization, and potential green alternative for plant productivity, viability of co-inoculating, plant microorganism interactions, and mechanisms of root colonization.

Keeping in mind, the role of AMF and PGPR in growth promotion and disease management of different plants, the present study was undertaken to generate possible information regarding the diversity of AMF in one of the most important plantation crop of the region i.e. Tea (Camellia sinensis) in comparison with the AMF diversity of other plantation crops of the region. The study has also been focused to utilize AMF along with PGPR to improve growth and control root disease of tea.

I take this opportunity to thank all those who made this thesis possible. At the very outset, I would like to pay my utmost sense of gratitude to my supervisor, Prof. B.N. Chakraborty, Immuno-Phytopathology Laboratory, Dept. of Botany, North Bengal University for their guidance, undivided attention, kind and valuable suggestions and wise counseling throughout the execution of this endeavor.

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Chapter 1
Introduction

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.

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).

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).

Arbuscular mycorrhiza (AM), a symbiosis between plants and members of an ancient phylum of fungi, the Glomeromycota, improves the supply of water and nutrients, such as phosphate and nitrogen, to the host plant. In return, up to 20% of plant-fixed carbon is transferred to the fungus. Nutrient transport occurs through symbiotic structures inside plant root cells known as arbuscules. AM development is accompanied by an exchange of signaling molecules between the symbionts. Novel classes of plant hormones known as strigolactones are exuded by the plant roots. On the one hand, strigolactones stimulate fungal metabolism and branching. On the other hand, they also trigger seed germination of parasitic plants. Fungi release signaling molecules, in the form of 'Myco factors' that trigger symbiotic root responses. Plant genes required for AM development

have been characterized. During evolution, the genetic programme for AM has been recruited for other plant root symbioses: functional adaptation of a plant receptor kinase that is essential for AM symbiosis paved the way for nitrogen-fixing bacteria to form intracellular symbioses with plant cells (Parniske, 2008).

Since Arbuscular mycorrhizas are mutualistic associations formed between the roots of 80 % of terrestrial plant species and fungi from the small phylum Glomeromycota (Schüßler *et al*., 2001), their associated interactions with plants are thought to reduce the damage caused by plant pathogens (Singh *et al* 2013). 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, 1995).

Fig.1. A glimpse of five plantation crops and Arbuscular Mycorrhizal Spores

Of the various mechanisms proposed for biocontrol of plant diseases, effective bioprotection is a cumulative result of all mechanisms working either separately or together. In order to apply AM fungi in sustainable agriculture, knowledge of factors such as fertilizer inputs, pesticide use, and soil management practices which influence AM fungi is essential (Linderman, 1992). In addition, efficient inoculants should be identified and employed as biofertilizers, bioprotectants, and biostimulants for sustainable agriculture and forestry. Conventional farming systems using lower application rates of fertilizers and pesticides have been developed, but are used only minimally in grain production in India, perhaps due to insufficient understanding of agricultural soils dynamics (Ryan, and Delhaize, 2001). AM symbiosis also increases resistance to biotic and abiotic stresses and reduces disease incidence, representing a key component of sustainable agriculture (St-Arnaud and Vujanovic, 2007; Subramanian and Charest, 1999; Aliasgarzad *et al.*, 2006, Maya *et al*. 2013). Appropriate management of mycorrhizae in agriculture should ultimately result in a substantial reduction in chemical use and production costs.

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).

Tea (*Camellia sinensis*), having a lots of medicinal value (Chakraborty and Chakraborty 1998**)** is a perennial plant and is always challenged by pests and pathogens during various stages of growth and development. The most devastating are the root diseases caused by various fungal pathogens of which sclerotial blight (*Sclerotium rolfsii* [teleomorph = *Athelia rolfsii*]) brown root rot (*Fomes lamaënsis*), red root rot (*Poria hypobrunnea*,), black root rot (*Rosellinia arcuata*), and charcoal stump rot (*Ustulina zonata*) are common (Chakraborty, *et al* 2007).

Such root diseases can be controlled by manipulation of indigenous microbes or by introducing antagonists to reduce the disease-producing propagules (Linderman, 1994). Arbuscular Mycorrhizal Fungi and their associated interactions with plants reduce the damage caused by plant pathogens (Chakraborty *et al*, 2004, Chakraborty *et al*, 2011, Chakraborty *et al*, 2013, Chakraborty *et al*, 2012, Singh *et al* 2013). 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* 2012). 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).

Keeping in mind, the role of AMF and PGPR in growth promotion and disease management of different plants, the following major objectives were undertaken in this

present investigation to generate possible information regarding the diversity of AMF in one of the most important plantation crop of the region i.e. Tea (*Camellia sinensis*) in comparison with the AMF diversity of other plantation crops of the region (Figure 1). The study has also been focused to utilize AMF along with PGPR to improve growth and control root disease of tea.

1. Isolation of microorganisms (AMF and PGPR) from rhizosphere of plantation crops and their identification
2. Screening of isolates and characterization as phosphate solubilizers and plant growth promoter as well as biocontrol agents.
3. *In vitro* interaction study among dominant AMF and PGPR of tea and root pathogen
4. Assessment of the efficacy of AMF and PGPR singly and in combinations on plant growth promotion and biocontrol of sclerotial blight diseases of tea caused by *Sclerotium rolfsii*.
5. Determination of cell defense responses in tea plants associated with induction of resistance toward *S. rolfsii* by microbial formulation.

Literature Review

In agricultural management two of the most important goals are to ensure the crop of enough nutrients and to prevent it from diseases and goals have been achieved by using pesticides and fertilizers indiscriminately. However, these management practices lead to a high loss of biodiversity all over the landscape. Therefore, other approaches should be investigated. Soil organisms play an important role in nutrient cycling and can reduce diseases; a new approach is to manage the system by increasing the soil biodiversity. Since the below ground organisms are for a large part dependent on input from above ground organisms are for a large part dependent on input from above ground organisms, this may be achieved by modifying the rhizosphere.

Arbuscular Mycorrhizas are multifaceted associations comprising diverse morphological, functional and evolutionary categories (Brundrett, 2002) that form highly evolved, mutualistic associations between soil fungi and plant roots where the host plant receives mineral nutrients while the fungus obtains photosynthetically derived carbon compounds (Harley and Smith, 1983, Stürmer 2012). Of the seven types of mycorrhizae described (arbuscular, ecto, ectendo, arbutoid, monotropoid, ericoid and orchidaceous mycorrhizae), arbuscular mycorrhizae and ectomycorrhizae are the most abundant and widespread (Allen *et al*., 2003).

Most AMF form spores in the soil from the extraradical hyphae which are able to germinate and grow in response to different edaphic and environmental conditions, but are unable to produce extensive mycelia and complete their life cycle without establishing a functional symbiosis within a host plant. The key developmental switches occurring in the fungal organism, from the germination of an individual spore to the formation of an extensive hyphal network in the soil, that involves spore germination, pre-symbiotic mycelial growth, differential hyphal branching pattern in the presence of host roots, pre penetration apparatus formation, appressorium formation, root colonization, arbuscule development, extraradical mycelial growth and spore production (Giovannetti, 2000). The phenomenon of spore dormancy has concerned researchers since Godfrey's early studies on spore germination (Godfrey, 1957). In the year 1959,

Mosse suggested the storage of collected spores on damp filter paper at 5°C for 6 weeks in order to obtain the regular germination of resting spores of an *Endogone* sp. (presumably *Glomus mosseae*) (Mosse, 1959). Eighty percent of spores treated in this way germinated within 3–4 days. The relief of dormancy by storage was reported by many authors. Hepper and Smith, (1976) found that spores of *G. mosseae* from freshly harvested sporocarps germinated slowly compared to spores detached from sporocarps and stored at 6°C for 5 weeks. The same results were obtained with a North American isolate of *G. mosseae*, which showed a marked difference in germinability between freshly isolated and 10°C-stored spores (Daniels and Graham, 1976). Diverse species of the genus *Glomus* exhibited spore dormancy, such as *Glomus intraradices, Glomus clarum, Glomus caledonium, Glomus monosporum* (Juge *et al*., 2002). Other species, such as *Glomus coronatum*, showed erratic germination even after cold treatments lasting 1 year (Giovannetti *et al*., 1991). Germtubes of *G. margarita* emerged after 72 h incubation on water agar or within 3–5 days on agar media without any storage treatment (Sward, 1981). Similarly, spores of *Scutellospora fulgida* and *Scutellospora persica* did not possess any dormancy, showing mycelial growth and the formation of auxiliary cells after 2 weeks in the dark at 24°C (Turrini *et al*., 2008). Many germination factors have been identified which play important roles in growth activation of quiescent spores. Although complex interactions among different factors probably play the most important role in spore germination in nature, many investigators have studied germination factors such as pH, temperature, moisture, mineral and organic nutrients, host plants, and microorganisms. Metabolites present in the liquid secreted (exudate) by the host plant roots contain constitutive compounds that stimulate the growth/hyphal branching of AM fungi (Nagahashi and Douds, 2000). These compounds are elevated in the exudates of plant roots grown under phosphorus (Pi) stress (Yoneyama *et al*., 2007), and the enhanced exudation of these compounds can increase appressoria formation and therefore enhance the colonization of the host root by AM fungi. Some of these exuded compounds stimulate elongation growth (Nagahashi and Douds, 2007) which probably allows the fungus to explore the soil at farther distances from the germinated spore. Other compounds stimulate extensive hyphal branching (Nagahashi and Douds, 2000) and have been referred to as branching factors (BFs). These compounds are most effective near or

at the root surface and increase the chances for the fungus to come in physical contact to form appressoria in the cell wall grooves between epidermal cells. Recently, a BF has been identified in *Lotus japonicus* (Akiyama *et al*., 2005) as 5-deoxy-strigol. Two other strigolactones, strigol from *Menispermum dauricum* root culture (Yasuda *et al*., 2003) and sorgolactone from *Sorghum* exudates (Besserer *et al*., 2006) were also shown to be BFs.

Symbiotic Benefits and Nutrient Uptake

When it was demonstrated that AM fungi increased productivity in AM plants compared to non-mycorrhizal plants, interest in AM fungal symbioses arose in agriculture, forestry, rehabilitation and in environments were managerial practices have altered the soils native state **(**Friberg, 2001, Ba L *et al* 2012). The major benefits of AM fungi to symbionts includes enhanced nutrient uptake, increased tolerance to root pathogens, drought resistance, tolerance to toxic heavy metals and improved soil aggregation and structure. Macro and micronutrients are required for plant growth in varying amounts. Various levels of micronutrient have been reported to affect the yield of crops such as rice, wheat and legumes (Johnson *et al.*, 2005). Heavy metals on the other hand are soil pollutants that are present in quantities greater than 5-6g / cm3. But at very low concentrations, some of these heavy metals become micronutrients that are essential for plant growth e.g. lead and nickel (Ashman and Puri, 2002). AM fungi are known to enhance mainly the uptake of the macronutrient phosphorus P from the soil, which is then translocated to the host plant through hyphal networks in the soil. Their ability to also take up other micronutrients such as Cu, Zn, Ni, Pb and Fe etc; has been demonstrated by researchers using different host plants and soil type management. Furthermore, it has been proposed that AM fungi also have the ability to sequester these nutrients and minimise transfer to the plant roots when nutrients are in high concentrations. However, the mechanism of this ability has not been proved (Turnau *et al.*, 1993). Phosphorus is the second essential nutrient after nitrogen (N) required for plant growth and is found in many soils in organic and complex inorganic forms (phytic acid). Due to its low solubility and mobility, plants cannot readily utilise P in an organic or complex inorganic form (Schachtman and Reid, 1998, Reidinger *et al* 2012). Inorganic phosphate present in soluble forms in the soil can be readily utilised by plants but usually in limited amounts.

Thus, AM fungi intervene to enhance nutrient uptake through the spread of extraradical hyphae into the surrounding soil and hydrolysing any unavailable sources of P with the aid of secreted enzymes such as phosphatise (Koide and Kabir, 2000). The enzyme phosphatase, produced by AM fungal extraradical hyphae hydrolyses and releases P from organic P complexes and facilitates the absorption of P and other nutrients thereby creating a depletion zone around the roots (Li *et al.*, 1991). These depletion zones limits the rate of phosphorus uptake by non-mycorrhizal plants but gives mycorrhizal plants a greater advantage because of the ability of the AM fungal ERH to extend past this nutrient depletion zone to enhance absorption (Sylvia *et al.*, 2001) reported that under nutrient deficient conditions the effectiveness of AM fungi is exercised by the ability of the ERH to bridge the nutrient depletion zones of host plant roots. But when nutrients are available to the plant, root length growth is increased and the mycorrhizal dependency of the plant to take up nutrient is reduced.

Although P is the main nutrient transported by AM fungi to plants, N is of great importance for plant growth and should not be over-looked. Nitrogen is obtained by the extraradical hyphae of AM fungi in different forms ranging from amino acids, peptides, ions (NO3 - or NH4 +) to recalcitrant organic nitrogen forms (Hawkins *et al.*, 2000). It has been recorded that the extraradical hyphae of different *Glomus sp.* can assimilate and metabolise both organic and inorganic sources of nitrogen perhaps by glutamate synthetase activity (Hawkins *et al.*, 2000). It can be stated therefore that the concentration of P and N in the soil can determine the rate of other micro (Fe, Cu, Mn, Zn) and macronutrient (K, Ca) uptake by mycorrhizal plants. Liu *et al.*, (2000) confirmed this in their study which determined the role of AM fungi in the uptake of Cu, Zn, Mn and Fe in maize which showed that the uptake of these nutrients was significantly influenced by soil P nutrition. Due to the potential of mycorrhizal fungi to enhance nutrient uptake, this benefit has however brought about the suggested use of AM inoculum instead of some chemical fertilisers for plant productivity, growth and restoration of polluted soils or in revegetation (Cardoso and Kuyper, 2006).

Plant Protections

AM fungal colonisation of plant roots has been suggested to increase plants tolerance to pathogens thereby acting as a biocontrol agent. A biocontrol agent is defined

as the use of a biologically friendly resource from the ecosystem that can target and protect plants against pathogens. The role of Mycorrhizal fungi in control of various soil borne plant diseases has been reviewed by many workers (Hafez *et al* 2012, Chakraborty et al 1995, Chakraborty et al 2007) and recently Adholeya (2012) has discussed positive impact of AMF in sustainable agriculture. Besides the association of AM fungi with plant nematodes and the beneficial effect of mycorrhizal symbiosis on plant growth had also led to investigations into the potential of AM fungi to limit yield losses due to nematodes (Bhargava *et al*. 2008). Several mechanisms or combination of mechanisms could account for the observed bio protection of plants by AM fungi. Some of these pathogens can be root-infecting fungi that are antagonistic and capable of feeding on their host as necrotrophs, wilt pathogens such as *Fusarium oxysporum*, or root rotting pathogens like *Phytophthora* and *Rhizoctonia* that are common soil borne pathogens. Primarily, the ability of AM fungi to enhance plant vigour due to increased nutrient uptake enables it to resist pathogen infection. It has been now established that the interaction of AM fungi with soil root pathogens has everything to do with the enhanced nutritional uptake of P and other nutrients. And through this action, the fungus increases the plant's tolerance to pathogens through mechanisms such as alteration of root exudates, increased root growth and function and competition for space or infection sites. Bainton *et al.*, (1992), reported that increased nutritional status of plants with AM fungi might increase tolerance to root pathogens. But no effect on the development of leaf diseases in maize caused by *Helminthosporium maydis* and *Acremonium kiliense* was observed. Besides, AM fungi were found to increase *Zea mays* tolerance to leaf rust with control plants having 80% leaf rust as compared to AM inoculated plants, which had less than 5% leaf rust. AM fungi have direct access to plant photosynthetic product while pathogens, which are not obligate biotrophs can only obtain C from decomposing organic sources. This automatically gives AM fungi a growth advantage over pathogens like *Fusarium* that must access organic sources for carbon on their own. However, it is not yet confirmed if competition for carbon and other nutrients induces pathogen resistance (Linderman, 1994). Similarly, competition for colonisation sites within the roots has been suggested to occur, as some pathogenic fungal infections colonise similar plant tissues (Smith, 1988). For example, *Fusarium* infects the vascular tissues of plants, but requires

passage of the hyphae through the root cortical cells. If root cortical cells are colonised by AM fungi this will limit the entry of the *Fusarium* pathogen (Agrios, 2005). However, this is a proposed localised mechanism by which AM fungi exerts biocontrol activity (Barea, 1991). In addition, microbial changes in the mycorrhizosphere and anatomical changes in the root induced by AM formation may bring about stimulation of specific functional groups in the microbiota that are antagonistic towards pathogens (Ferrol *et al.*, 2002). However, these mechanisms are said not to be effective for all pathogens and are influenced by soil and environmental conditions (Azcón-Aguilar and Barea, 2002). A study on the biocontrol potential of AM fungi on *Fusarium* using different cultivars of maize proved to increase the plant's tolerance to the pathogen when used as an inoculant (Mukasa-Mugerwa, 2005). The actual mechanism by which AM fungi confers localised or induced systemic protection against pathogens to plants remains unidentified (Dumas-Gaudot *et al.*, 2000). Though there are indications that this mechanism is signalled by modulations such as lignifications, induction of cell wall appositions containing callose, accumulation of pathogenic related proteins or phenolic compounds (Pozo *et al.*, 2002). Lignification caused by AM fungal colonisation involves the thickening of the exodermis and cortical root cell walls which makes penetration of pathogenic hyphae difficult (Cordier *et al.*, 1996). As such, pathogens that target plants through this way will likely not penetrate and infect the plant root due to anatomical changes in root structure (Dumas-Gaudot *et al.*, 2000). Similarly the accumulation of phenols in response to AM fungal colonisation has been reported to cause both localised and systemic induced resistance to pathogens. A study by Zhu and Yao (2004) confirmed this when they examined the ability of *Gl. versiforme* to inhibit *Ralstonia solanacearum* when inoculated together in tomato roots. It was observed that *Gl. versiforme* increased the soluble phenol contents in the tomato roots thereby decreasing the population of *Ralstonia solanacearum* in the rhizosphere and in the xylem tissues of the plant. Pozo *et al.*, (2002) also used tomato plants and demonstrated similar effects using the pathogen *Phytophthora parasitca* and two species of AM fungi (*Gl. mosseae* and *Gl. intraradices*). They observed that *Gl. mosseae* had the ability to reduce infection of *P. parasitica* in tomato roots by inducing the mycorrhizal related hydrolytic enzymes such as chitosanases and ß-1, 3 glucanase that have lytic activity against *Phytophthora* cell walls.

Table 1. AM fungi and their effects on plant pathogenic fungi

AM fungi	Pathogenic fungus	Effect	Reference
G. mosseae	*F. oxysporum*	Significantly reduced *Fusarium* wilt on tomato and pepper	Al-Momany and Al-Raddad, 1988
G. mosseae	*Verticillium albo-atrum F. oxysporum* f. sp. *medicaginis*	Seedlings inoculated with AM fungi had lower incidence of wilt in alfalfa than did nonmycorrhizal species	Hwang *et al*., 1992
G. mosseae	*Verticillium dahliae*	Inoculation of AM fungi reduced disease indices in cotton	Liu, 1995
G. mosseae	*Fusarium udum*	Reduced disease severity on pigeon pea	Siddiqui and Mahmood, 1996
G. mosseae	*Phytophthora nicotianae var. parasitica*	Reduced root necrosis, and necrotic root apices ranged between 63–89 %	Trotta *et al*., 1996
G. mosseae	*Fusarium solani*	Significantly reduced disease severity in chickpea	Siddiqui and Mahmood, 1997
G. mosseae	*Fusarium udum*	Reduced disease severity in pigeon pea	Siddiqui *et al*., 1998
G. mosseae	*F. solani R. solani*	Significantly reduced severity of diseases on peanut	Elsayed Abdalla and Abdel-Fattah, 2000
G. mosseae	*Fusarium chlamydosporium*	Reduced disease severity but best management was obtained when used with *T. viridae*	Boby and Bagyaraj, 2003
G. mosseae	*Alternaria triticina*	Reduced percent infected leaf area on wheat	Siddiqui and Singh, 2005
G. mosseae	*C. orbiculare*	AM fungus had no significant effect on disease development	Chandanie *et al*., 2006
G. fasciculatum	*Aphanomyces euteiches*	Reduced root rot on pea	Rosendahl, 1985
G. fasciculatum	*Macrophomina phaseolina*	Prior inoculation of AM fungus reduced disease on cowpea	Devi and Goswami, 1992
G. fasciculatum	*F. oxysporum f. sp. ciceris*	Reduced the disease severity in chickpea	Siddiqui and Singh, 2004
G. fasciculatum	*M. phaseolina*	Reduced disease severity in chickpea	Akhtar and Siddiqui, 2007
G. intraradices	*Fusarium oxysporum* f. sp. *radicislycopersici*	AM fungus significantly reduced *Fusarium* root rot on tomato	Caron *et al*., 1985

G. intraradices	*Pythium ultimum*	Reduced populations of *P. ultimum* on *Tagetes patula*	St-Arnaud *et al*., 1994
G. intraradices	*F. oxysporum* f. sp. *lycopersici*	Significantly reduced disease severity but is most effective when applied with *T. harizianum*	Datnoff *et al*., 1995
G. intraradices	*Rhizoctonia solani*	Defense response elicited by R. solani significantly suppressed by AM fungus in alfalfa	Guenoune *et al*., 2001
G. intraradices	*F. oxysporum f. sp. lycopersici*	Reduced severity of disease in tomato	Akkopru and Demir, 2005
G. intraradices	*Rhizoctonia solani*	Significantly decreased epiphytic and parasitic growth of pathogen in tomato	Berta *et al*., 2005
G. intraradices	*M. phaseolina*	Inoculation of AM fungus with *A. niger* and *Bacillus* (B22) caused a geater reduction in root-rot of chickpea	Akhtar and Siddiqui, 2006
G. intraradices	*M. phaseolina*	Significantly reduced disease severity in chickpea	Akhtar and Siddiqui, 2007
G. intraradices	*M. phaseolina*	Combined inoculation of AM fungus with *Pseudomonas straita* and *Rhizobium* caused a greater reduction in the root-rot of chickpea	Akhtar and Siddiqui, 2008
G. intraradices	*Aphanomyces euteiches*	Reduced disease severity in pea	Kjoller and Rosendahl, 1997
G. intraradices	*Aphanomyces euteiches*	Reduced disease severity in pea	Bodker *et al*., 1998
G. intraradices	*P. parasitica*	G. mosseae was most effective in reducing disease symptoms produced by *P. parasitica* on tomato	Pozo *et al*., 2002
G. etunicatum	*Pythium ultimum*	Prior or simultaneous inoculation of AM fungus with *P. ultimum* reduced disease severity on cucumber	Rosendahl and Rosendahl, 1990
G. etunicatum	*F. oxysporum f.*	Reduced disease severity in tomato	Ozgonen *et al*., 1999
G. etunicatum	*F. oxysporum f. sp. lycopersici*	Reduced disease severity on tomato	Bhagawati *et al*., 2000
G. etunicatum,	*Phytophthora capsici*	AM fungi significantly increased plant growth and	Ozgonen and Erkilic, 2007

		reduced disease severity in pepper but *G. mosseae* reduced disease severity to a greater extent	
G. claroideum	*Aphanomyces euteiches*	Reduced disease severity on pea but effects were more pronounced in plant inoculated with *G. intraradices* than with *G. claroideum*	Thygesen *et al*., 2004
Glomus sp.	*Sclerotium cepivorum*	AM fungi reduced white rot incidence and delayed disease development on onion	Torres-Barragan *et al*., 1996
G. clarum	*Rhizoctonia solani*	Significantly reduced root necrosis and number of sclerotia on cowpea	Abdel-Fattah and Shabana, 2002
Gigaspora margarita	*Fusarium udum*	Reduced wilt indices in pigeon pea	Siddiqui and Mahmood, 1995
G. versiforme	*Cylindroclad-ium spathiphylli*	AM fungi significantly increased growth and reduced disease severity in banana. *Glomus* sp. and *G. proliferum* caused greatest increase in plant growth compared to that caused by *G. intraradices* and *G. versiforme*	Declerck *et al*., 2002

Plant Growth Promoting Rhizobacteria and their effects on plant health improvement

The associations that occur between plant roots and soil microorganisms have been known for many decades. Considerable efforts have been devoted to study ectomycorrhizal fungi, nitrogen-fixing bacteria, soil-borne pathogenic fungi, and other microorganisms. As a consequence of the many investigations of the variable response of plants to different soils, an awareness of the complexity of the interactions between roots and soil microbes has been developed. When seeds germinate and roots grow through the soil, the loss of organic material provides the driving force for the development of active microbial populations around the root. The rhizosphere of plants is a zone of intense microbial activity, and some bacteria from this zone, termed rhizobacteria, exhibit different functions. The rhizosphere contains an increased microbial biomass and activity compared with nonrhizosphere soil: the number of microorganisms in the rhizosphere is

19–32 times larger than in root-free soil (Bodelier *et al.* 1997). Rhizobacteria that exert beneficial effects on plant development are referred to as plant growth-promoting rhizobacteria (PGPR) because their application is often associated with increased rates of plant growth (Kloepper and Schroth 1978). The well-known PGPR include members of the genera *Arthrobacter, Azoarcus, Azospirillum, Bacillus, Burkholderia, Enterobacter, Gluconacetobacter, Herbaspirillum, Klebsiella, Paenibacillus, Pseudomonas*, and *Serratia*, among others. PGPR can affect plant growth either directly by providing plants with a compound synthesized by the bacterium or by facilitating the uptake of certain nutrients from the environment or indirectly by decreasing or preventing the deleterious effects of one or more phytopathogenic organisms (Glick 1995). In order to exert their function, PGPR must colonize the rhizosphere around the roots, the rhizoplane or within root tissues (Glick 1995).

Non-pathogenic rhizobacteria can induce a systemic resistance in plants that is phenotypically similar to the pathogen-induced systemic acquired resistance (SAR). Rhizobacteria-mediated induced systemic resistance (ISR) has been demonstrated against fungi, bacteria, and viruses in bean, carnation, cucumber, radish, tobacco, and tomato under conditions in which the inducing bacteria and the challenging pathogen remained spatially separated (van Loon *et al.* 1998). ISR elicited by PGPR has suppressed plant diseases caused by a range of pathogens in both greenhouse and field conditions. However, fewer reports have been published on PGPR as elicitors of tolerance to abiotic stresses, such as drought, salt and nutrient deficiency or excess. Yang *et al.* (2007) have proposed the term “induced systemic tolerance” (IST) for PGPR-induced physical and chemical changes in plants that result in enhanced tolerance to abiotic stresses. Beneficial bacteria that are able to establish a nitrogen-fixing symbiotic relationship with leguminous plants (collectively called rhizobia) are usually not considered as PGPR. Endosymbiotic interactions between legume plants and the genera *Azorhizobium*, *Bradyrhizobium*, *Mesorhizobium*, *Rhizobium* and *Sinorhizobium* have been intensively studied (Graham, 2000). Legumes are particularly important and are used in sylvopastoral and agroforestry systems (Subba Rao, 1983). Intricate signaling between the host and rhizobial symbiont is required for successful symbiotic interactions, which result in the reduction of atmospheric N2 to ammonia by the bacteroids. Some of these bacteria have been shown to be plant-growth promoting on nonlegumes, through

mechanisms different from nitrogen fixation. Nevertheless, these will not be further considered, as the mechanisms involved are not different from those of the wellknown and better-documented PGPR (Spaepen *et al.*, 2009). Thus, in the broadest sense, PGPR include the N2-fixing rhizobacteria that colonize the rhizosphere and provide N to plants. Rhizosphere interactions are based on complex exchanges that take place round plant roots. Beneficial, detrimental, and neutral relationships between plant roots and microorganisms are all regulated by complex molecular signaling. It is clear that all the biological community, rather than only the immediate micro-flora, plays a role in the interaction of the rhizosphere. The existence of both microbial responses to plants and plant responses to the presence of microbes suggests a degree of coevolution between two partners. Two of the best-studied interactions between plant hosts and bacteria include the root nodule inhabiting *Rhizobium* spp. and tumor-forming *Agrobacterium* spp. The study of these systems has led to the discovery that plants and bacteria communicate by using chemical signals, which are involved in a successful interaction (Dardanelli *et al.,* 2009). Chemical signaling between plant roots and other soil organisms, including the roots of neighboring plants, is often based on root-derived chemicals. Forty to ninety percent of the carbon transferred to the roots is lost and is called rhizodeposition (Kennedy, 2010). In the rhizosphere environment, rhizodeposition includes different fractions: root exudates, lysates, mucilage, secretions, and dead cell material (Lynch and Whipps, 1990). A substantial portion of the root exudates consists of carbon and energy sources readily available for microbial growth development and the physiology of microbial cell populations (Sommers *et al.,* 2004). PGPR influence direct growth promotion of plants by fixing atmospheric nitrogen solubilizing insoluble phosphates, secreting hormones such as IAA, GAs, and Kinetins besides ACC deaminase production, which helps in regulation of ethylene.

Several stages of plant growth and development such as cell elongation, cell division, tissue differentiation, and apical dominance are controlled by the plant hormones, especially auxins and cytokinins. The biosynthesis and the underlying mechanism of auxins and cytokinins action are subjects of intense investigation. Auxins and cytokinins can be synthesized by both the plants and the microorganisms. Although the role of phytohormone biosynthesis by microorganisms is not fully explained, it is stated that direct mechanisms of plant growth by PGPR include production of plant

hormones such as auxins, cytokinins, GAs, and lowering of plant ethylene levels (Glick, 1995; Lucy *et al.,* 2004). On the other hand, some pathogenic bacteria such as *Pseudomonas syringae*, *Agrobacterium tumefaciens*, and *Erwinia herbicola* synthesize IAA predominantly via the indole-3-acetamide (IAM) pathway (Dobbelaere *et al.* 2003). The role of IAA in the observed plant growth promotion was obtained by attempting to mimic the effect of the bacterium for the root growth by the direct application of IAA on the roots. Inoculation with *Bacillus* RC23, *Paenibacillus polymyxa* RC05, *B. subtilis* OSU142, *Bacillus* RC03, *Comamonas acidovorans* RC41, *B. megaterium* RC01, and *B. simplex* RC19 with tea (*Camellia sinensis*) cuttings enhanced rooting percentages when compared with control because of IAA production of bacteria. Similarly, treatments of hardwood stem cuttings of kiwifruit cv. Hayward, stem cuttings of two rose selections (ERS 14, *Rosa canina*, and ERS15, *Rosa dumalis*), sour cherry (*Prunus cerasus*) softwood and semi-hardwood cuttings and *Pistacia vera* cuttings with *Agrobacterium rubi* (A1, A16, and A18) and *Bacillus subtilis* OSU142 promoted rooting ratio and increased the numbers of lateral roots (Orhan *et al.,* 2007). In addition, *Azospirillum* is not only capable of nitrogen fixation but also codes for plant growth hormone auxins in addition strains of *Azospirillum* showed that production depended on the type of culture media and availability of tryptophan as a precursor. *A. brasilense* Cd produced the highest level of IAA among the *Azospirillum* strains tested (Bashan *et al.,* 2004). The isolation and quantification of cytokinins in nonpathogenic soil bacteria in general and diazotrophic bacteria in particular has received a little attention.

Cytokinins are a diverse group of labile compounds that are usually presented in small amounts in biological samples and are often difficult to identify and quantify (Dobbelaere *et al.,* 2003). Cytokinins are produced by bacteria such as *Azospirillum* and *Pseudomonas* spp. Moreover, a few PGPR strains were reported to produce cytokinins, such as *Rhizobium leguminosarum*, *Paenibacillus polymyxa*, and *Pseudomonas fluorescens* (Vessey, 2003). These studies sufficiently cloud the production of cytokinins, compared with IAA or GAs, in PGPR. Also, it appears that more work is necessary before proving for the role of PGPR-produced cytokinins in plant growth promotion. Also in the case of GAs, the bacterial genetic determinants have not been identified so far. Therefore, no mutants are available to demonstrate the role of this phytohormone in plant growth promotion (Dobbelaere *et al.,* 2003). Also the evidence of GA production by PGPR is

rare (Vessey, 2003). On the other hand, PGPR such as *Azotospirillum brasilense, Acetobacter diazotropicus, Herbospirillum seropedicae, Bacillus licheniformis, B. pumilus, B. cereus* MJ-1 and *B. macroides* CJ-29 were reported to produce GAs (Joo *et al.,* 2004). However, this is not a strong evidence of GA production in a common method of growth promotion by PGPR. Nevertheless, Gutierrez-Manero *et al.* (2001) provide an evidence that four different forms of GAs are produced by *B. pumilus* and *B. licheniformis*. Inoculation of alder (*Alnus glutinosa*) with these PGPR could effectively reverse a chemically induced inhibition of stem growth. In addition to this research, Joo *et al.,* (2004) reported that the growth of red pepper plug seedlings was increased by *B. cereus* MJ-1, *B. macroides* CJ-29, and *B. pumilus* CJ-69, though the number of leaves and stem diameter were not significantly changed. The greatest increase is in the height and the root fresh weight of the seedlings was by *B. pumilus*, which could increase the height by 12% and the root fresh weight by 20%. In the last few years, a new mechanism of plant growth promotion involving ethylene has been proposed by Burdman *et al.,* (2000). Showing that some soil bacteria contain 1-aminocyclopropane-1-carboxylate (ACC) deaminase Glick *et al*. (1998) put forward the theory that the mode of action of some PGPR was the production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, an enzyme that could cleave ACC, the immediate precursor to ethylene in the biosynthetic pathway for ethylene in plants. They submitted that ACC deaminase activity would decrease ethylene production in the roots of host plants and results in root lengthening. In some cases, the growth promotion effects of ACC deaminase producing PGPR is the best expressed in stress conditions including drought and salt (Nadeem *et al.,* 2007; Zahir *et al.,* 2009) stress. PGPR (containing ACC deaminase) boost plant growth particularly under stressed conditions by the regulation of accelerated ethylene production in response to a multitude of abiotic and biotic stresses such as salinity, drought, waterlogging, temperature, pathogenicity, and contaminants (Saleem *et al.,* 2007). For example, under salinity stress, 1-aminocyclopropane-1-carboxylic acid-deaminase activity of *P. putida* (N21), *P. aeruginosa* (N39) and *Serratia proteamaculans* (M35) might have caused reduction in the synthesis of stress (salt)-induced inhibitory levels of ethylene (Zahir *et al.,* 2009). Similarly, inoculation with *Variovorax paradoxus* 5C- 2 improved growth, yield, and water-use efficiency of droughty peas (Belimov *et al.,* 2009). It is reported that inoculation with *P. fluorescens* was found to be more effective

in promoting root growth than that with *P. putida* as it caused up to 46% increase in root elongation and up to 94% increase in root weight of pea over the respective uninoculated drought stressed control (Arshad *et al.,* 2008). Since the last few decades, the response of agriculturally important crops to inoculation with PGPR was investigated in numerous field and greenhouse experiments carried out in various countries. On the basis of the given data, it was concluded that inoculation with PGPR resulted in significant yield increases in different crops, enhanced rooting of hardwood and semi-hardwood cuttings, seed germination and emergence under different conditions. In other words, they can affect plant growth and yield in a number of ways and enhancement of vegetative and reproductive growth is documented in a range of crops such as cereals or vegetables. Treatments with PGPR increase germination percentage, seedling vigor, emergence, plant stand, root and shoot growth, total biomass of the plants, seed weight, early flowering, grains, fodder and fruit yields, etc., (van Loon *et al.,* 1998; Ramamoorthy *et al.,* 2001). In crop production, there is a continuous demand of increasing crop productivity and quality. There are lot of agricultural practices applied for increasing the yield and the yield components. Recently, one of them is applications of PGPR for increasing yield and environment friendly crop production. Floral and foliar applications of PGPR strains *Pseudomonas* BA-8 and *Bacillus* OSU-142 on apple trees significantly increased yield per trunk cross-section area (13.3–118.5%), fruit weight (4.2–7.5%), shoot length (20.8–30.1%), and shoot diameter (9.0–19.8%) in "Starkrimson" and yield per trunk cross-sectional area (TCSA; 14.9%) and fruit weight (6.5–8.7%) in "Granny Smith" compared with the control (Pırlak *et al.,* 2007). Karlıdag˘ *et al.* (2007) reported similar results in apple.

The term mycorrhizosphere is referred to as the zone of mycorrhizal colonisation in and outside the plant root (Andrade *et al.*, 1997). In general mycorrhizal fungi, through modifications to the plant root system, interact with beneficial soil organisms such as N2-fixing bacteria, P solubilising bacteria, fungi and root inhabiting nematodes. These interactions are important in the natural ecosystem for nutrient cycling (Gryndler, 2000;). Some bacteria are known to facilitate mycorrhizal formation by affecting spore germination or root colonisation (Fitter and Garbaye, 1994). An *in vitro* experiment with *Klebsiella pneumoniae* grown in compartments next to those containing *Gl. deserticola* showed no effect of the bacteria on number of spores germinated but 9 days later, there

was an increased AM fungal hyphal extension away from germinated spores (Will and Sylvia, 1990). This indicated the potential of *Klebsiella pneumonia* to influence hyphal extension through the production of volatile compounds such as long chain alcohols-1decanol or 1dodecanol. The interaction of AM fungi with *Rhizobium* has received considerable attention due to the high P demand for N2 fixation. Studies have shown that the co-inoculation of legumes with rhizobia to fix nitrogen and AM fungi increased plant growth than when inoculated with rhizobia alone. This was attributed to the fact that under limiting conditions of N and P, AM fungi improves P uptake thereby enhancing the plants nitrogenase activity, which in turn promotes root and mycorrhizal development (Abdalla *et al.,* 2000). Thus the symbiotic role of *Rhizobium* is said to be dependent on the beneficial nutrient effect of AM fungi. Apart from P, enhanced uptake of other nutrients such as Zn, Cu and Ca by AM fungi can influence the symbiotic effectiveness of *Rhizobium* as well as other microbial processes that occurs at root or nodule level (Barea *et al.*, 2002). Though *Rhizobium* is well known inoculants for legumes,they have also been used as inoculants for non-leguminous plants. Galal *et al.* (2003) studied the effect of P and N fertilisation on the growth and yield of wheat inoculated with AM fungi and *Rhizobium* using radiolabelled 15N technique. They observed an increase in growth of wheat when both *Rhizobium* and AM fungi were inoculated together at high levels of N and P. This dual inoculation also facilitated the uptake of N and P; while the single inoculation of plants with AM fungi increased yield of wheat grain. This indicated the ability of both organisms to stimulate plant growth and accumulate P and N (Requena *et al.*, 1997). Species of *Azobacter, Azospirillum, Derxia* and *Clostridium* are well known free-living diazotrophs that fix atmospheric nitrogen (Linderman, 1992). A synergistic effect was observed between *Gl. fasciculatum* and *Azotobacter chroococcum* in tomato plants. The latter helped to enhance fungal colonisation and spore production, while the former increased the bacterial population in the rhizosphere (Bagyaraj, 1984). Biró *et al*. (2000) observed an increase in nodulation of alfalfa plants with co-inoculations of *Gl. fasciculatum*, *Azospirillum* and *Rhizobium* under sterile and normal soil conditions. Phosphate solubilising bacteria (PSB) have great prospects to improve plant growth under given conditions such as in P deficient soils when used in conjunction with AM fungi (Gryndler, 2000). They are known to mobilise phosphate ions from sparingly soluble organic and inorganic P sources. However, the released P does not reach the root

surface as a result of inadequate diffusion (Barea *et al.*, 2005; Azcón-Aguilar and Barea, 1992). It was proposed that AM fungi could improve the uptake of the solubilised P; hence, this combined interaction should improve P nutrition and supply to plants (Barea *et al.*, 2002). The interactive effects of AM fungi and PSB on plant use of soil P in the form of either endogenous or added rock P was studied using a soil microcosm system integrated with 32P isotopic dilution. Results revealed that the PSB (*Enterobacter sp*. and *Bacillus subtilis*) promoted mycorrhizal establishment of *Gl. intraradices* and their combined inoculation increased biomass, N and P accumulation in the onion plant tissues (Toro *et al.*, 1997). Thus, the inoulation of organisms may result in utilisation of P fertilisers that quickly become unavailable in soils (Picini and Azcon, 1987). Multi-microbial interactions between AM fungi, PSB and *Azospirillum* have been reported to be synergistic when inoculated together (Muthukumar *et al.*, 2001). Muthukumar *et al.*, (2001) confirmed this by inoculating the Neem tree seedlings with *Gl. intraradices*, *Gl. geosporum*, *Azospirillum brasilense* and isolated PSB individually or in various combinations under nursery conditions. Mycorrhizal colonisation, leaf area and number, plant height and biomass, nutrient content (N, P and K) and seedling quality were found to be significantly increased because of combined microbial inoculants. Some soil bacteria isolated from the rhizosphere possess the ability to produce compounds such as antibiotics or siderophores which are Fe chelators that may act as inhibitors against pathogens or stimulate plant growth. *Pseudomonas* strains that produce non-volatile diffusible compounds such as methane, acetaldehyde, acetoin and diacetyl that may or may not reduce mycorrhizal volume (Aspray *et al*., 2006; Gryndler, 2000; Linderman, 1992). Vázquez *et al.*, (2000) demonstrated that the incorporation of a fungus, *Trichoderma harzianum* with *Pseudomonas fluorescens*, *Azospirillum* spp. and AM fungal species (*G. mosseae* and *G. Deserticola*) did not affect the establishment of AM fungal species in maize. However, an increase in phosphatase, esterase, trehalase and chitinase enzymatic activity was observed. These soil enzymes are mainly used as an indicator to detect microbial functioning in the rhizosphere as influenced by AM fungi and differ in their activity. Phosphatase which is produced by bacteria and AM fungi catalyses organic bound P into inorganic P (Häusling and Marschner, 1989). Esterase indicates catabolic activity in the soil which is directly correlated to microbial acivity (Vázquez *et al.*, 2000). The enzyme trehalase hydrolyses trehalose a common sugar

found in plant symbioses, while chitinase degrades chitin, a major component of fungal cell walls that plays a role in plant defence mechanisms (Pozo *et al.*, 2002). Similarly, the dual inoculation of subterranean clover and maize with *Pseudomonas putida* and different species of AM fungi were found to enhance plant growth and AM fungal colonisation (Gryndler and Vosátka, 1996; Paulitz and Lindermam, 1989). It was suggested that before commercial bacterial inoculants are considered, resultant changes in the mycorrhizosphere should be studied. Walley and Germida (1997) proposed this when they observed that the interaction of five pseudomonads selected as PGPR under laboratory conditions affected plant growth and AM fungi root colonisation when tested in the field. This effect was found to vary (positive or negative) based on the bacterial inoculant strain, harvest date and growth parameter. In addition, Ravnskov *et al.* (1999), observed under controlled conditions that the fungus *Gl. intraradices* had a negative effect on the growth and survival of *Pseudomonas fluorescens* DF57 which was likely to be due to competition for nutrients. PGPR exert direct or indirect effects on plant growth and belong mainly to the genera *Paenibacillus*, *Burkholderia*, *Pseudomonas* and *Bacillus* sp. The direct effects are through the release of phytohormones, nitrogen fixation and mineralisation of organic phosphates into available forms for plants. While the indirect effect on plant growth is realised by decreasing or preventing deleterious effects of pathogenic organisms mainly through the synthesis of antibiotics or production of siderophores.

Solubilisation of P is reported to be the most common mode of action for PGPR. Singh and Kapoor (1998) showed that phosphate solubilising bacteria (PSB) such as *Bacillus circulans* together with AM fungi increased plant yield and P uptake of wheat. There are some inconsistencies in reports of the effects of PGPR on AM fungi as well as in their mode of action. *Bacillus substilis* and *Enterobacter* sp. were found to promote the establishment of *G. intraradices*, increase plant biomass and N and P contents of onion (Toro *et al.*, 1997). While studies by Walley and Germida (1997) using different *Pseudomonas* strains with the co-inoculation of AM fungi observed varying effects i.e some strains of *Pseudomonas* hindered AM fungal germination. Hence it can be argued that not all PGPR are mycorrhizal helper bacteria (MHB) or vice versa. So it will be worthy to have a brief idea about MHB.

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 (Schrey *et al.*, 2005; Garbaye, 1994). When PGPR are found to stimulate mycorrhizal formation they can be regarded as MHB (Fitter and Garbaye, 1994). This interchangeable characteristic brings about the overlap that exists between the two groups. Similarly, not all P solubilising PGPR promote plant growth by P availability to the host. Studies by De Freitas *et al.* (1997) revealed that a number of *Bacillus* strains and *Xanthomonas maltophilia* isolated from the rhizosphere of canola a non-mycorrhizal plant had positive effects on plant growth but not on P content of the host plant. This indicated that P solubilisation was not responsible for the plant growth response. 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. For example, P solubilisers having additional functions (Linderman, 1988). 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 solubilisers or grouped as PGPR or MHB (Rodriguez and Fraga, 1999). For example, Pandey *et al.* (2005) isolated an organism coded as MSSP from the root nodules of *Mimosa pudica*. This organism was found to belong to the *Burkholderia* genus and had the ability to fix N, solubilise P and had all the characteristics of PGPR. Similarly, studies and have also reported species of the genera *Bradyrhyzobium*, *Sinorhizobium*, *Rhizobium and Azorhizobium* as PGPR and phosphate solubilisers (Vessey, 2003; Rodriguez and Fraga, 1999; Antoun *et al.*, 1998). Few studies have been carried out on the interaction between AM fungi 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 (Bagyaraj, 1984). The production of inhibitory compounds by actinomycetes could be seen as the organism's way of competing with others organisms for nutrients. However, other species of Actinomyces belonging to the genus *Frankia* were able to form a synergistic relationship with AM fungi when

inoculated together in actinorrhizal plants such as tibetan seabuckthorn (*Hippophae tibetana*) and *Discaria trinervis* (Tian *et al.*, 2002; Gryndler, 2000). *Streptomyces* is a common soil organism belonging to the actinomycetes. Their effect on AM fungi varies according to species. For example, the colonisation of finger millet roots by *Gl. fasciculatum* was shown to be inhibited by *Streptomyces cinnamomeous* (Krishna *et al.*, 1982) while *Streptomyces orientalis* produced volatile compounds that stimulated germination of the resting spores of *Gl. mosseae*, *G. margarita* and *Scutellospora heterogama* when cultured auxenically (Tylka *et al.*, 1991). 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 symbiosis (Johannson *et al.,* 2004; Minerdi *et al.,*2002). To demonstrate this symbiosis, a combination of morphological and molecular techniques were conducted and it was concluded that the AM fungal spores of *G. margarita, Gl. versiforme* and *A. laevis* spores harboured these BLOs in their cytoplasm (Bianciotto *et al.* 1996b; Minerdi *et al.,* 2002). 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* (Minerdi *et al.,* 2002). Investigation of two geographically separated isolates of *Gigaspora margarita* and four other isolates, *G. gigantea*, *G. rosea*, *G. margarita/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 (Minerdi *et al.,* 2002). Bianciotto *et al.* (2003) further analysed the morphological and molecular similarities between the endosymbionts found in *G. magarita*, *S. persica and S. castenea*. It was observed through the amplification and sequence of partially complete 16S rRNA that all endosymbionts obtained from the three AM fungal species were over 98% similar to each other. This genomic similarity in their ribosomal sequence led to their being referred to as 'Candidatus Glomeribacter gigasporarum'.

AM fungal colonisation of plant roots has been suggested to increase plants tolerance to pathogens thereby acting as a biocontrol agent (Azcón-Aguilar and Barea, 1996). A biocontrol agent is defined as the use of a biologically friendly resource from the ecosystem that can target and protect plants against pathogens (Azcón-Aguilar et al., 2002). Several mechanisms or combination of mechanisms could account for the observed bio protection of plants by AM fungi. Some of these pathogens can be root-infecting fungi that are antagonistic and capable of feeding on their host as necrotrophs, wilt pathogens such as *Fusarium oxysporum*, or root rotting pathogens like *Phytophthora* and *Rhizoctonia* that are common soil borne pathogens (Smith, 1988).

AM fungi, the most widespread symbionts on earth, are receiving attention because of the increasing range of their application in sustainable agriculture and ecosystem management. Since AM fungi are obligate symbionts, most studies have been conducted on a host plant grown in a sterilized medium using potculture methods. Procedures such as single-spore culture isolates of AM fungi have been a valuable resource, not only for plant growth experiments, but also for taxonomic and biochemical studies. Several techniques for establishing single-spore isolate have used germinated and ungerminated spores. There are also some basic techniques in AM fungal research such as the isolation of AM fungal spores from soil, their identification, the establishment of pot cultures in the greenhouse, methods for isolating extra-radical mycelium from soil, vital staining of mycorrhizal roots and methods for AM fungal inoculums production. New approaches to the study of the biology of AM fungi have also been developed, involving growing these fungi in Ri T-DNA transformed root cultures in which some AM fungus species develop profusely and form viable spores. On the other hand, only those microorganisms which can grow in the rhizosphere are suitable for use as biocontrol agents, as the rhizosphere provides the first line of defense for the roots of a plant against attack by soil-borne pathogens. Also the introduction of bioinoculants having plant growth-promoting and biocontrol activity will be successful if they have rhizosphere competence to exert the desired effect to the plants. In last few decades a large array of bacteria including species of *Pseudomonas, Azospirillum, Azotobacter, Klebsiella, Enterobacter, Alcaligenes, Arthrobacter, Burkholderia, Bacillus, Rhizobium* and *Serratia* have reported to enhance plant growth. The direct promotion by PGPR entails either providing the plant with plant growth promoting substances that are synthesized by the bacterium or facilitating the

uptake of certain plant nutrients from the environment. The indirect promotion of plant growth occurs when PGPR prevent deleterious effects of one or more phytopathogenic microorganisms. The exact mechanisms by which PGPR promote plant growth are not fully understood, but are thought to include (i) the ability to produce or change the concentration of plant growth regulators like indoleacetic acid, gibberellic acid, cytokinins and ethylene (ii) asymbiotic N2 fixation (iii) antagonism against phytopathogenic microorganisms by production of siderophores, antibiotics and cyanide (iv) solubilization of mineral phosphates and other nutrients. Some PGPR may promote plant growth indirectly by affecting symbiotic N2 fixation, nodulation or nodule occupancy. However, role of cyanide production is contradictory as it may be associated with deleterious as well as beneficial rhizobacteria. In addition to these traits, plant growth promoting bacterial strains must be rhizospheric competent, able to survive and colonize in the rhizospheric soil. Unfortunately, the interaction between associative PGPR and plants can be unstable. The good results obtained *in vitro* cannot always be dependably reproduced under field conditions. The variability in the performance of PGPR may be due to various environmental factors that may affect their growth and exert their effects on plant. The environmental factors include climate, weather conditions, soil characteristics or the composition or activity of the indigenous microbial flora of the soil. To achieve the maximum growth promoting interaction between PGPR and nursery seedlings it is important to discover how the rhizobacteria exerting their effects on plant and whether the effects are altered by various environmental factors, including the presence of other microorganisms. The mechanism of achieving optimum affect by using AMF and PGPR in singly or in combined form is not clear but the dual activity have proved undoubtly a boon for the sustainable agriculture in the coming decades.

Chapter 3
Material and methods

3.1. Plant Material

Fifteen tea (Camelia sinensis. Kuntz) varieties, of which six UPASI varieties (UP-2, UP-3, UP-8, UP-9, UP-26 and BSS-2) and nine Tocklai varieties (TV-18, TV-9, T-17, TV-22, TV-23, TV-25, TV-26, TV-29 and TV-30) being grown in the experimental field (15 year old bush) were taken into consideration for assessing mycorrhization along with seedlings of ten tea varieties (TV-25, TV-26, TV-9, TV-20, TV-18, T-17, AV-2, T-78, UP-3, UP-26) were also obtained from Gayaganga Tea Estate, situated 18 km from University of North Bengal. The selected seedlings were planted in the experimental field. Suitable management practices were adopted in the field throughout the years.

3.2. Isolation and identification of Arbuscular Mycorrhizal Fungi (AMF)

Tea [*Camellia sinensis* (L.) O. Kuntze, Family: Theaceae], **Rubber** [*Hevea brasiliensis* (Willd. ex A. Juss.) Müll. Arg. Family: Euphorbiaceae], **Areca** (*Areca catechu* L. Family: Arecaceae), **Coffee** (*Coffea arabica* L. Family: Rubiaceae) and **Cinnamon** (*Cinnamomum zeylanicum* Blume, family Lauraceae) were selected for AMF isolation. Spores of arbuscular mycorrhizal fungi were isolated from rhizosphere soil by wet sieving and decanting method (Gerdemann and Nicolson, 1963). Approximately 250 g of soil was suspended in 1 L water. Heavier particles were allowed to settle for a few seconds and the liquid was decanted through sieves of decreasing size (BS 60, BS 80, BS 100, BS 150 and BS 200). Pores are fine enough to remove the larger particles of organic matter, but coarse enough to allow the desired spores to pass through. The suspension that passed through these sieves was saved and stirred to resuspend all particles. The heavier particles were allowed to settle for a few seconds and the liquid decanted again through the sieve and spores collected by fine brushes and were kept in different Petri plates according to their size and colours. Moreover for further observations or purification of AMF spores sucrose gradient centrifugation method was used. In sucrose gradient centrifugation (Daniels and Skipper, 1982), spores and minimal amount of organic particles were further purified by suspending sieving in 40% sucrose solution and centrifuging at 2000 rpm (approximate 370 x g) for 1 minute. The supernatant (with spores) was passed through a sieve of 400 mesh and rinsed with distilled water to remove

sucrose residue. With the help of a simple microscope (10X), followed by dissecting microscope parasitized spores, plant debris etc were separated and clean spores were stained with PVLG 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 (Schenck and Perez, 1990).

3.3. Root colonization and histopathology

The method of Philip and Haymann (1970) was used to determine AMF association with the roots of five plants. Roots were cleared in 2% KOH for 15 min at 120ºC (1h at 90ºC in water bath) followed by rinsing with distilled H_20 thrice on a fine sieve or using a mesh and forceps. The roots were then kept in 2% HCl for 30 mins. HCl was decanted and roots were covered with 0.05% trypan blue in lacto glycerol (1:1:1 lactic acid, glycerol and water) for 15 min at 120ºC in water bath. Roots were placed into a Petri dish with 50% glycerol for distaining and were viewed under stereo dissecting microscope. Each plantation crop was studied from minimum three sites to find out the average spore population, and % root colonization.

3.4. Isolation of bacterial isolates

Soil was collected from healthy tea, coffee, areca, cinnamon and rubber plant rhizosphere.

3.4.1. Plant Growth Promoting Rhizobacteria (PGPR)

Five grams of soil particles loosely adhering to the roots were collected and suspended in 30 ml of sterile distilled water by constant stirring for one hour. The soil suspension was allowed to settle down till the two distinct layers were clearly visible. Then the upper light brown colored layer was used for isolation by dilution plate technique (Kobayashi *et al*., 2000). Nutrient Agar (NA) was used for isolation of bacteria and potato dextrose agar (PDA) for fungi. Isolated microorganisms were then placed in an incubator in the dark for observation of the microbial growth after 24, 48 and 96 hour in dark or maximum up to 10 days. Colony forming units (CFUs) were recorded. The representative colonies of bacteria and fungi were isolated and purified from the soil

dilution plates by repeated sub culturing until pure individual cultures were obtained. The isolates were identified up to the group or genus level using standard morphological, microscopic, cultural, and biochemical methods.

3.4.2. Mycorrhiza Helper Bacteria (MHB)

Mycorrhiza Helper Bacteria (MHB) were isolated as per the protocol of Budi et al (1999). Spores of *G. mosseae* (45-50) were 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.

3.5. Biochemical tests of bacterial isolates

3.5.1. Gram reaction

Smears of test organisms prepared from 24h old culture (on nutrient agar slant) with sterile distilled water were made in the centre of clean grease-free slides. The smears were air dried, heat fixed with crystal violet (crystal violet – 2.0g, 95% alcohol- 20ml, ammonium oxalate 1% W/V, aqueous solution – 80ml) stain for 1 min, washed with tap water for 5 sec, flooded with Burke's iodine solution (Iodine 1.0g, KI- 2.0g, distilled water 100ml) and allowed to react for 1 min. Slides were washed for 5 sec in 95% ethanol which was poured drop by drop by holding the slides in slanting position till the smears becomes decolorised, 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.

3.5.2. 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.

3.5.3. Urease activity

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.

3.5.4. H_2S Production

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

3.6. *In vitro* tests for plant growth promoting activity of bacterial isolates

3.6.1. 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.

3.6.2. 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.

3.6.3. 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.

3.6.4. 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 in side 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.

3.6.5. Chitinase production

For detecting the chitinolytic behavior of the bacteria chitinase detection agar (CDA) plates were prepared by mixing 1.0% (w/v) colloidal chitin with 15 g of agar in a medium consisted of (Na_2HPO_4 6.0 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g, yeast extract 0.05g and distilled water 1 L; pH 6.5). The CDA plate was spot inoculated with organism followed by incubation at 30°C for 7-10 days. Colonies showing zones of clearance against the creamy background were regarded as chitinase producing strains (Kamil *et al*. 2007). The colloidal chitin was prepared by following the method described by Roberts and Selitrennikoff (1988). 5 g of chitin powder was slowly added to 60 ml of concentrated HCl and left at 4°C overnight with vigorous stirring. The mixture was added to 2 L of ice cold 95 % ethanol with rapid stirring and kept overnight at 25°C. The precipitation formed was collected by centrifugation at 7000 rpm for 20 min at 4°C and washed with sterile distilled water until the colloidal solution became neutral (pH 7). The prepared colloidal chitin solution (5 %) was stored at 4°C until further use.

3.6.6. Protease production

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

3.7. Scanning Electron Microscopy of AMF and PGPR

3.7.1. Arbuscular mycorrhizal fungi

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.

3.7.2. Plant Growth Promoting Rhizobacteria

For scanning electron microscopy of the bacterial cells, 2 days old culture grown in nutrient broth medium were centrifuged at 3000 rpm. The pellet were collected and washed with 0.1 M phosphate buffer saline then the samples were prefixed in 2.5 % glutaraldehyde in 0.1 M phosphate buffer pH 6.8 under vacuum followed by dehydrolysis of the sample by different ethanol volumes starting; 30%, 50%, 70%, 80%, 90% and 100% and for each ethanol volume incubated for 10 minutes. After stepwise dehydration in graded alcohol, the samples were critical point dried in CO_2 (CPD 030; BAL TEC, Vaduz, Liechtenstein), mounted onto the sample stubs and were coated with 20 nm silver-palladium alloy in a mini sputter coater (SC7620) and examined in a JEOL JSM 5200 Scanning Electron Microscope (Tokyo Japan).

3.8. Assessment of growth of PGPR

For assessment of bacterial growth in liquid medium,1ml of bacterial suspension was inoculated into the medium and allowed to grow for desired period. Following growth, absorbance was noted in a colorimeter at 600nm. Absorbance was converted into cfu/ml from a standard where known concentration of bacterial suspension was used. The

cfu values were counted to log whenever needed. For assessment of bacterial growth, different media were used. These are as follows;

A. Nutrient broth (NB):

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

B. Nutrient agar medium (NA):

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

C. Luria broth (LB):

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

pH 7.2 ± 0.2

D. Nutrient sucrose broth (NSB):

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

3.9. Mycelial growth and assessment of fungal pathogen (*S. rolfsii*)

3.9.1. Solid media

Mycellial growth of *S. rolfsii* was observed in Potato Dextrose Agar (PDA).

3.10. Antifungal activity of PGPR against tea root pathogen

3.10.1. Fungus

3.10.1.1 Solid medium

The efficacy of individual fungal isolates, from tea rhizosphere was tested *in vitro* for inhibiting growth of the pathogen in dual culture using PDA or NA. Each fungal isolate was placed at one side of the agar plate about 1cm away from the edge and 7mm diameter block of the pathogen taken from growing edge of the fungal culture was inoculated at the other half of the Petri plate. For each test three replicate plates were used. The plates were incubated for 7 days (depending upon the growth of the pathogen) at 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.

3.10.2. Bacteria

3.10.2.1 Solid medium

The obtained bacterial isolates were evaluated against tea root pathogens- *Ustulina zonata, Fomes lamaoensis, Sclerotium rolfsii* and*, Fusarium oxysporum,* in dual culture using NA medium. The bacteria were streaked on one side of the Petri plate, 5mm fungal pathogen block was placed at the other side of the plate, incubated 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 the pathogen, were selected for further evaluation and identification.

3.11. *In vitro* germination of AMF

In vitro culture of AM fungi is still a big challenge for mycorrhizologists. The pre-symbiotique growth of AM is evident by the formation of a running hyphae. After some weeks without additional host partner, growth of germinated AM propagules ceases to grow. Factors such as nutrition, chemical elicitors and genetical factors might be responsible for the lack of growth of the extraradical phase of AM fungi. The selected spores were separated from soil by wet sieving followed by rinsing three times with distilled water. The isolated spores were transferred to filter paper in a funnel where they were surface-sterilized with 5% chloramine-T for 3 mins and were again washed with distilled water. Simultaneously use of a dissecting microscope (20x) situated in a clean air hood, spores were transferred with forceps to Petri dishes (5 cm diam) containing the water agar media. 500 µl of root extracts of three days old maize seedlings were added. The spores were then incubated in the dark at 28° C. In another set of experiments surface sterilised AMF spores were kept on moist filter paper with three days old seedlings of maize plant in a petri dish (8 sets). The set up was kept in dark at 28° for two weeks. The whole set up was regularly checked for any hyphal growth.

3.12. Preparation of inoculum and inoculation technique

Cultures of *Sclerotium rolfsii* were grown in sand- maize meal medium (maize meal: sand: water- 1:9:1.5 w:w:v); (Biswas and Sen 2000) 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. Selected varieties of tea plants were then inoculated by adding 100g of prepared inoculum of *S. rolfsii* to the rhizosphere of each plant. Inoculation was done 3 days after final application of bacteria.

3.13. Disease assessment in tea plants

To determine the disease assessment sick plot was prepared. For this, roots of infected plants were chopped and mixed with the soil, along with inocula of *S.rolfsii*. Separate plots were earmarked for control set. The percentage of disease incidence was calculated by dividing the number of diseased plants by total number of plants and then multiplying by hundred while disease intensity was calculated by using 0 - 6 scale as adopted by Chakraborty *et al.* (2006) after 15, 30 and 45 days of inoculation. The disease infection observation were recorded in a continuous 0-6 scale, where 0 = no symptoms; 1 = small roots turn brownish and start rotting; 2 = leaves start withering and 20 - 30 % of root turns brown; 3 = leaves withered and 50 % of leaves affected; 4 = shoot tips also start withering and 60-70 % root affected; 5 = shoot withered with defoliation of lower withered leaves and 80 % roots affected; 6 = whole plant die with upper withered leaves still remaining attached and roots fully rotted.

3.14. Mass multiplication of bioinoculants and their *in vivo* application.

3.14.1. AMF

Maize (*Zea mays*), sorghum (*Sorghum bicolor*) and common tuff grass (*Cynodon dactylon*) are being used for the mass multiplication of AM fungi. Black plastic pots (12inch) were filled with sterilized soil to discard the presence of other fungal propagules. After the plantation adequate water was given. Spores of *Glomus mosseae* and Glomus *fasciculatum* were separated from the mass of other AM spores by fine tweezers and needles under dissecting microscope and were washed by distilled water several times to remove the adhered debris followed by inoculation in the roots (7-10 days old). After 45 days the presence of spores of *G. mosseae* and Glomus *fasciculatum* were verified and inocula were prepared by mixing the chopped roots of sorghum / maize /tuff grass plants with the potted soil where extra radical spores of required spores were present. Approximately > 175 spores / 100gms could be considered as potent inocula for application.

3.14.1.1. Single cell-line culture

In order to develop pure cell line culture of two specific AM fungi (*Glomus fasciculatum* and *Glomus mosseae*) 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 *Glomus fasciculatum* and *Glomus mosseae* were carefully put in 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 **(Fig. 3)**

3.14.2. PGPR

3.14.1.1. 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 waater. The optical density of the suspension was adjusted using UV-VIS spectrophotometer following method to obtain a final density of 3 X 10^6cfu ml^{-1}. The bacterial suspension was applied to the soil adjacent to the main root system. Applications were done @ 0f 100 ml per plant at regular interval of one month for three months subsequently

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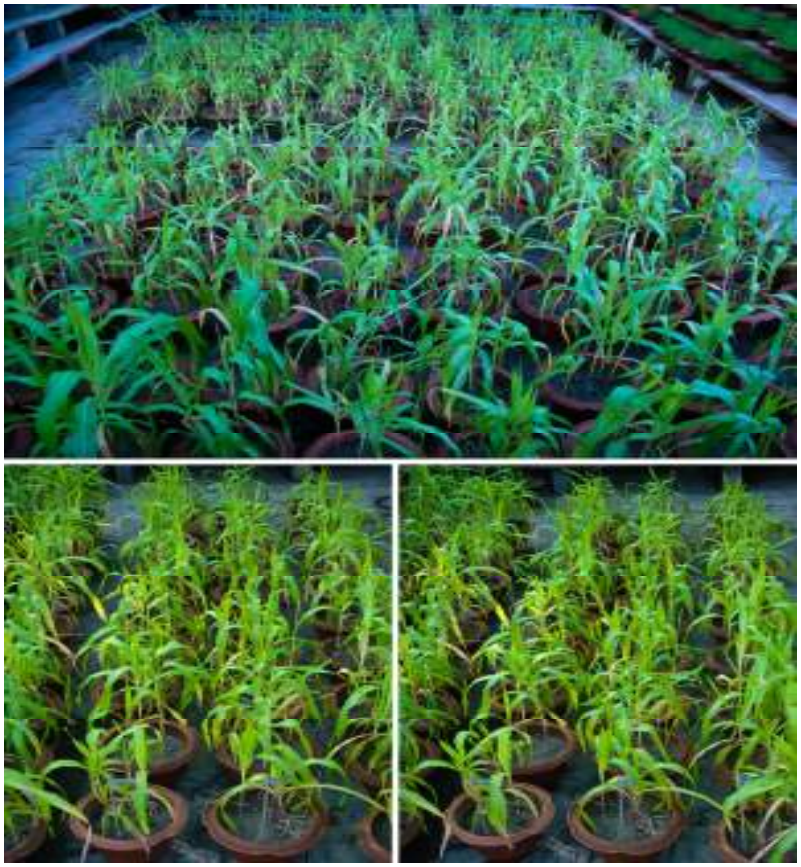

Fig2. Mass multiplication of AMF spores in *Zea mays*

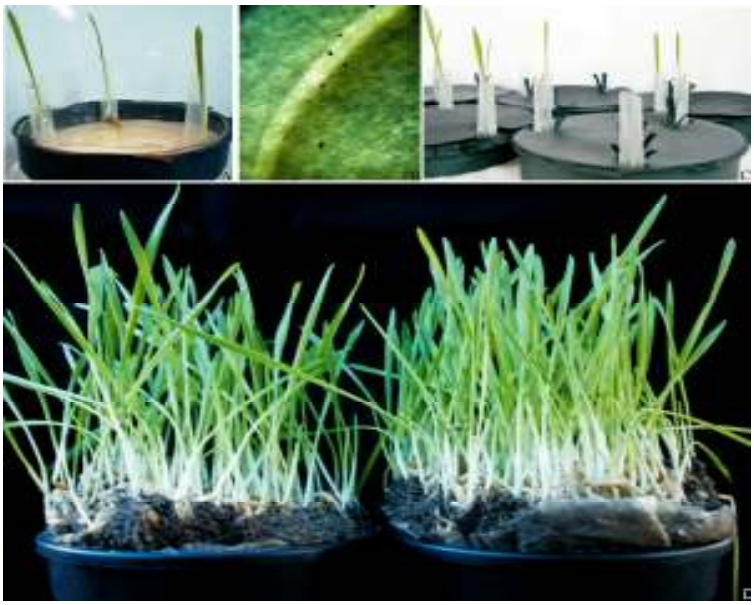

Fig3. Mass multiplication of AMF spores in *Sorghum bicolor*. Single spore culture (A-C). Mass multiplication in *S. bicolor* in sterile soil (D)

3.14.1.2. 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 selected plants for experimental purposes. The spraying was done forth nightly till the new shoots started appearing. The growth parameters such as number of leaves, branches and height were observed.

3.15. Assessment of plant growth promotion following application of AMF and PGPR

3.15.1. Root phosphate estimation

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). 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.

3.15.1.1. Leaf and root phosphate estimation

In case of root and leaf samples, oven dried plant material (leaf/root) was taken. Extraction and estimation of leaf phosphate contents was similar to the procedure that was followed in case of determination of soil phosphate contents.

3.15.2. Estimation of micro and macro elements

Estimation of micro and macro elements was done by the soil testing unit, Uttar Banga Krishi Vishwa Vidyalaya, Cooch Behar.

3.16. Extraction and assay of defense enzyme activity in tea plants following application of AMF and PGPR singly and jointly and artificially challenged with *S. rolfsii*.

3.16.1. 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.

3.16.2. 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.

3.16.3. 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.

3.16.4. 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).

3.16.5. Extraction and estimation of phenols from leaves

3.16.5.1. Extraction of phenol

Phenol was extracted from the fresh young leaves following the method of Mahadevan and Sridhar (1982). One g of leaf tissues 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.

3.16.5.2. Estimation

3.16.5.2.1. 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.

3.16.5.2.1.2. 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.

3.17. 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.

3.18. Analysis of tea leaf catechins

3.18.1. Extraction

Extraction from tea leaf tissues was done following the method of Obanda and Owuor (1994) with slight modification. Leaf samples (10 g) were extracted with 100ml of acetone at 45°C in water bath for 30 min. Extracts were decanted and filtered through Whatman No.1 filter paper. Acetone extract was concentrated to dryness and finally the residue was dissolved in 20 ml distilled water. Aqueous solution was extracted with equal volume of chloroform for four times. The pH of the water layer was adjusted to 2 by 2 drops of 2 N HCl and finally extracted with methyl isobutyl ketone. The extract was concentrated to dryness and finally dissolved in 3 ml of 2 % acetic acid. The samples were finally filtered through milipore filter (Milipore 0.4µm HA filter paper).

3.18.2. HPLC analysis

Catechin analysis of the extract was carried out on HPLC (Shimadzu Advanced VP Binary Gradient) using C-18 hypersil column with linear gradient elution system as follows- mobile phase A 100 % acetonitrile; mobile phase B 2 % acetic acid in water. Elution: 88 % B for 6 min then linear gradient to 75 % B over 5 min. The elution was complete after 25 min. Flow rate was fixed as 1 ml min^{-1} with sensitivity of 0.5 aufs. Injection volume was 20 µl and monitored at 278 nm.

3.19. Extraction and estimation of soluble proteins

3.19.1. 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± 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 fro the preparation of antiserum and other experiments.

3.19.2. Root

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

3.19.3. Estimation of protein content

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

optical density was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA).

3.20. SDS-PAGE analysis of soluble proteins

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

For the preparation of gel the following stock solutions were prepared:

(1) Acrylamide and N' N'- methylene bis acrylamide

A stock solution containing 29 % acrylamide and 1% bisacrylamide was prepared in water. The pH of the solution was kept below 7.0. The stock solution was then filtered through Whatman No. 1 filter paper, kept in brown bottle and stored at 4ºC and used within one month.

(2) Sodium Dodecyl Sulphate (SDS)

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

(3) Tris Buffer

(i) 1.5 M 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 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.

(4) Ammonium Persulphate (APS)

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

(5) Tris –Glycine electrophoresis buffer

Tris running buffer consists of 25 mM Tris base, 250 mM 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 1 L of distilled water.

(6) SDS gel loading buffer

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

(7) Preparation of gel

Mini slab gel (plate size 8 cm x10 cm) was prepared for the analysis of protein patterns by SDS-PAGE. For gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm thick spacers were placed between the glass plates at three sides and sealed with high vacuum grease and clipped tightly to prevent any leakage of the gel solution during pouring. 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 overlayered with isobutanol and kept for polymerization for 2 hours. 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 overlayered 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	0.50
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 1 M Tris pH 6.8 in stacking gel ** N, N, N', N'- Tetramethyl ethylene diamine		

(8) Sample preparation

Sample (50 µl) was prepared by mixing the sample protein (35 µl) with 1X SDS gel loading buffer (15 µl) in cyclomixer. All the samples were floated in boiling water bath for 4 min to denature the protein sample. The samples were immediately loaded in a pre-determined order into the bottom of the wells with a microloter syringe. Along with the samples, protein markers consisting of a mixture of six proteins ranging from high to low molecular mass (Phosphorylase b- 97,4000; Bovine Serum Albumin- 68,000; Ovalbumin- 43,000; Carbolic Anhydrase- 29,000; Soyabean Trypsin inhibitor-20,000; Lysozyme- 14,300) was treated as the other sample and loaded in a separate well.

(9) Electrophoresis

Electrophoresis was performed at a constant 15 mA current for a period of three hours until the dye front reached the bottom of the gel.

(10) 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 fixed in glacial acetic acid: methanol: water (10:20:70) for overnight. The staining solution was prepared by dissolving 250 mg of Coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. After the stain was completely dissolved, 45 ml of water and 10 ml 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 hours at 37°C with constant shaking at low speed. After staining the gel was finally destained with destaining solution containing methanol, water and acetic acid (4.5: 4.5: 1) at 40°C with constant shaking until the background became clear.

3.21. Immunological studies

3.21.1. Preparation of antigen

3.21.1.1. Fungal antigen

3.21.1.1.1. Pathogen

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.

3.21.1.1.2. AMF

Spores of *Glomus mosseae* and *G. fasciculatum* were isolated from rhizosphere soil of tea 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 *Glomus mosseae* 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.

3.21.1.2. Root antigen

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

3.21.2. Raising of polyclonal antibodies

3.21.2.1. Rabbits and their maintenance

Polyclonal antibodies were prepared against fungal 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.

3.21.2.2. 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. 21.2.3. 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)

3.22. Purification of IgG

3.22.1. Precipitation

IgG was purified as described by Clausen (1988). Crude antiserum (2 ml) was first diluted with two volume of distilled water and an equal volume of 4 M ammonium sulphate. The pH was adjusted to 6.8 and mixture was stirred for 16 h at 20°C. The precipitate thus formed was collected by centrifugation at 12000 rpm at 22°C for 1 h. Then the precipitate was dissolved in 2 ml of 0.02 M sodium phosphate buffer, pH 8.0.

3.22.2. 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

3.22.3. Fraction collection

At the top of the column, 2 ml of ammonium sulphate precipitatewas 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 buffer (pH 8.0). The buffer was applied in the flask on which rubber connection from its bottom was supplying the 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 mixed into buffer (1) thereby producing a continuous raise in molarity. Ultimately, 40 fractions each of 5 ml were collected and the optical density values were recorded at 280 nm using UV-Vis spectrophotometer (SYSTRONICS).

3.23. Immunological assays

3.23.1. Agar gel double diffusion

3.23.1.1. 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.

3.23.1.2. 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.

3.23.1.3. 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

3.23.2. 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.

Carbonate –bicarbonate (0.05 M, pH 9.6) coating buffer.

Tris buffer saline (10mM pH 7.4) with 0.9% NaCl and 0.5% Tween 20 for washing.

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.

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.

3.23.3. Fluorescence antibody staining and microscopy

Fluorescence antibody staining and microscopy were done following the method of (Chakraborty and Saha, 1994). Fungal mycelia were grown in liquid Richards's medium as described earlier. After five days of inoculation young mycelia were taken out from flask and taken in Eppendorf tube and washed with PBS (pH 7.2) by centrifugation at slow speed. Then mycelia was treated with normal sera or antisera diluted (1:50) in PBS and incubated for 1 h at room temperature. The mycelia was washed thrice with PBS- Tween (pH 7.2) as mentioned above and treated with Goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC) (Sigma chemicals) diluted 1:40 with PBS (pH 7.2) and incubated in dark for 45 min at room temperature. After incubation mycelia was washed thrice in PBS and mounted in 10% glycerol. A cover slip was placed and sealed. The slides were then ready to observe. Indirect fluorescence staining of AMF spres, arbuscules under tea roots, maize roots were also done using FITC and RITC labeled goat antirabbit IgG. PAbs raised against sonicated spores of *Glomus mosseae* and goat antisera specific to rabbit globulins conjugated with Fluorescein isothiocyanate (FITC) were used for indirect immunofluorescence study to detect the AMF in soil samples and to determine the cellular location of major cross reactive antigens (CRA) shared by AMF in healthy tea root tissues. Besides root colonization and cellular location of AMF was also observed both in mass multiplied host roots (maize) as well as tea roots

following colonization with AMF. 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 Scope Photo Image Software by Catalyst Biotech which can be directly attached to the microscope and the computer.

3.23.4. Localization of chitinase by immunofluorescence

Indirect fluorescence staining of cross-section of tea leaves was done using FITC labelled goat antirabbit IgG following the method of Chakraborty and Saha (1994). Initially, cross sections of healthy and infected tea roots were cut and immersed in phosphate buffer saline (PBS), pH 7.2. These sections were treated with normal serum or antiserum diluted (1:50) in PBS and incubated for 1h. at room temperature. After incubation, sections were washed thrice with PBS-Tween (pH 7.2) for 15 min and transferred to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC). The sections were incubated for 30 min 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. Photographs were taken by scope photo instrument directly attached to a computer. Fluorescence 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.

3.23.5. Western blot

Immunoblotting was also determined using Western Blot technique as described by Wakeham and White (1996). The following buffers were used for Western blotting-

(i) All the stock solutions and buffers used in SDS-Gel preparation for Western blotting were as mentioned earlier in SDS-PAGE protein.

(ii) Tranfer buffer (Towbin buffer):

(25mM Tris, 192mM glycine 20% reagent grade Methanol, pH 8.3).

Tris- 3.03g; Glycine- 14.4g; 200 ml Methanol (adjusted to 1lit. with dist. Water).

(iii) Phosphate buffer Saline, PBS, (0.15M, pH 7.2)

Stocks

A. Sodium dihydrogen phosphate- 23.40g in 1000ml dist. water

B. Disodium hydrogen phosphate- 21.294g in 1000ml dist. water

280 ml of stock A was mixed with 720 ml of stock B and the pH was adjusted to 7.2. Then

0.8% NaCl and 0.02% KCl was added to the solution.

(iv) Blocking solution

5% non fat dried milk + 0.02% sodium azide in PBS with 0.02% Tween 20.

(v) Washing buffers:

(a) Washing buffer-1: PBS

(b) Washing buffer-2: (50mM Tris-HCl, 150 mM NaCl, pH 7.5).

Tris- 6.07 g; NaCl- 8.78g; made up to 1lit. with distilled water.

(vi) Alkaline phosphatase buffer:

(100mM NaCl, 5mM $MgCl_2$, Tris- HCl, pH 9.5).

Tris- 12.14g; NaCl- 5.84g; $MgCl_2$- 1.015g; made up to 1 lit. with double distilled water.

(vii) Substrate

NBT: 5mg NBT in 100µl of 70% N,N-dimethyl formamide

BCIP: 5mg BCIP in 100µl of 70% N,N-dimethyl formamide

Substrate solution was prepared by adding 66µl NBT and 33µl BCIP in 10ml alkaline

phosphatase buffer.

Or, 1 tablet of NBT/BCIP (Sigma Chemical, USA) in 10ml of double distilled water.

(viii) Stop solution: (0.5M EDTA solution in PBS, pH 8.0)

EDTA sodium salt- 0.0372g in 200µl distilled water, added in 50ml of PBS.

Extraction and estimation of protein

Protein extraction and estimation was done as described earlier.

3.23.5.1. SDS PAGE of protein

SDS-PAGE was carried out as mentioned earlier.

3.23.5.2. Blot transfer process

Following the SDS-PAGE, the gel was transferred in prechilled transfer (Towbin) buffer for 1h. The nitrocellulose membrane (BIO-RAD, 0.45µm) and the filter paper (BIO-RAD, 2mm thickness) were cut to gel size, wearing gloves and soaked in Towbin buffer for 15 min. The transfer process was done in Trans- Blot SD Semi-Dry

Transfer cell (BIO-RAD) through BIO-RAD power pack. The presoaked filter paper was placed on the platinum anode of the semi-dry cell. A pipette glass (or glass rod) was rolled over the surface of the filter paper to exclude all air bubbles. The prewetted membrane was placed on top of the filter paper and air bubbles were rolled out. The equilibrated gel was carefully placed on the membrane and air bubbles were rolled out. Finally another presoaked filter paper was placed on the top of gel and air bubbles were removed. The cathode was carefully placed on the sandwich and pressed to engage the latches with the guide posts without disturbing the filter paper stack. The blot unit was run for 45 min at a constant volt (15V). After the run the membrane was removed and dried on a clean piece of 3mm filter paper for 1h. and proceeded for immunological probing.

3.23.5.3. Immunoprobing

Following drying, blocking was done by 5% non fat dried milk in a heat sealable plastic bag and incubated for 90 min with gentle shaking on a platform shaker at room temperature. Subsequently, the membrane was incubated with antibody (IgG) solution (blocking solution: PBS [1:1, v/v + IgG, diluted as 1:100 or as per requirement]. The bag was sealed leaving space for few air bubbles and incubated at 4°C overnight. All the processes were done with gentle shaking. Next day the membrane was washed thrice in 250 ml PBS (washing buffer -1). Final washing was done in 200ml washing buffer-2 to remove azide and phosphate from the membrane before enzyme coupled reactions. The enzyme, alkaline phosphatase tagged with antirabbit goat IgG (Sigma Chemicals) diluted (1:10,000) in alkaline phosphatase buffer, was added and incubated for 1h. at room temperature. After enzyme reaction, membrane was washed four times in washing buffer-2. Then 10ml substrate was added and the reaction was monitored carefully. When bands were observed up to the desired intensity, the membrane was transferred to tray of 50ml stop solution.

3.24. 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.

3.24.1. 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	**Genomic DNA Buffer**	**CTAB Buffer**
50 mM Tris, pH 8.0 100 mM EDTA 100mM NaCl 1% SDS	10 mM Tris, pH 8.0 0.1 mM EDTA	2% CTAB 1.5% PVP K 30 1.4 mM Nacl 20 mM EDTA 100mM Tris HCL pH 8.0 0.1% B-mercaptoethanol

3.24.2. 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 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.

3.24.3. 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.

3.24.4. Measure DNA Concentration using Spectrophotometer

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.

3.24.5. Agarose gel electrophoresis 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. Preparing an agarose gel involves melting a specified amount (0.8%) of agarose in 1X TBE buffer, cooling the solution, and pouring it into the gel casting tray with ethidium bromide. Gels solidify in 15-20 minutes.

3.24.6. Run gel electrophoresis for DNA fraction

The electrical lead of the gel tank was attached firmly and applied electric supply at constant current 90 mA and voltage 75 volt (BioRAD Power Pac 3000) at least for 90 minutes. 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.

3.25. ITS PCR analysis

Genomic DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase Chain Reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq pol buffer, 1 µl of 1 U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM reverse and forward primers and 1 µl of 50 ng template DNA. PCR was programmed

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

3.25.1. PCR primers

ITS-Primers pairs	**Primer Seq 5'-3'**	**Mer**	**TM**	**% GC**
T/ITS 1	TCTGTAGGTGAACCTGCGG	19	63.9	57%
T/ITS4	TCCTCCGCTTATTGATATGC	20	61.5	45%

3.25.2. Amplification conditions

Temperature profile, 94°C for 4 min. followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 60°C for 30 sec and extension at 70°C for 5 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler.

3.25.3. Sequencing of rDNA gene

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

3.26. Sequence analysis

DNA sequence information was analyzed using bioinformatic algorithms tools e.g. Bioedit, MEGA 4.

3.26.1. Chomatogram of sequence

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

3.26.2. Editing and alignment of sequence data

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

3.27. BLAST of Sequence

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.

3.28. 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.

3.29. Multiple sequence alignment and phylogenetic analysis

The sequenced PCR product was aligned with ex-type strain sequences from NCBI Gene Bank and established fungal taxonomy for identification. Sequences were aligned following the Clustal W algorithm (Thompson *et al.,* 1994). Phylogenetic inference was performed by the UPGMA (Unweighted Pair Group Method with Arithmetic Mean) and Neighbour Joining (NJ) method (Sneath and Sokal, 1973). Bootstrap tests with 1,000 replications were conducted to examine the reliability of the interior branches and the validity of the trees obtained (Tamura *et. al*., 2007).

Chapter 4
Results

4.1. Association of Arbuscular Mycorrhizal Fungi (AMF) in plantation crops

Arbuscular mycorrhizal fungal spores from the five major plantation crops *viz.* Tea (*Camellia sinensis*), Rubber (*Havea brasiliensis*), Areca (*Areca catechu*), Coffee (*Coffea arabica*) and Cinnamomum (*Cinnamomum zeylanicum*) were isolated and their average spore population and percent root colonization were determined. Morphological features of isolated AMF spores were critically examined with special reference to 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. Tea (*Camellia sinensis*)

AMF associations in tea plants from three geographically different tea gardens *viz,* Happy Valley, Darjeeling Hills (27°3′00″N 88°16′00″E, 2,100 metres above sea level), Hasqua Tea Garden, Siliguri (26°36'58"N 88°18'45"E, 126 metres above sea level) and Karala Valley Tea Garden, Jalpaiguri (26°31'3"N 88°42'13"E, 75 metres above sea level) were studied. Eleven different species of *Glomus*, six different species of *Acaulospora*, three different species of *Gigaspora* and four species of *Scutellospora* were found to be dominant in all the soil samples collected from different tea gardens. In addition few species of *Sclerocystis* were also documented.**.** Microscopical views of the spores are presented in Fig 4. Average population of AMF spores obtained from different tea rhizosphere have been presented in Table 2 and Fig 5**.** Among all the genera, the genus *Glomus* was predominant followed by *Acaulospora*, *Gigaspora*, *Scutellospora*, *Sclerocystis* and *Entrophospora*. *Glomus aggregatum*, *G. constrictum*, *G. mosseae, G. intraradices, G. fasciculatum* are most abundant species of the genus *Glomus* in tea rhizosphere collected from all the sites. The most common species of *Acaulospora* were found to be *A. bireticulata, A. capsicula* and *A. spinosa*. Large dark red *Scutellospora* spores, mainly S. calospora, S. rubra and S. pellucida are present throughout the year. Sporocarps of Sclerocystis with its conspicuous beaded amorphous structures are also present.

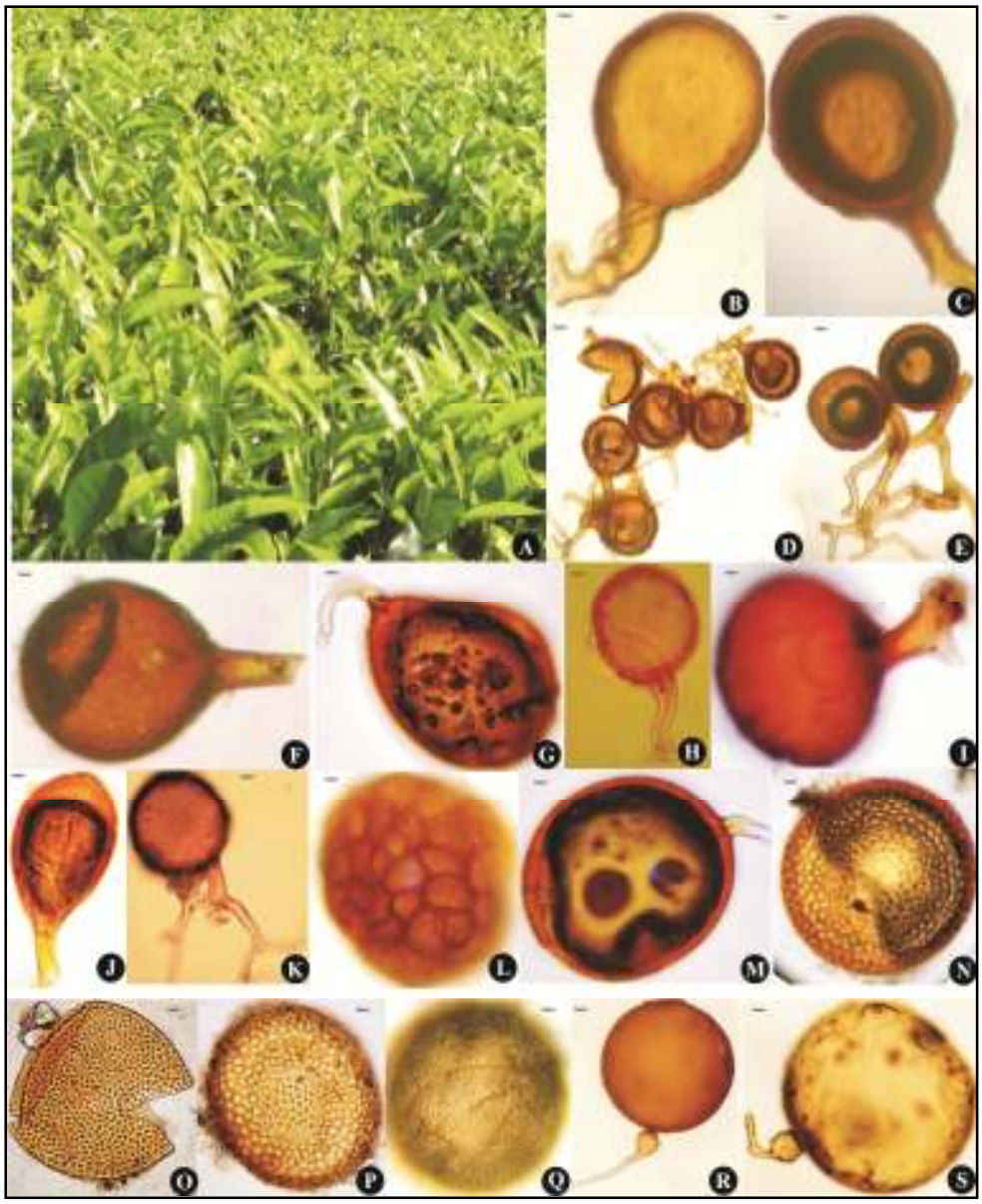

Fig.4. Compound microscopic observations of Arbuscular Mycorrhizal Fungal spores obtained from different tea gardens. General view of tea garden canopy located at Hasqua TE (A); Young spore of *Glomus mosseae* (B). Mature spore of *Glomus mosseae* (C); *G. intraradisces* (D);.*G. fasciculatum* (E); *G. constrictum* (F); *G. ambiosporum* (G); *Glomus* sp. (H) ; *Glomus badium* (I); *Glomus* sp (J) ;*Glomus* sp. (K); *Glomus* sporocarp (L); *Glomus* sp. (M); *Acaulospora* sp. (N); *Acaulospora* bireticulata. (O); *Acaulospora* sp. (P); *Acaulospora* sp. (Q); *Gigaspora* sp (R); *Scutellospora* sp. (S).

Table 2. Occurrence of different AMF in the rhizosphere soil of *Camellia sinensis*			
Name of AMF	**Percentage of spores in 100 gm soil**		
Acaulospora	Happy Valley, Darjeeling Hills (27°3′00″N 88°16′00″E)	Hasqua Tea Garden, Siliguri (26°36'58"N 88°18'45"E)	Karala Valley Tea Garden, Jalpaiguri (26°31'3"N 88°42'13"E))
A. bireticulata	22±2.30	18±4.61	27±0.58
A. delicata	12±0.57	02±0.75	14±1.50
A. capsicula	21±1.15	18±4.61	11±1.44
A. scrobiculata	18±4.61	12±0.58	14±1.50
A. spinosa	16±0.33	11±1.44	23±0.58
Acaulospora sp. I	22±0.58	18±4.61	27±0.33
Glomus			
Glomus aggregatum	58±1.90	67±2.88	51±2.88
G. constrictum	80±1.73	76±1.15	39±3.17
G. fasciculatum	45±2.30	39±0.92	52±0.80
G. intraradices	23±0.57	28±2.30	18±4.61
G. mosseae	46±2.59	41±0.92	48±1.73
G. badium	12±0.57	18±4.61	09±3.46
G. versiforme	05±1.21	08±0.33	00±0.00
G. ambiosporum	12±0.57	09±3.46	07±0.63
G. deserticola	05±1.21	02±0.75	04±1.15
G. drummondii	02±0.75	00±0.00	08±0.33
Glomus sp.I	04±1.15	09±3.46	07±0.63
Gigaspora			
Gi. rosea	32±1.38	34±1.32	41±0.75
Gi. gigantea	24±0.57	28±2.30	35±0.63
Gi. margarita	15±1.54	12±0.57	21±0.57
Scutellospora			
S. calospora	08±0.33	02±0.75	12±0.58
S. rubra	02±0.75	06±1.09	06±1.09
S. pellucida	04±1.15	02±0.75	07±0.63
S. persica	07±0.63	04±1.15	04±1.15
Sclerocystis			
Sclerocystis sp. I	26±0.75	21±1.15	23±0.58
Sclerocystis sp. II	05±1.21	08±0.33	03±1.73
Average of three replicate experiments, ±= SE			

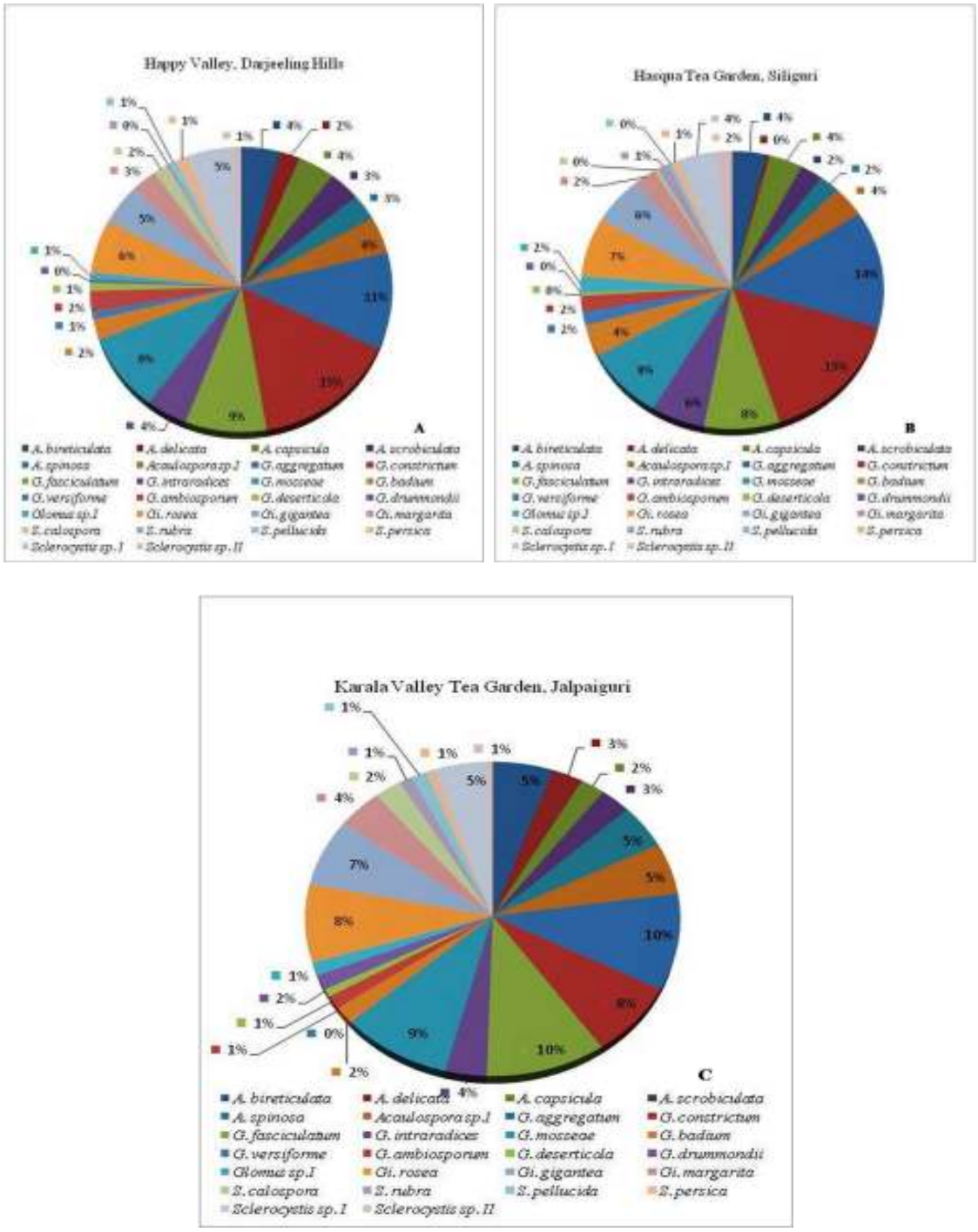

Fig. 5. Population of AMF isolated from tea rhizosphere from Happy Valley, Darjeeling Hills (A), Hasqua Tea Garden, Siliguri (B) and Karala Valley Tea Garden, Jalpaiguri (C).

4.1.2. Rubber (*Havea brasiliensis*)

AMF association in Rubber plants from three different sites from the Rubber plantation garden of University of North Bengal was studied and average spore population and percent colonization determined. Over all spore count showed fourteen different kinds of *Glomus* species to be dominant in all the soil samples. Among them *Glomus aggregatum*, *G. constrictum*, *G. fasciculatum*, *G. albidum*, *G. ambisporum*, *G. intraradisces*, *G. mosseae* were most abundant. The genus *Acaulospora* comprises of *A. alpina*, *A.bireticulata, A.capsicula* and *A.delicata*. Among *Gigaspora* species of *Gi. gigantea*, *Gi. margarita* and *Gi. albida* are common. *Acaulospora* includes *A. alpina*, *A. bireticulata*, *A capsicula*, *A. delicata* and few unidentified spores of Acaulospora. Scutellospora and Sclerocystis are fewer in comparison to others. Average population of AMF spores obtained from different rubber plant rhizosphere have been presented in Fig. 6 and Table 3. and Fig 5. Microscopical views of the spores are presented in Fig 7.

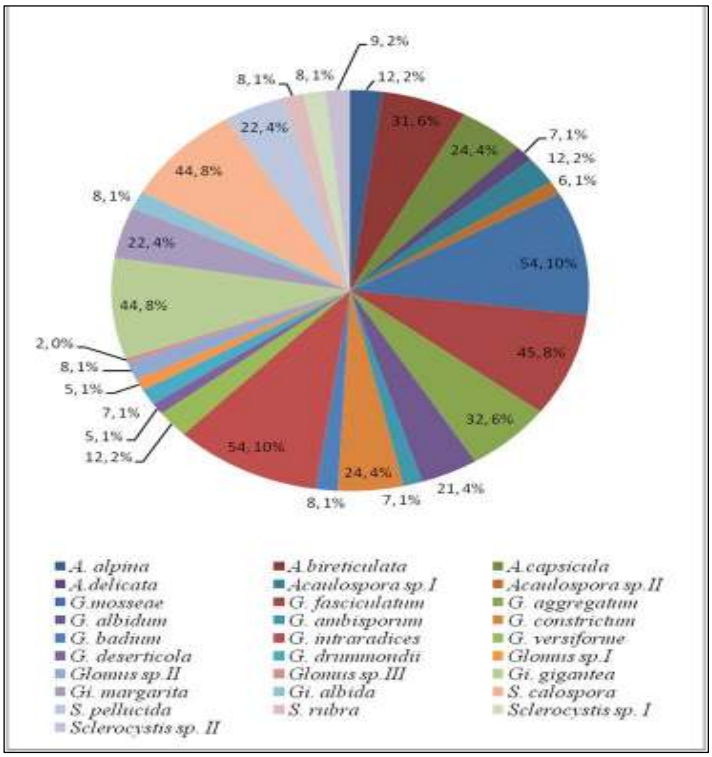

Fig.6. Average spore percentage in 100 gm of soil in rubber rhizosphere

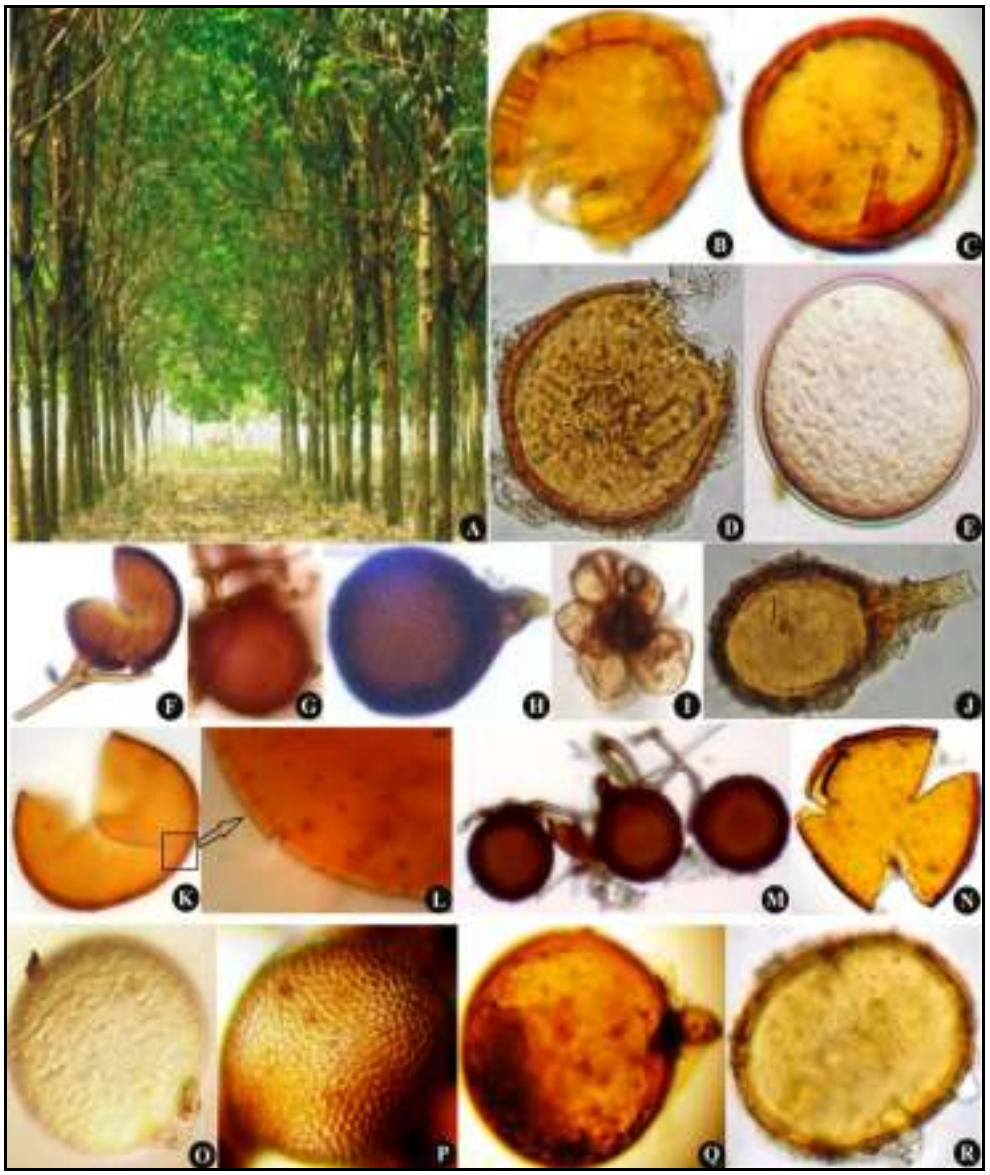

Fig. 7. Compound microscopic observations of Arbuscular Mycorrhizal Fungal spores obtained from rubber plants. General view of Rubber Plantation (A); *G. albidum* (B); *G. ambisporum* (C);*G. mosseae*(D); *G. fasciculatum* (E); *G. constrictum* (F); *G. ambiosporum* (G); *Glomus* sp.(H); *Glomus badium* (I); *Glomus* sp (J); *Glomus* sp (K); *Glomus* sporocarp (L); *Glomus* sp. (M); *Acaulospora* sp.(N); *Acaulospora* sp. (O); *Acaulospora* sp. (P); *Acaulospora* sp.(Q); *Gigaspora* sp.(R)

Table 3. AMF spores found in Rubber (*Havea brasiliensis*)

Name of AMF	Percentage of spores in 100 gm soil		
Acaulospora	Site 1 (26°42'33"N 88°21'11"E)	Site 2 (26°42'36"N 88°21'9"E)	Site 3 (26°42'36"N 88°21'13"E)
A. alpina	12±0.57	16±0.92	10±1.44
A.bireticulata	31±1.38	26±2.88	22±1.15
A.capsicula	24±2.50	27±1.73	27±1.73
A.delicata	07±0.63	09±0.33	06±1.09
Acaulospora sp.I	12±0.58	20±1.15	18±2.88
*Acaulospora sp.*II	06±1.09	02±0.58	05±1.21
Glomus			
G.mosseae	54±2.59	46±1.38	41±1.90
G. fasciculatum	45±1.38	34±1.15	37±1.15
G. aggregatum	32±1.73	28±2.88	41±0.58
G. albidum	21±0.98	17±0.92	15±0.57
G. ambisporum	07±0.63	08±0.33	04±0.75
G. constrictum	24±2.50	28±0.63	19±0.69
G. badium	08±0.33	02±0.75	03±1.73
G. intraradices	54±2.59	48±0.80	38±1.15
G. versiforme	12±0.57	09±3.46	09±3.46
G. deserticola	05±1.21	09±3.46	06±1.09
G. drummondii	07±0.63	05±1.21	04±1.15
Glomus sp.I	05±1.21	03±1.73	02±0.75
Glomus sp.II	08±0.33	06±1.09	04±1.15
Glomus sp.III	02±0.75	06±1.09	05±1.21
Gigaspora			
Gi. gigantea	44±2.30	35±1.32	38±2.30
Gi. margarita	22±0.58	28±1.15	31±2.88
Gi. albida	08±0.33	07±0.63	11±0.58
Scutellospora			
S. calospora	05±1.21	06±1.09	02±0.57
S. pellucida	07±0.63	05±1.21	05±1.21
S. rubra	12±0.58	17±4.61	18±4.61
Sclerocystis			
Sclerocystis sp. I	08±0.33	11±1.44	16±1.44
Sclerocystis sp. II	09±3.46	12±0.57	11±0.57

Average of three replicate experiments, ±= SE

4.1.3. Areca (*Areca catechu*)

AMF association in Areca (*Areca catechu*) plants from plantation garden located at Padmaja park of University of North Bengal was studied and average spore population and percent colonization determined. The genus *Glomus* is predominant among other the genus *Acaulospora*, *Gigaspora*, and *Sclerocystis*. *G. albidum, G. fasciculatum, G. mosseae, G. constrictum, G. intraradices, G aggregatum* are most abundant among other *Glomus* spores. Few *Glomus* spores could not be identified upto species level. *Gigaspora gigantea* and *Gi. margarita* are abundant in areca rhizosphere. Dark red coloured *Scutellospora* and *Sclerocystis* are also present. The average percentage of spores in Areca plants have been presented in Fig.8 and Table 4. Microscopical views of the spores are presented in Fig 8.

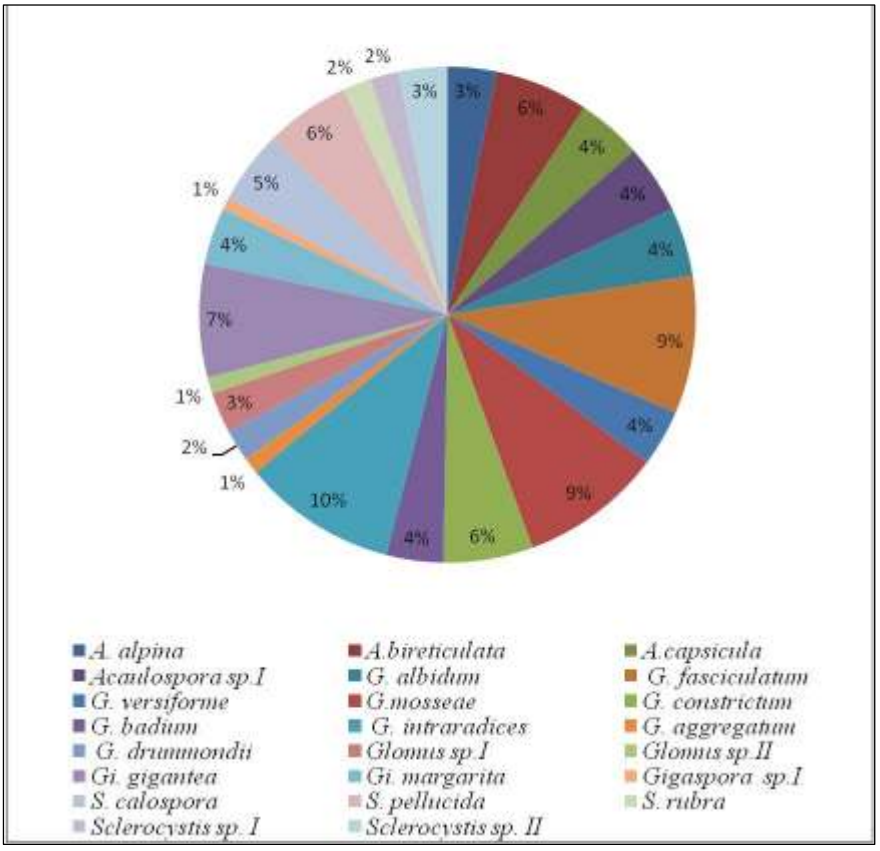

Fig.8. Average spore percentage in 100 gm of soil in areca rhizosphere

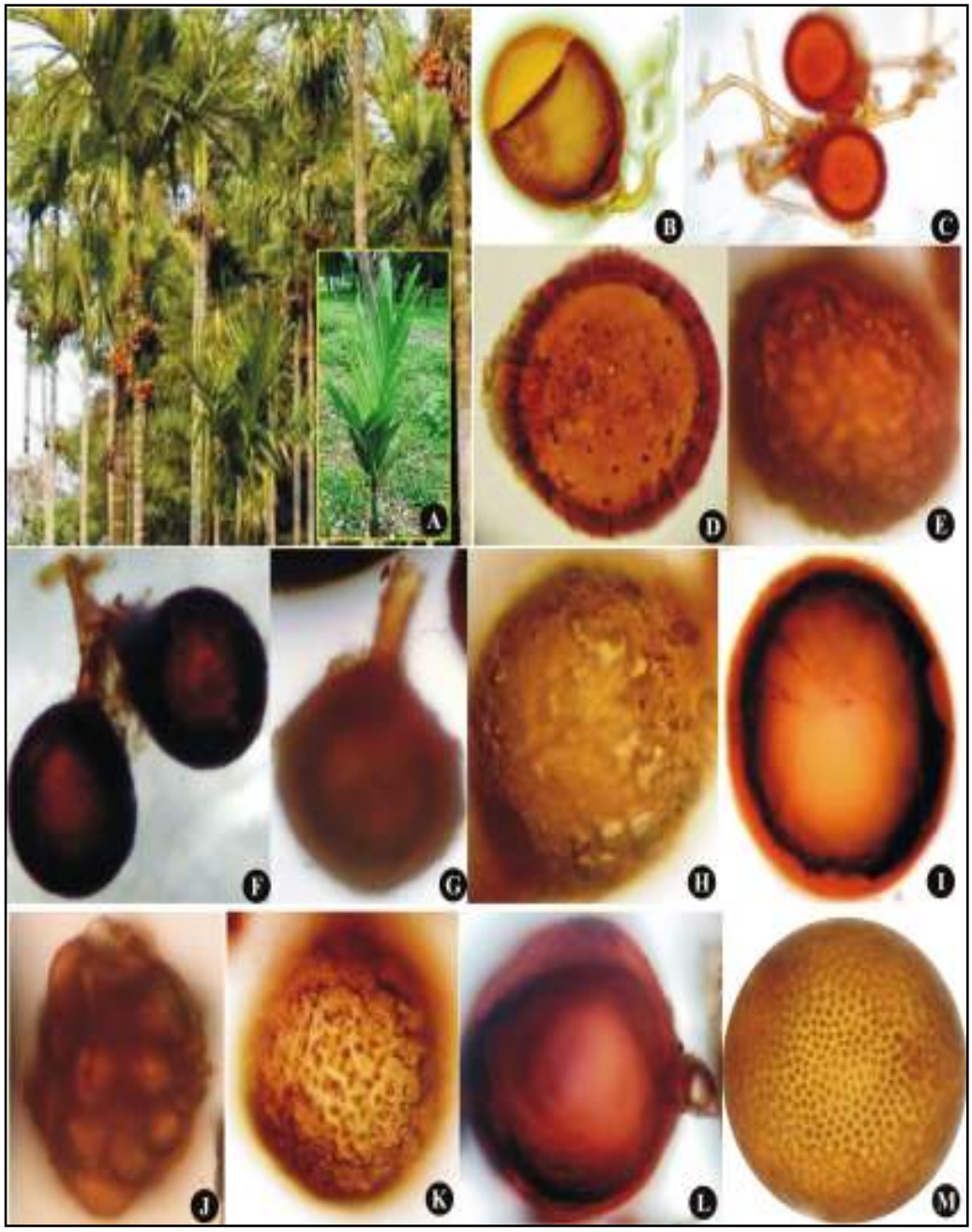

Fig.9. Compound microscopic observations of Arbuscular Mycorrhizal Fungal spores obtained from areca. General view of Areca plants.(A); *G. albidum* (B); *G. ambisporum* (C);*G. mosseae*(D); *G. fasciculatum* (E); *G. constrictum* (F); *G. ambiosporum* (G); *Glomus* sp.(H); *Glomus badium* (I); *Glomus* sporocarp (J); *Scutellospora* sp. (K); *Gigaspora margarita* (L); *Acaulospora capsicula* (M);

Table 4. AMF spores found in Areca (*Areca catechu*)

Name of AMF	Percentage of spores in 100 gm soil		
	Site 1 (26°42'40"N 88°21'14"E)	Site 2 (26°42'42"N 88°21'16"E)	Site 3 (26°42'43"N 88°21'17"E)
Acaulospora			
A. alpina	12±0.57	10±1.44	06±1.09
A.bireticulata	23±1.73	27±2.50	34±1.15
A.capsicula	16±1.44	19±4.61	10±1.44
Acaulospora sp.I	17±4.61	11±1.15	14±0.57
Glomus			
G. albidum	17±4.61	21±0.98	19±4.61
G. fasciculatum	34±1.15	46±1.15	45±1.15
G. versiforme	14±0.57	11±0.57	09±3.46
G.mosseae	35±1.32	38±1.44	42±0.33
G. constrictum	22±1.15	27±2.50	21±0.98
G. badium	14±1.50	19±1.73	20±2.30
G. intraradices	38±1.21	28±0.58	22±0.57
G. aggregatum	04±1.21	06±1.09	06±1.09
G. drummondii	08±3.46	07±0.63	10±1.44
Glomus sp.I	10±1.44	11±1.15	07±0.63
Glomus sp.II	04±1.21	08±0.33	11±0.75
Gigaspora			
Gi. gigantea	28±2.50	31±1.32	42±1.84
Gi. margarita	14±1.50	17±4.61	23±0.99
Gigaspora sp.I	03±0.75	02±0.58	08±0.33
Scutellospora			
S. calospora	18±2.30	22±1.15	15±1.15
S. pellucida	21±0.98	25±2.30	14±1.50
S. rubra	07±0.63	04±0.75	06±1.09
Sclerocystis			
Sclerocystis sp. I	07±0.63	08±0.33	11±1.15
Sclerocystis sp. II	12±0.58	09±3.46	10±1.44

Average of three replicate experiments ,±= SE

4.1.4. Coffee (*Coffea arabica*)

AMF association in Coffee plants growing in Coffee plantation field situated in Bagdogra, Siliguri was studied and average spore population and percent colonization determined. The genus of *Glomus* and *Acaulospora* are most abundant. Among them *G. intraradisces*, *G. albidum*, *G. mosseae*, *G. fasciculatum*, *G. constrictum* from the genus *Glomus* and *A. bireticulata, A. spinosa* from the genus *Acaulospora* are found to be abundant. The other AMF spores from *Gigaspora* genus are *Gigaspora gigantean, Gi. margarita* and *Gi. albida* in which *Gi. gigantea* is highest among all *Gigaspora* spores. *Scutellospora calospora* was found to be highest among other Scutellospora species. The average percentages of spores in Coffee plants have been presented in Fig. 10 and Table 5. Microscopical studies of the spores are given in Fig. 11.

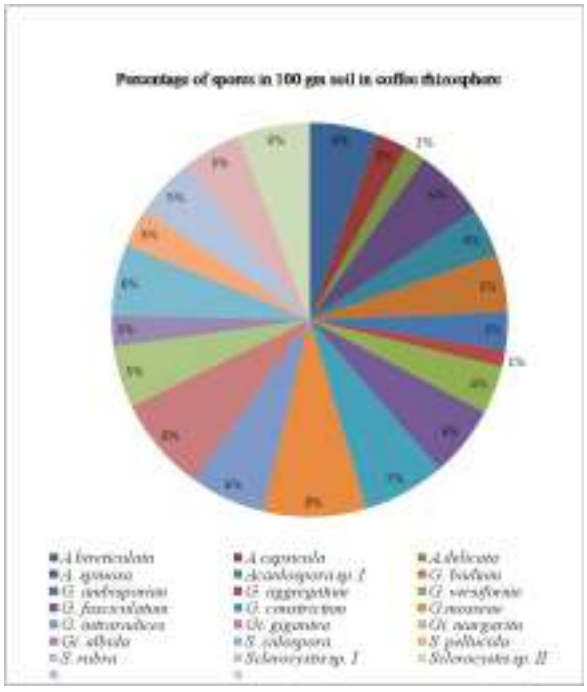

Fig.10. Average spore percentage in 100 gm of soil in coffee rhizosphere.

Fig.11 .Compound microscopic observations of Arbuscular Mycorrhizal Fungal spores obtained from coffee. General view of coffee plants.(A); *G. mosseae* (B); *G. ambisporum* (C);*G. mosseae*(D); *G. fasciculatum* (E); *Glomus* sp (F); Hyphal attachment of *Glomus* sp. (G); *Glomus* sp.(H); *Glomus badium* (I); *Glomus* sporocarp (J); *Scutellospora* sp. (K); *Gigaspora margarita* (L); *Acaulospora capsicula* (M)

Table 5. AMF spores found in Coffee (*Coffea arabica*)

Name of AMF	Percentage of spores in 100 gm soil		
	Site 1 (26°42'48"N 88°21'22"E)	**Site 2 (26°42'50"N 88°21'17"E)**	**Site 3 (26°42'47"N 88°21'13"E)**
Acaulospora			
A.bireticulata	20±0.69	16±1.44	23±0.33
A.capsicula	09±3.46	13±0.57	17±4.61
A.delicata	06±1.09	13±0.58	10±1.15
A. spinosa	21±0.98	16±1.44	17±4.61
Acaulospora sp. I	14±1.50	18±2.30	21±0.98
Glomus			
G. badium	17±4.61	24±2.30	18±1.15
G. ambisporum	11±0.57	07±1.09	13±0.58
G. aggregatum	05±1.21	08±3.46	11±0.57
G. versiforme	14±1.50	11±0.57	09±3.46
G. fasciculatum	21±0.98	19±1.73	25±1.73
G. constrictum	24±2.30	27±0.33	21±0.98
G.mosseae	30±2.88	28±0.58	29±1.15
G. intraradices	22±0.69	27±0.33	21±0.98
Gigaspora			
Gi. gigantea	28±0.58	27±0.33	34±0.75
Gi. margarita	18±2.30	15±1.73	15±1.73
Gi. albida	09±3.46	06±1.09	04±1.15
Scutellospora			
S. calospora	21±0.98	28±0.58	19±1.73
S. pellucida	11±0.57	17±4.61	14±1.50
S. rubra	18±2.30	15±1.73	21±0.98
Sclerocystis			
Sclerocystis sp. I	17±4.61	09±3.46	02±0.75
Sclerocystis sp. II	21±0.98	15±1.73	19±1.73

Average of three replicate experiments ,±= SE

4.1.5. Cinnamomum (*Cinnamomum zeylanicum*)

Soil samples were collected from three sites from the rhizosphere of *Cinnamomum* garden situated in Padmaja Park, University of North Bengal. It has been found that the genus *Glomus* and *Acaulospora* are mostly abundant followed by Gigaspora, Scutellospora and Sclerocystis. The genus Glomus comprises of *G. mosseae, G. fasciculatum, G. aggregatum, G. albidum, G. ambiosporum, G. constrictum, G. badium, G. intraradices, G. versiforme* and *G. drummondii*. *Glomus constrictum, G. fasciculatum* are most common to be found. *Archaeospora trappei* of the genus *Archaeospora was* found in all the sites. The average percentages of spores in Coffee plants have been presented in Fig**.** 12 and Table 6. Microscopical studies of the spores are given in Fig. 13.

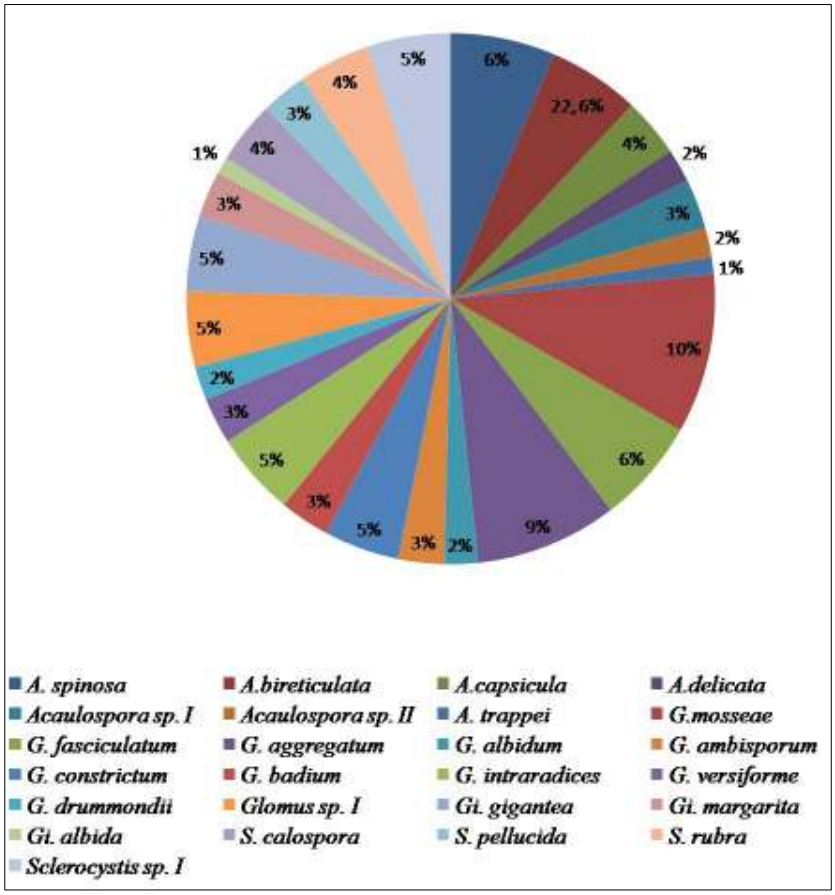

Fig.12. Average spore percentage in 100 gm of soil in Cinnamomum rhizosphere

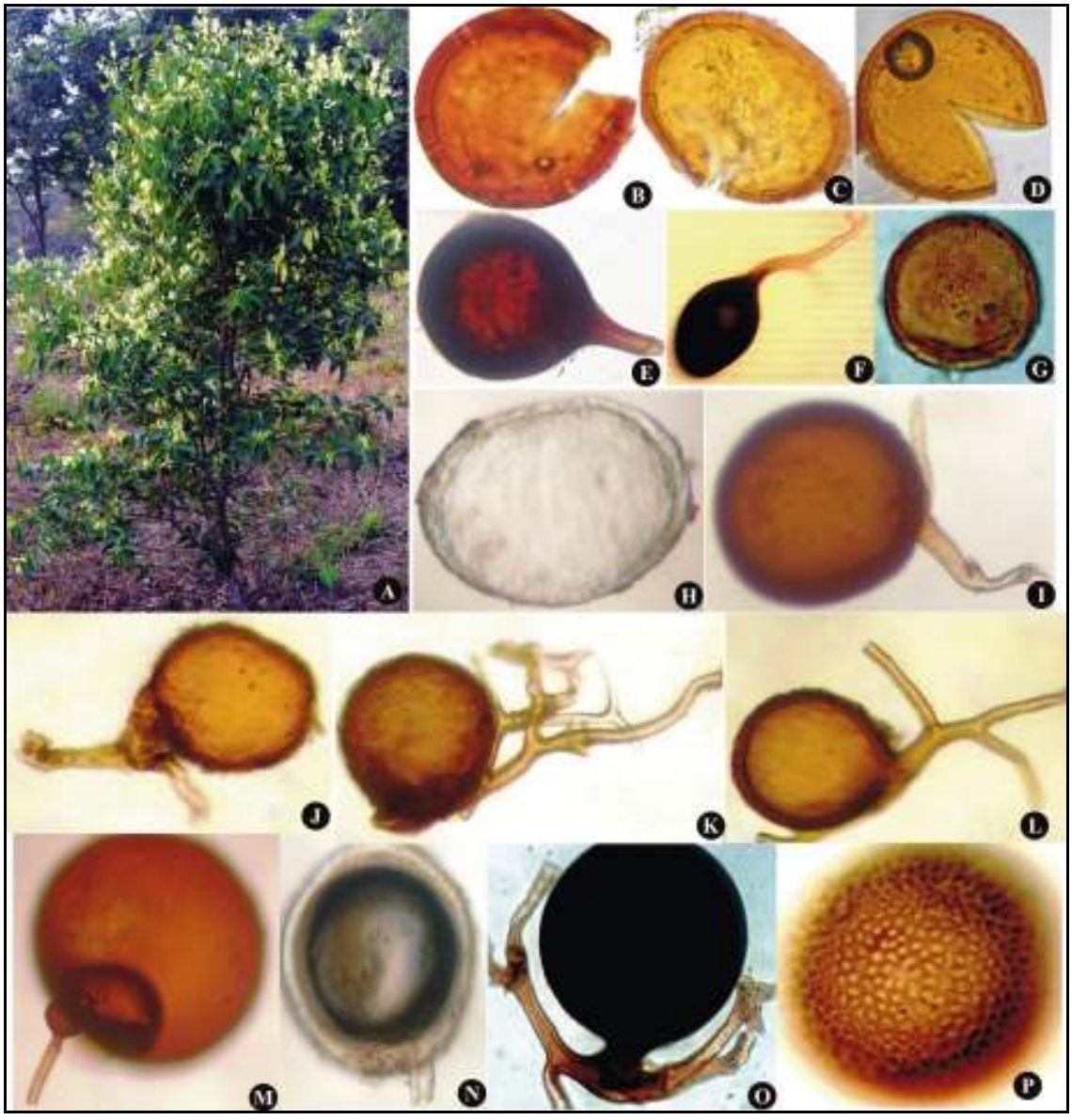

Fig.13. Compound microscopic observations of Arbuscular Mycorrhizal Fungal spores obtained from Cinnamomum. General view of Cinnamomum plants.(A); *G. albidum* (B); *G. ambisporum* (C);*G. mosseae*(D); *G. fasciculatum* (E); *G. constrictum* (F); *G. ambiosporum* (G); *Glomus* sp.(H); *Glomus badium* (I); *Glomus* sp (J); *Glomus* sp (K); *Glomus* sporocarp (L); *Glomus* sp. (M); *Scutellospora* sp.(N); *Acaulospora* sp. (O); *Acaulospora* sp. (P);

Table 6. AMF spores found in *Cinnamomum* (*Cinnamomum zeylanicum*)

Name of AMF	Percentage of spores in 100 gm soil		
	Site 1 (26°42'36"N 88°21'9"E)	**Site 2 (26°42'36"N 88°21'12"E)**	**Site 3 (26°42'36"N 88°21'11"E)**
Acaulospora			
A. spinosa	25±1.73	17±4.61	30±2.59
A.bireticulata	22±0.69	16±1.44	19±1.73
A.capsicula	14±1.50	19±1.73	23±0.58
A.delicata	08±3.46	07±0.63	11±0.57
Acaulospora sp. I	12±0.57	15±1.73	18±2.30
Acaulospora sp. II	07±0.63	08±3.46	03±0.75
Archaeospora			
Archaeospora trappei	04±1.15	02±0.75	00±0.00
Glomus			
G.mosseae	38±1.44	28±0.58	32±1.44
G. fasciculatum	25±2.30	28±0.58	30±2.59
G. aggregatum	34±1.84	37±1.44	30±2.59
G. albidum	08±3.46	03±0.75	07±0.63
G. ambisporum	11±0.58	10±1.15	09±3.46
G. constrictum	18±2.30	23±0.58	26±1.09
G. badium	12±0.57	11±0.57	13±0.58
G. intraradices	20±1.15	22±0.98	18±2.30
G. versiforme	11±0.57	17±4.61	18±2.30
G. drummondii	08±3.46	03±0.75	00±0.00
Glomus sp. I	18±2.30	14±1.50	21±0.98
Gigaspora			
Gi. gigantea	18±2.30	21±0.98	23±0.58
Gi. margarita	11±0.57	12±0.57	17±4.61
Gi. albida	04±1.15	08±3.46	01±0.57
Scutellospora			
S. calospora	15±1.73	22±0.69	25±1.73
S. pellucida	11±0.57	09±3.46	04±1.15
S. rubra	17±4.61	14±1.50	10±1.15
Sclerocystis			
Sclerocystis sp. I	20±2.30	14±1.50	12±0.57

Average of three replicate experiments ,±= SE

4.2. Histopathology and root colonization with AMF in plantation crops

4.2.1. Tea (*Camellia sinensis*)

Fifteen tea varieties, of which six UPASI varieties (UP-2, UP-3, UP-8, UP-9, UP-26 and BSS-2) and nine Tocklai varieties (TV-18, TV-9, T-17, TV-22, TV-23, TV-25, TV-26, TV-29 and TV-30) being grown in the experimental field (15 year old bush) were studied extensively to explore the diversity and mycorrhization. 86-88% percentage root colonisations were noticed in two UPASI varieties (UPASI-9 and BSS-2), of these biclonal seed stock variety BSS-2 yielded highest root colonization. Besides, among nine Tocklai tea varieties tested, TV-29 yielded highest (87%) root colonization. Root samples taken from each of the fifteen varieties 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 of these tea varieties. The arbuscules characters that were found during the investigation in tea roots are full of diversities. Vesicles are formed by the hyphal swellings and they may be inter or intra cellular. Vesicles formed by *Glomus* sp. are ovoid in shape and contain oil globules. Both Arum and Paris type hyphae are abundant in all the varieties. In Arum type association hyphae had proliferated in the cortex longitudinally in the tea root system. In paris type hyphae have spread and formed several coiled structures. Both intra radical and extra radical hyphae are present. Extraradical hyphae are spore bearing hyphae. The DSE (Dark Septate Endophyte) also formed arbuscules that are coiled in structure are known as microsclerotia. DSE did not take the cotton blue stain. During the onset of germination of AM spores a fine structure called the Pre-penetrating Apparatus is produced that ultimately triggers the entry into the host cell. In tea roots artificially inoculated with *Glomus mosseae* spores follow the same pattern during the entry and approximately 20-25 days are required for sporulation. Coiled and fine thread like arbuscules is abundant in tea roots. Besides known AM fungi plenty of other hyphal structures that did not take stain were revealed. These Dark Septate Hyphae produce arbuscules that were found in large number is in BSS-2, UP-3, UP-8, TV-18, T-17, TV-22 and TV-26(Fig. 14, Fig.15 and Table 7).

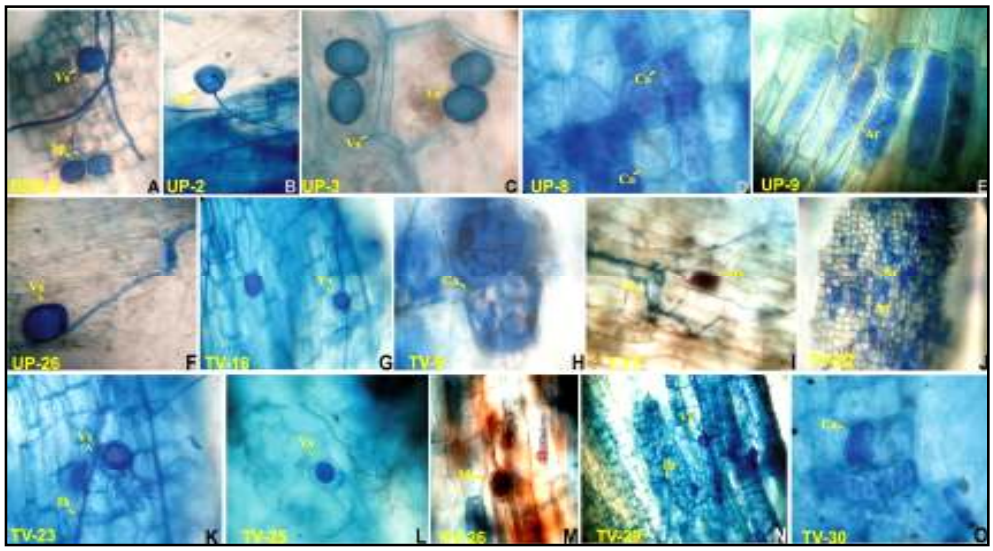

Fig.14. Histopathological study of all the 15 tea varieties showing different shapes of vesicles, hyphae and arbuscules (Vs-vesicles, Sp-spore, Ca-coiled arbuscules, Ar-arbuscules, Ih-intraradical hyphae)

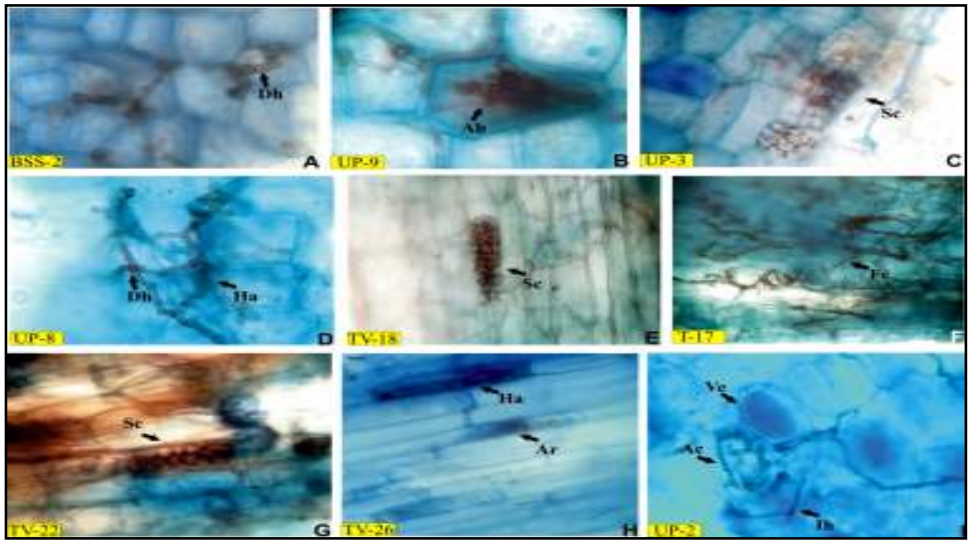

Fig. 15. A-H Different structures of endophyte obtained from tea varieties. A. BSS-2; B.UP-3; C.UP-8; D.TV -18; E.T-17; F. TV-22; G. TV-26; H. UP-2 and I. UP-9. (Dh- Dark hyphae, Ab- Arbuscules, Sc – Sclerotium formed by DSE, Ha – Hyphae growing intercellular, Fe – Fine endophytic mycelium, Ar – Terminally formed arbuscules, Ve – Vesicle formed inside a cortical cell, Ac – Arbuscules formed in coils, Ih – Intercellular hyphae.)

Table. 7. AMF and DSE associations of Tea varieties

Tea variety	pH of soil	No. of spores / 100gm soil	% colonization	No. of Root vesicles/root	Vesicle / cm root	DSE
BSS-2	6.8	142	88	123	03	+++
UP-2	6.5	125	64	88	04	+++
UP-3	7.2	123	55	94	01	+++
UP-8	6.8	132	48	79	04	+++
UP-9	7.6	85	86	113	06	+++
UP-26	6.5	75	47	61	02	+
TV-18	6.9	65	50	72	05	+++
TV-9	7.2	45	50	77	01	+
T-17	6.6	50	58	90	02	+++
TV-22	6.8	49	68	122	02	+++
TV-23	6.5	78	49	95	04	+
TV-25	7.2	72	35	88	01	+
TV-26	6.8	87	47	104	03	+++
TV-29	7.6	95	87	116	06	+
TV-30	6.5	134	69	112	01	+

4.2.2. Rubber (*Havea brasiliensis*)

Arbuscules found in the roots of rubber are haustoria-like structures that are formed by profuse dichotomous hyphae branching after penetration into inner plant cortical cell walls, forming an interface. These arbuscules are the exchange site of nutrients. In rubber the arbuscules formed are highly coiled with swollen trunks and is formed either singly or in clusters. Vesicles are absent nearer to the arbuscules which indicate that these arbuscules are formed by *Gigaspora* sp. Arum and Paris types of hyphae are abundant. The genus *Glomus* produced relatively short and straight hyphae and dense coloured vesicles. Some arbuscules are compact and took deep stain. Thick walled and deep stained hyphae are formed by *Gigaspora* and/or *Scutellospora* sp. In some root fragments deep blue in coloured, thin walled, ellipsoidal structures were found in abundant. These are vesicles. Dark Septate Endophytes are abundant in root cells which sometimes are changed into microsclerotia. Auxiliary cells are formed by short ramifications occurring at one or simultaneously at both sides of extra radical hyphae**.(Fig.16)** Percent colonization is more than 80 in lateral roots in rubber.

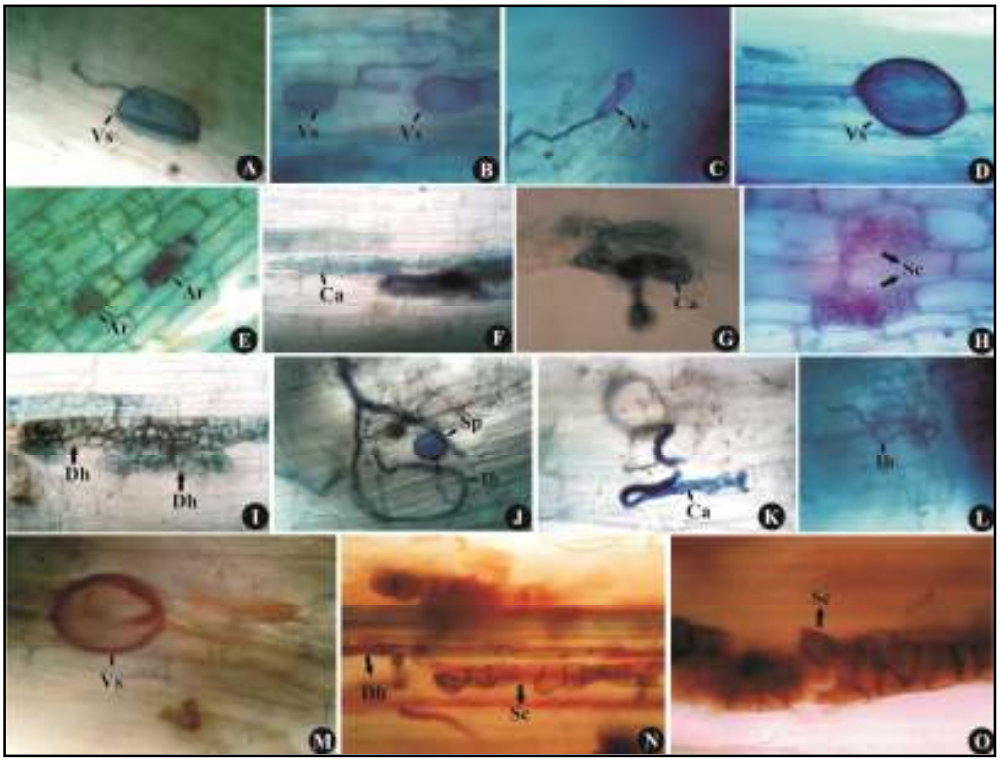

Fig. 16. Histopathological study of rubber roots showing different shapes of vesicles, hyphae and arbuscules (Vs-vesicles, Sp-spore, Ca-coiled arbuscules, Ar-arbuscules, Ih-intraradical hyphae)

4.2.3. Areca (*Areca catechu*)

Percent colonization is 75-80 % in all the studied root samples.. The root structures showed diverse characters forming both arum and paris type of hyphae. Arum hyphae run longitudinally with the root axis and forms fine coiled structures inside the root cells. These are mainly formed by the species of *Glomus*. On the other hand there are several coiled structures of Paris type in addition with deep stained dark hyphae of other endophytes. Both Dark Septate Endophyte and mycorrhizal hyphae run parallel to the root axis. Microsclerotia formed by DSE are evident. Some arbuscules are coiled and some are thin and heavily branched. Genera from the order Glomerales or spores with Glomus type morphology were the most common at all study sites. Presence of Dark Septate Hyphae is also evident in some root samples. Dark coloured vesicles are abundant in Areca roots (Fig.17).

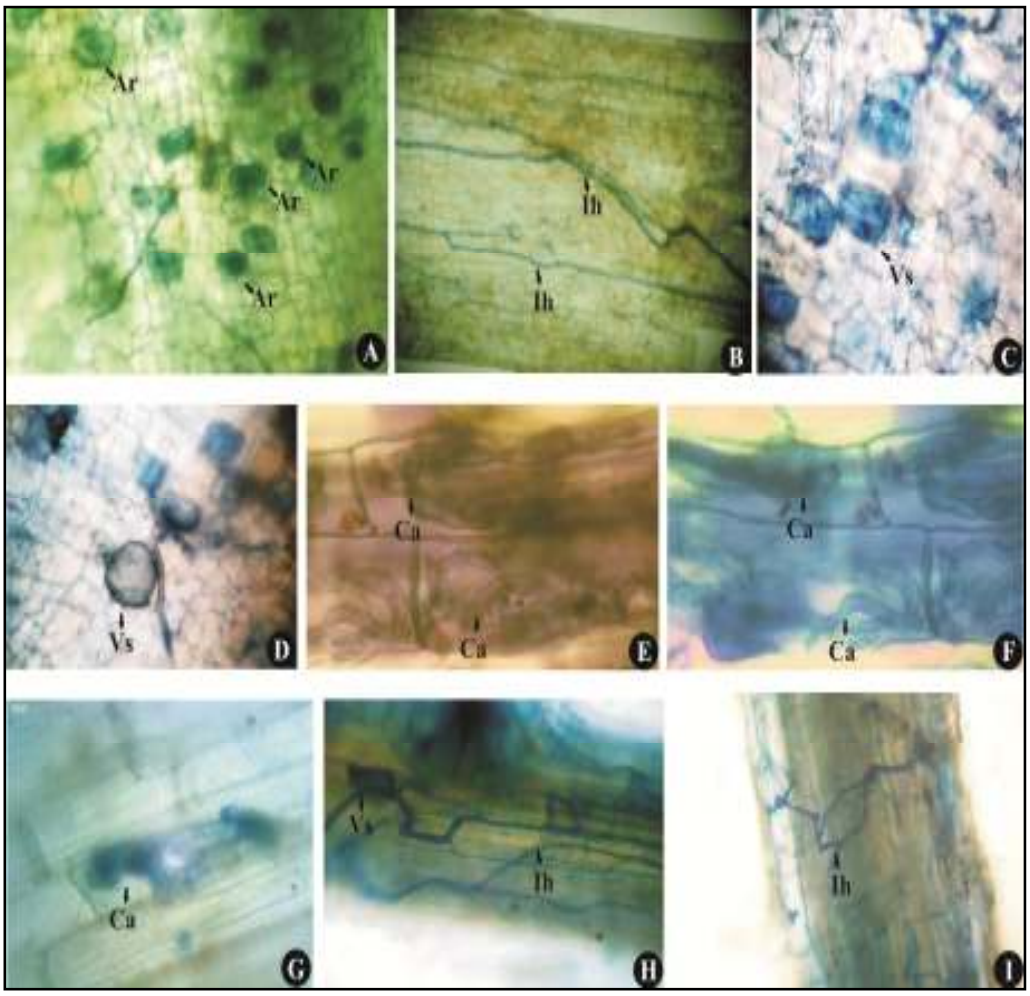

Fig.17 Histopathological study of areca roots showing different shapes of vesicles, hyphae and arbuscules (Vs-vesicles, Sp-spore, Ca-coiled arbuscules, Ar-arbuscules, Ih-intraradical hyphae)

4.2.4. Coffee (*Coffea arabica*)

The root system showed diverse characters of colonization. Coiled and branched arbuscules are plenty. Both arum and paris type of hyphae are present that suggests the presence of both *Glomus* and *Gigaspora* infestation. Round to globose shaped vesicles are abundant but vesicle structures are smaller in young roots than those of old roots. Both intra radical and extra radical hyphae are profuse. Spores are formed from these extraradical hyphae. Auxiliary cells are sometimes present formed by *Gigaspora* sp. Arbuscules with elongated trunk hyphae and tufts of fine branch hyphae are present that give the evidence of infestation by *Gigaspora* sp. (Fig.18) Percent colonisation is 70-75% in average.

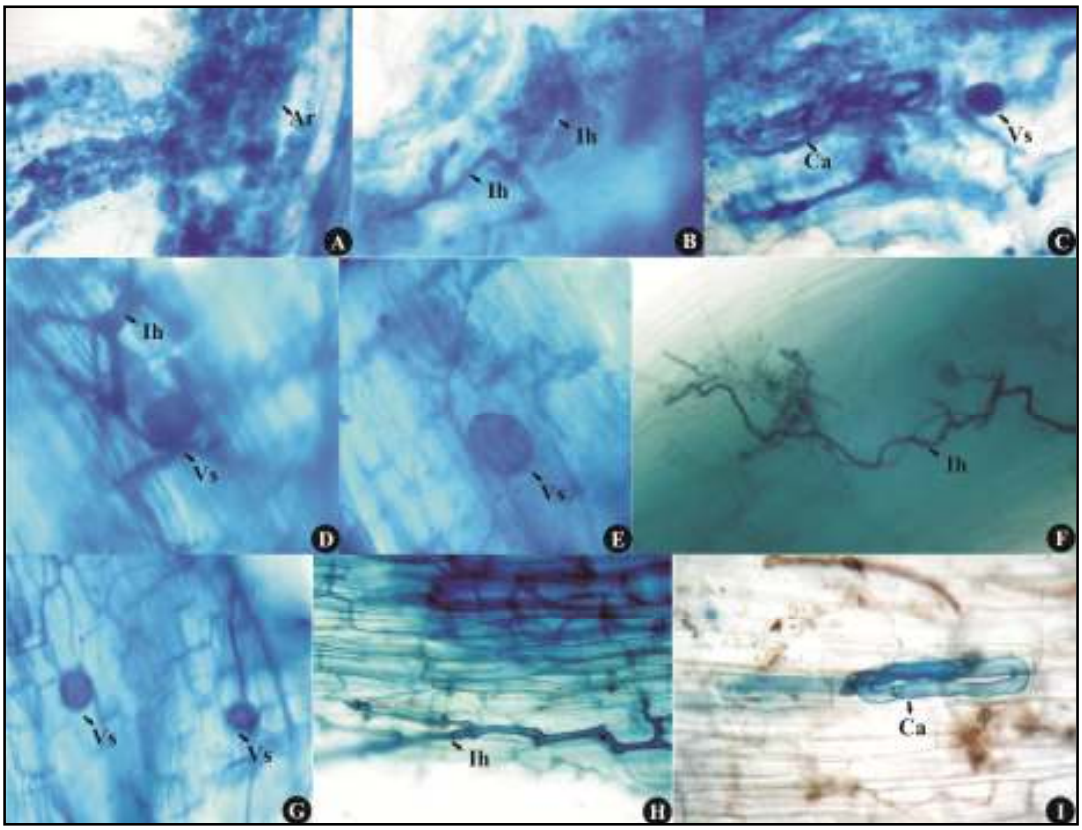

Fig.18. Histopathological study of coffee roots showing different shapes of vesicles, hyphae and arbuscules (Vs-vesicles, Sp-spore, Ca-coiled arbuscules, Ar-arbuscules, Ih-intraradical hyphae)

4.2.5. Cinnamomum (*Cinnamomum zeylanicum*)

Arbuscules are found to originate from both Arum and Paris type hyphae. These are abundant in all the samples. In Arum type association hyphae had proliferated in the cortex longitudinally in the root system. In Paris type, hyphae have spread and formed several coiled structures. Both intra radical and extra radical hyphae are abundant. Vesicles are deep in colour and globose to subglobose in shape. Both intra and extracellular vesicles are found. Arbuscules formed by *Glomus* have numerous fine branches and are only visible under 100X magnification. Thick flat coiled arbuscules are also present. DSE are abundant in root samples and forms microsclerotia (Fig.19). DSE and mycorrhizal hyphae are sometime coiled with each other to form deep coloured region that is hard to distinguish. Percent colonisation ranges between 65-70 %.

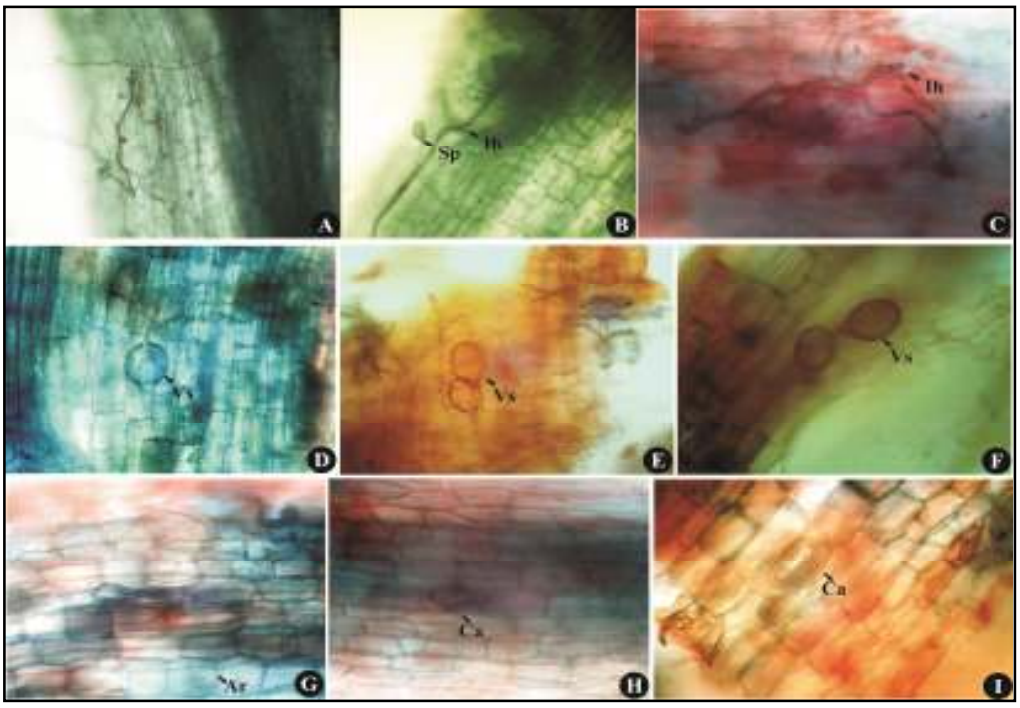

Fig.19. Histopathological study of cinnamomum roots showing different shapes of vesicles, hyphae and arbuscules (Vs-vesicles, Sp-spore, Ca-coiled arbuscules, Ar-arbuscules, Ih-intraradical hyphae)

4.3. Characterization and identification of AMF

AMF spores collected from various hosts were initially characterized on the basis of their morphological features like size, shape, spore wall texture, subtending hyphe, colour, hyphal attachment, spore wall ornamentation as well a microscopical characters like wall layers, wall thickness, peridium structures as well as presence or absence of lipids granules. The detailed descriptions of the most dominant spores have been presented in Table 8.

Table 8. Morphological and microscopical characters of AMF spores

Glomus mosseae Nicolson and Gerdemann	**Spore colour:** Brown to orange-brown
	Spore size and shape: Size ranges from 80-180 µm. Globose to sub-globose, sometimes irregular. Sporocarp contains 2-5 spores surrounded in a peridium.
	Subcelluar structure: Presence of three hyaline layers with subtending hyphae attached.
	Subtending hyphae: Funnel shaped double layered hyphae.
Glomus fasciculatum Gerdemann and Trappe	**Spore colour** : Pale yellow to bright brown
	Spore size and shape : Globose to subglobose, size ranges from 70-120µm in diam.
	Subcelluar structure: Spores produced directly with one or more subtending hyphae attached to it. Spore wall is continuous, consisting of three layers (L1, L2, and L3).
	Subtending hyphae: Single subtending hyphae attached with the spore.
Glomus aggregatum Koske, Gemma and Olexia	**Spore colour** : Pale yellow to dark orange
	Spore size and shape: Spores globose to oval in shape. Size ranges from 40-120µm. Formed singly or in Sporocarps that is formed in loose clusters, from a single stalk
	Subcelluar structure : Spore wall consist of 1-2 layers
	Subtending hyphae : single subtending hyphae attached with each spore
Glomus	**Spore colour** : golden yellow

drummondii Blaszk. & C. Renker	**Spore size and shape** : globose to subglobose, average diameter 70µm, occur singly in the soil
	Subcelluar structure: Spore wall consists of three distinct layers.
	Subtending hyphae: single subtending hypha attached with the spore. Spores; develops from the tip of extraradical hyphae of mycorrhizal roots.
Glomus constrictum Trappe	**Spore colour :** brownish orange to dark brown
	Spore size and shape : globose to subglobose; 160µm diam in average
	Subcelluar structure : consists of one wall containing two layers, most juvenile spores with spore wall layer 1 only
	Subtending hyphae : Subtending hyphae brownish orange to dark brown; straight or curved; usually markedly constricted at the spore base, sometimes cylindrical, flared to funnel-shaped
Glomus clarum Nicol. & Smith	**Spore colour :** hyaline to pale yellow
	Spore size and shape : globose to subglobose; 150 µm diam; sometimes ovoid; 90-100 x 140-180 µm Spores are formed single in the soil
	Subcelluar structure: Spore wall consists of three distinct layers.
	Subtending hyphae: one subtending hyphae. hyaline to pale yellow straight to curved, thick at the spore base; composed of three layers
Glomus intraradisces N.C. Schenck & G.S. Sm.	**Spore colour :** white to pale cream, sometimes yellow brown with a green tint
	Spore size and shape : globose to subglobose, Size ranges from 40-140 µm
	Subcelluar structure : Three layers (L1, L2 and L3), with only the first layer present in juvenile spores
	Subtending hyphae : single subtending hyphae attached with the spore
Glomus albidum C. Walker & L.H. Rhodes.	**Spore colour :** dull yellow or orange-brown
	Spore size and shape : Spores are borne singly in soil; globose, subglobose, irregular, or ellipsoid; size ranges from 120-160 µm
	Subcelluar structure: The spore wall is composed of two distinct, separable layers (L1 and L2).

	Subtending hyphae: single, cylindrical, sometimes constricted or slightly flared toward the point of attachment, The hyphal wall consists of two layers.
Archaeospora trappei R.N. Ames & Linderman.	**Spore colour:** Usually completely hyaline, rarely creamy white.
	Spore size and shape :40-80 µm
	Subcelluar structure: Spore wall consists of three distinct layers**.**
	Subtending hyphae : Single subtending hyphae attached with the spore

Glomus badium Oehl, Redecker & Sieverd	**Spore colour :** brownish orange to reddish brown
	Spore size and shape : Spores occur in dense sporocarps; mainly ovoid to irregular; sometimes globose to subglobose; 250-320 µm diam
	Subcelluar structure : composed of two layers
	Subtending hyphae : from a hyphal plexus and separated by an interspore mycelium and occasionally by cystidium-like structures.
***Sclerocystis* sp.**	**Spore colour :** Brown to blackish brown
	Spore size and shape: Chlamydospores arranged side by side in a single layer radially arranged on a central plexus of hyphae. Globose to subglobose ,size ranges between 300-600 x 400-700 µm
	Subcelluar structure : NA
	Subtending hyphae :NA
Acaulospora bireticulata F.M. Rothwell & Trappe	**Spore colour :** light orange to yellowish brown
	Spore size and shape : Spores single in the soil; develop laterally; sessile;; globose to subglobose; approx. size is 190µm diam;
	Subcelluar structure: consists of a spore wall and two inner germination walls. Spore wall contains three layers. Layer 1, forming the spore surface, 1.1µm thick, closely attached to wall 2, continuous with the wall of a sporiferous saccule. Layer 2 laminate, ornamented, light orange yellowish brown, ornamentation consists of hyaline to yellowish white round-tipped polygonal structures.
	Subtending hyphae : NA
Acaulospora spinosa C.	**Spore colour :** cream to pale orange-brown
	Spore size and shape : Globose or subglobose, size distribution: 140-220

Walker & Trappe	µm
	Subcelluar structure: spore wall consists of two layers. Layer 1: Hyaline and1.2-1.6 µm thick. Layer 2: thickens by formation of pale yellow sub layers followed by synthesis of closely packed rounded spines. Layer 3: A single hyaline layer
	Subtending hyphae : NA
Acaulospora delicate C. Walker, C.M. Pfeiff. & Bloss	**Spore colour :** pale yellow with green tint
	Spore size and shape : Globose or subglobose, size distribution: 80-100µm
	Subcelluar structure: consists of two layers. Layer 1: Hyaline and1.2-1.6 µm thick. Layer 2: thickens by formation of pale yellow sub layers
	Subtending hyphae : NA
Acaulospora capsicula Błaszk	**Spore colour :** orange red
	Spore size and shape : globose to subglobose, varies from 150-300 µm
	Subcelluar structure : Spore wall contains three layers
	Subtending hyphae : NA
Acaulospora scrobiculata Trappe	**Spore colour :** pale yellow or straw-coloured
	Spore size and shape: Shape is globose, subglobose and occasionally irregular. Size ranges from 80-160 µm
	Subcelluar structure : Spore wall consists of 3 layers (L1, L2, and L3), the outer continuous with the wall of the neck of the parent sporiferous sacule
	Subtending hyphae : NA

Gigaspora gigantea (T.H. Nicolson & Gerd.) Gerd. & Trappe	**Spore colour :** greenish yellow
	Spore size and shape : globose to subglobose; 300µm diam; sometimes ovoid; 250x 270 µm
	Subcelluar structure: Spores single in the soil; formed terminally or laterally on a bulbous sporogenous cell Sub cellular structure of spores consists of a spore wall with two layers and one germinal wall.
	Subtending hyphae : single subtending hypha attached with the spore

Gigaspora margarita W.N. Becker & I.R. Hall	**Spore colour :** yellowish white to sunflower yellow
	Spore size and shape : globose to subglobose; 357 µm diam; sometimes ovoid; 320 X 370 µm.
	Subcelluar structure : Spores produced singly in the soil, blastically at the tip of a bulbous sporogenous cell that composed of two layers
	Subtending hyphae : single subtending hypha attached with the spore
Gigaspora rosea T.H. Nicolson & N.C. Schenck	**Spore colour :** Pale cream with a pale pink tint in new healthy spores
	Spore size and shape: Globose to subglobose. Size distribution: 160-280 µm,
	Subcelluar structure : Spore wall consists of three layers (L1, L2, and L3)
	Subtending hyphae : single subtending hyphae attached with the spore
Scutellospora pellucida (T.H. Nicolson & N.C. Schenck) C. Walker & F.E. Sanders	**Spore colour :** hyaline to yolk yellow
	Spore size and shape : globose to subglobose; 195µm diam; sometimes ovoid; 130-155 x 160-235 µm
	Subcelluar structure : NA
	Subtending hyphae : NA
Scutellospora rubra Stürmer & J.B. Morton	**Spore colour :** dark orange-brown to red-brown at maturity
	Spore size and shape : globose to subglobose. Size 180 µm in average
	Subcelluar structure : NA
	Subtending hyphae : NA
Scutellospora persica (Koske & C. Walker) C. Walker & F.E. Sanders	**Spore colour :** Pale to dark copper to slightly darker cream colour
	Spore size and shape : Globose to subglobose. Size distribution: 240-360 µm
	Subcelluar structure: Spore wall consists of two layers (L1 and L2) L1: An outer permanent rigid layer, L2: A layer consisting of orange-brown to dark orange-brown colour.
	Subtending hyphae : NA
Scutellospora calospora	**Spore colour :** Pale yellow with a greenish tint
	Spore size and shape : Subglobose to oblong, sometimes irregular. Size ranges between 120-220 µm

(Koske & C. Walker) C. Walker & F.E. Sanders	**Subcelluar structure :** Spore wall consists of two layers (L1 and L2) L1: An outer permanent rigid layer, smooth, pale yellow with a green tint.L2: A layer consisting of very fine sub layers
	Subtending hyphae : NA

4.3.1. Scanning Electron Microscopy of dominant AMF spores

Scanning electron microscopic observation was made of three most abundant genera *viz Glomus, Gigaspora* and *Acaulospora*. Different species of *Glomus*, *Glomus mosseae*, *Glomus constrictum* and *Glomus intraradisces* obtained from tea rhizosphere showed difference in theit wall characters and ornamentations. There basal attachments of all the different species were distinct which varied in their shape and size from one another (Fig. 20. Similarly *Gigaspora gigantean*, *Glomus fasciculatum* and *Acaulospora* sp. isolated from rubber root rhizosphere showed adhered hyphae with its sloughed and eroded outer hyaline layer covering the whole surface area. *G. constrictum* shows attached hyphae and few pores in the spore surface where MHB (Mycorrhiza Helper Bacteria) might persist. Image of *Gigaspora gigantean* reveals the outer hyaline layer and the conspicuous curved hyphal attachment. *Acaulospora bireticulata* with ornamentation consists of hyaline to round-tipped polygonal structures and the attached sporiferous sacule (Fig. 21).

4.4. *In vitro* germination of AMF spores

Germination of AMF spores *in vitro* was studied to screen their ability to grow in an artificial medium without any host. Clean and surface sterilised spore mass that were placed in the filter paper in close proximity of three days old germinated maize seeds showed germination of AMF spores after 7 days (Fig. 22). Germ tube emerged directly through the spore wall near the hyphal attachment. The germ tube often grew up to 3.5 cm, but no vegetative spores were observed during the experiment. Only one fourth of the spore mass (mixed spores) showed the emergence of the germ tubes. On the other hand surface sterilised *Glomus mosseae* spores started germination in water agar medium after 18 days but gradually the rate declines and stops completely Water agar when supplemented with three days old maize root extract showed a moderate increase in growth (Figure 22. E & F) with branch formation (Table 9).

Table 9. Germination percentage of AMF spores on two different types of media

Substrate	Germination (%)*	Lenght of germ tube (mm)
Water agar (1%)	18	15.62
Water agar (1%) supplemented with root extract	55	52.35
*average of 100 spores		

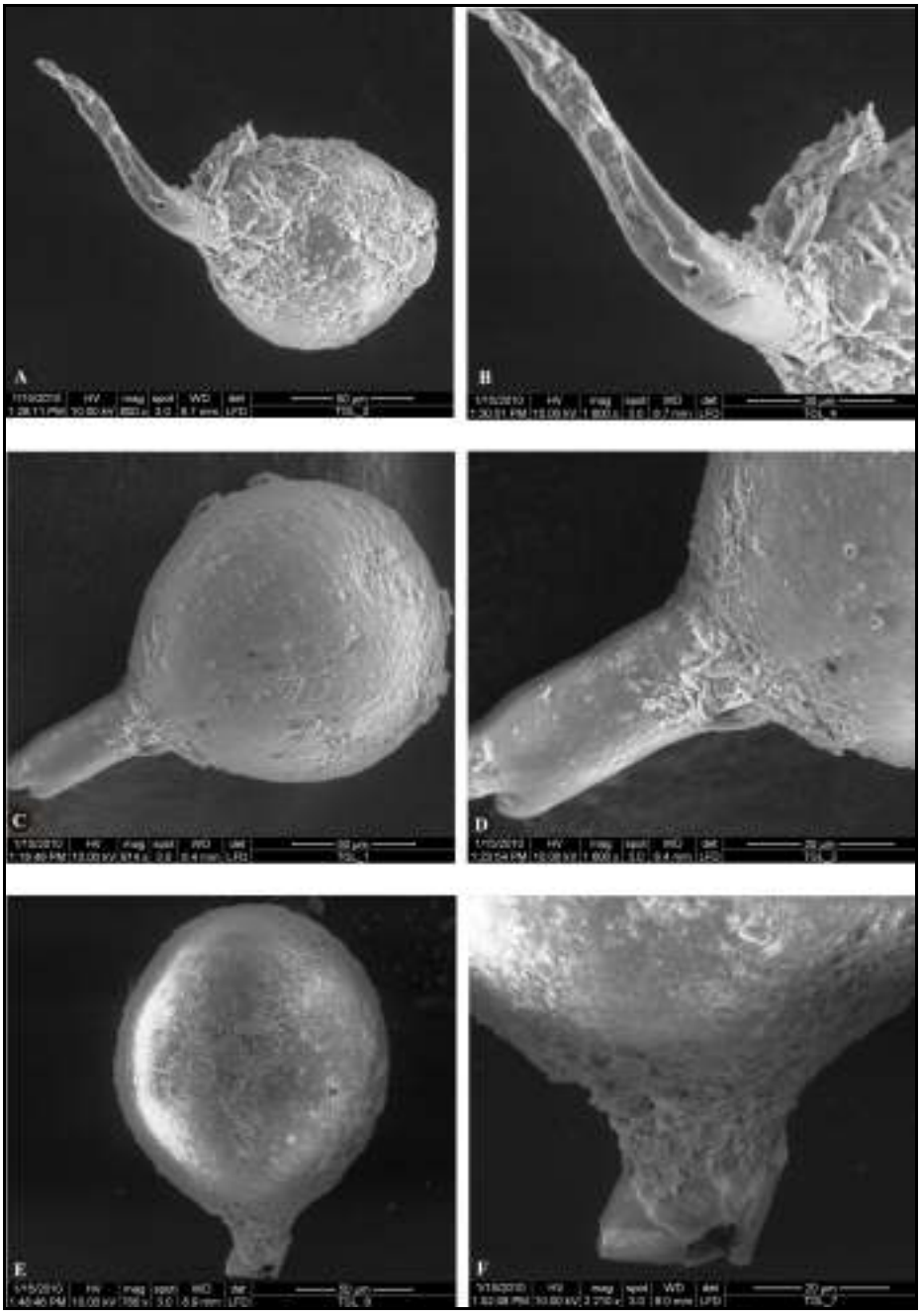

Fig.20. SEM image of *Glomus mosseae* (A-B), *Glomus constrictum* (C-D) and *Glomus fasciculatum*(E-F).

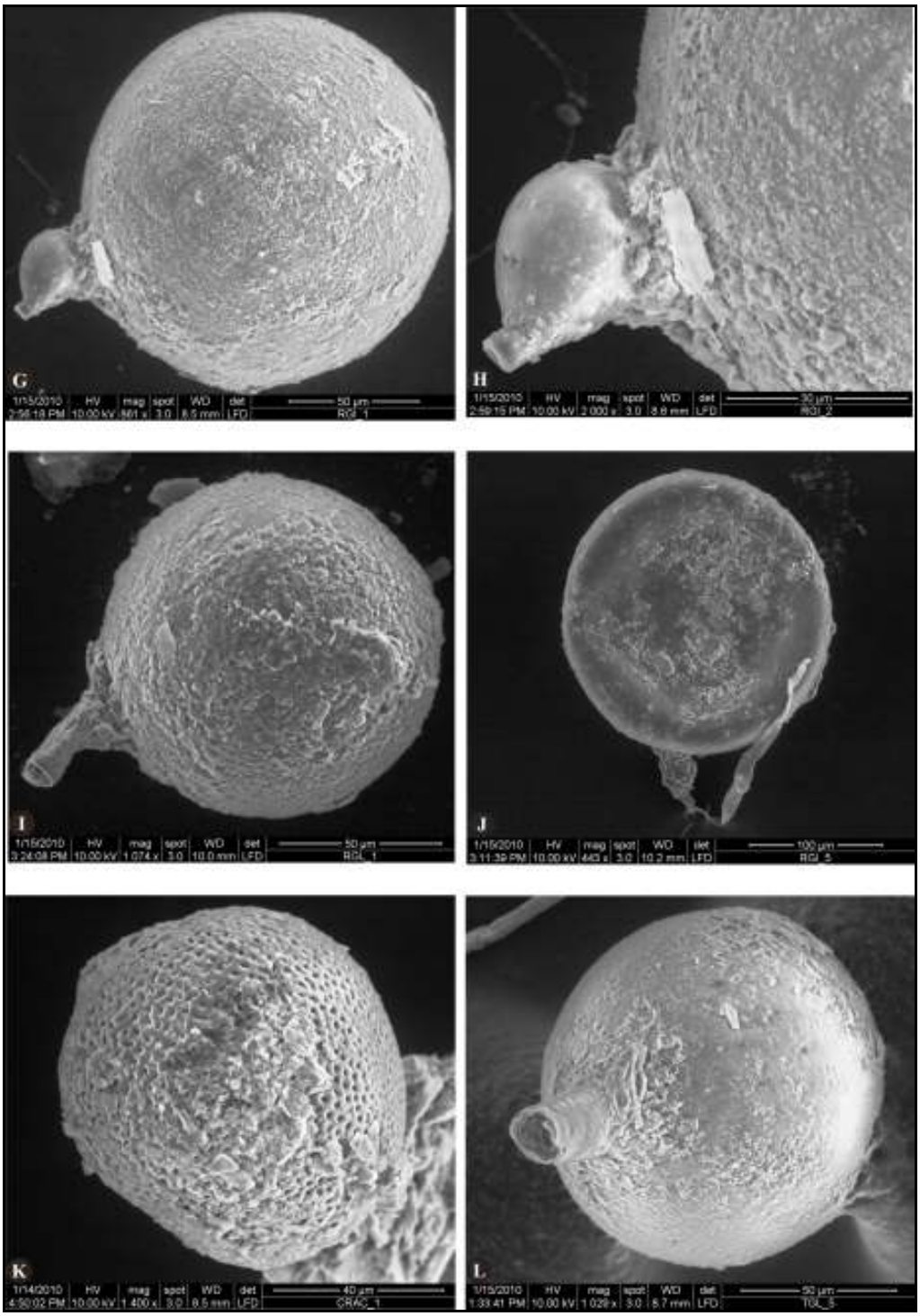

Fig.21. SEM image of *Gigaspora gigantia* (G-H), *Glomus* sp. (I, J and L) and Acaulospora bireticulata (K).

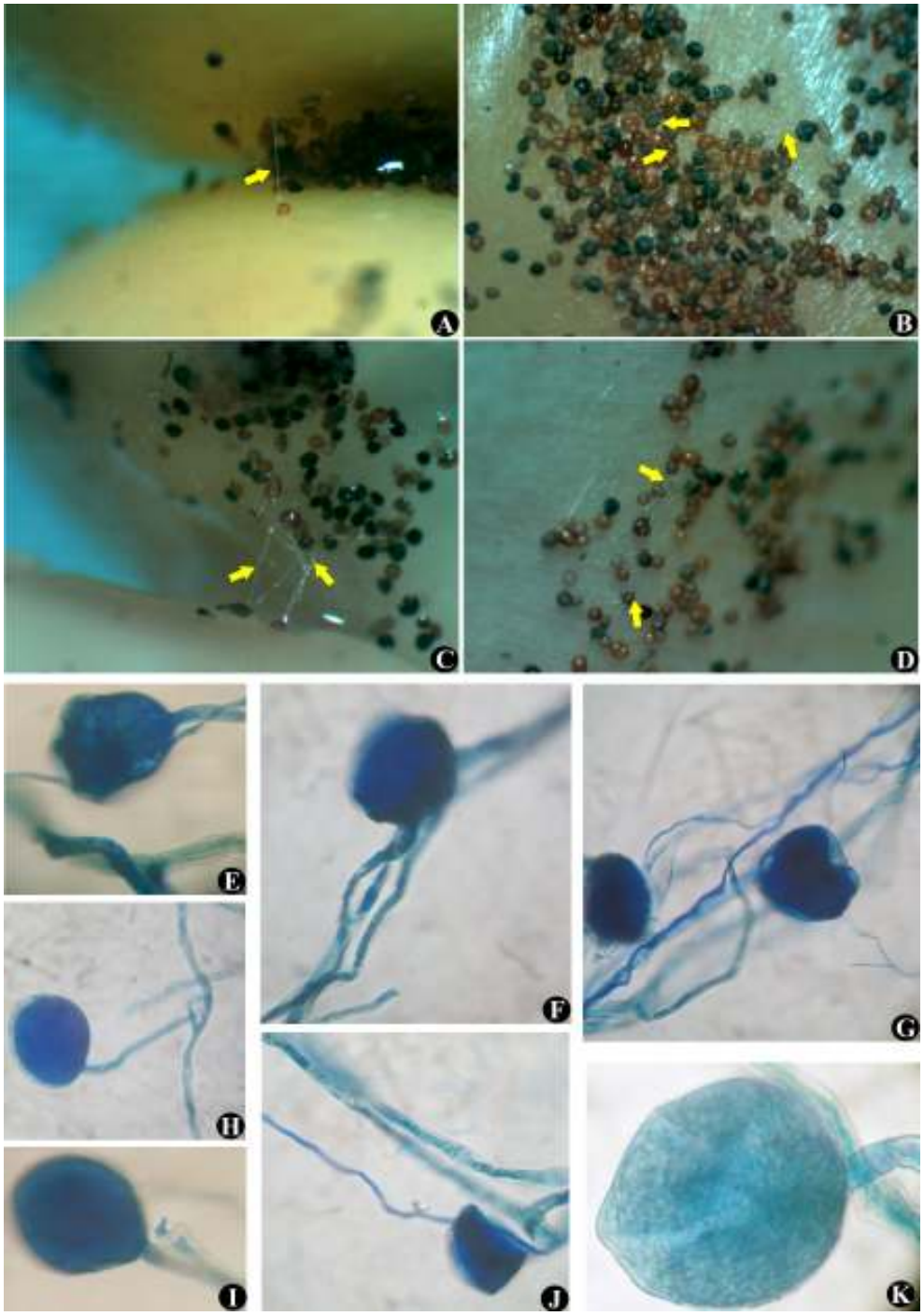

Fig.22. Germination of AMF spores in maize root (A-D) and germination of *G. mosseae* in water agar media

4.5. Screening of plant growth promoting rhizobacteria from rhizosphere of plantation crops

4.5.1. *In vitro* plant growth promoting activities

A total of twenty two (22) bacterial isolates were obtained from the rhizosphere soil of different plantation crops. Among them 10 bacterial isolates were obtained from Tea, 7 from rubber and 5 from Cinnamomum. Isolated bacteria were studied under microscope after suitable staining and characterized based on morphological and biochemical studies following Bergey's manual of Systematic Bacteriology. Overall 15 bacterial isolates were gram positive, rod shaped whereas 7 isolates were gram negative. The overall results for biochemical characterization of all the bacterial isolates have been presented in **Table 10.** Among all the bacterial isolates two bacterial isolates designated as RHS/T-382 and TRS-6 obtained from the rhizosphere of tea showed positive tests for all the PGPR tests like phosphate solubilization, protease, chitinase IAA and siderophore production (Fig. 23).

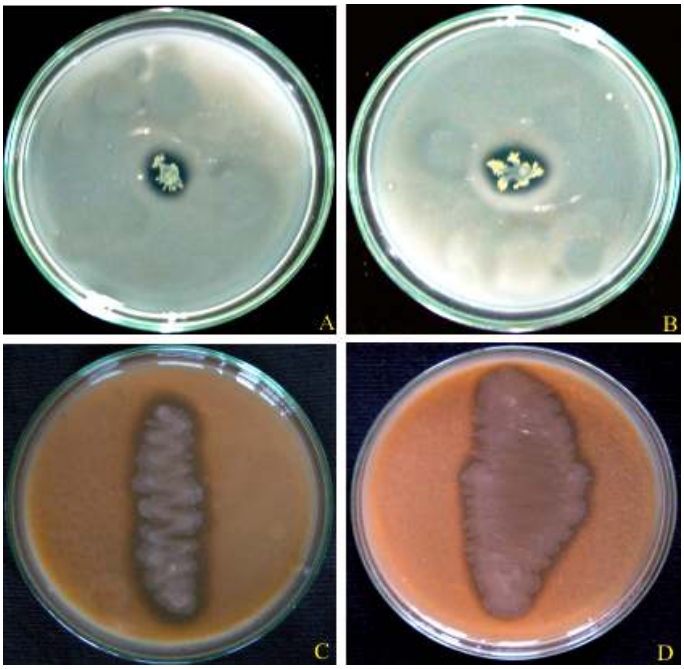

Fig.23. Screening of bacterial isolates for *in vitro* plant growth promoting activities. Phospphate solubilization by RHS/T-382 (A) & TRS-6 (B), Protease production by RHS/T-382 (C) & TRS-6 (D)

Table 10. Morphology and biochemical tests of bacterial isolates obtained from plantation crops

	Code	Shape	Spore	Gram reaction	H_2S production	Phosphate solubilization	Starch hydrolysis	Casein hydrolysis	Chitin degrading	Siderophore production	Catalase production	Protease production	Urase production	Cellulase Production	Indolae Production	Identification
Cm	**RHS/T-382**	**Rod**	+	+	-	+	+	+	+	+	+	+	-	+	+	***Bacillus pumilus***
Cm	**TRS6**	**Rod**	+	+	-	+	+	+	+	+	+	+	-	+	+	***Bacillus amyloliquefaciens***
Cm	T/RHS/01	Spherical	-	+	-	-	-	+	-	-	-	+	-	-	-	*Micrococcus* sp.
Cm	T/RHS/02	Rod	+	+	-	-	-	-	+	-	+	+	-	-	-	*Bacillus sp.*
Cm	T/RHS/03	Rod	+	+	-	-	+	+	+	-	+	+	-	-	-	*Bacillus sp.*
Cm	T/RHS/04	Rod	+	-	-	-	+	+	+	+	+	+	-	+	-	*Pseudomonas sp*
Cm	T/RHS/05	Rod	+	-	-	-	-	+	+	+	-	+	-	+	-	*Pseudomonas sp.*
Cm	T/RHS/02	Rod	+	-	-	-	-	+	+	-	-	+	-	+	-	*Pseudomonas sp.*
Cm	T/RHS/03	Rod	+	-	-	-	+	-	+	-	+	+	-	+	-	*Pseudomonas sp.*
Cm	T/RHS/04	Rod	+	+	-	-	-	+	+	-	-	-	-	+	-	*Bacillus cereus*
Hb	B/RHS/M11	Rod	+	+	-	-	+	-	+	-	+	+	-	-	-	*Bacillus sp.*
Hb	B/RHS/M12	Rod	+	+	-	-	+	+	+	-	+	+	-	-	-	*Bacillus sp.*
Hb	B/RHS/M13	Rod	+	+	-	-	-	-	+	-	+	+	-	-	-	*Bacillus sp.*
Hb	R/RHS/01	Rod	+	+	-	-	-	-	+	+	+	+	-	-	-	*Bacillus sp.*
Hb	R/RHS/02	Rod	+	-	-	-	+	+	+	+	+	+	-	+	-	*Pseudomonas sp.*
Hb	R/RHS/03	Rod	+	-	-	-	+	+	+	-	+	-	-	+	-	*Pseudomonas sp.*
Hb	R/RHS/04	Rod	+	-	-	-	+	+	+	-	+	-	-	+	-	*Pseudomonas sp.*
Cz	CZ/RHS/01	Spherical	-	+	-	-	-	-	-	-	-	+	-	-	-	*Micrococcus* sp.
Cz	CZ/RHS/02	Spherical	-	+	-	-	-	-	-	-	-	+	-	-	-	*Micrococcus* sp.
Cz	CZ/RHS/03	Rod	+	+	-	-	-	-	+	-	+	+	-	-	-	*Bacillus sp.*
Cz	CZ/RHS/04	Rod	+	+	-	-	+	+	+	-	+	+	-	-	-	*Bacillus sp.*
Cz	**CZ/RHS/05**	Rod	+	+	-	+	+	+	+	+	+	+	-	+	+	*Bacillus sp.*

Cm = *Camellia sinensis* (Tea) ; Hb= *Hevia brasiliensis* (Rubber) ; Cz = *Cinnamomum zeylanicum* (Cinnamomum)

4.5.2. Morphological characters and Scanning electron microscopic observations

Potential PGPR isolates RHS/T-382 and TRS-6 obtained from tea rhizosphere showed that both these isolates were gram positive, rod shaped and catalase positive. Scaning electron microscopic studies of both the bacterial isolates showed that they were characteristically rod shaped and the size of the bacterial cells ranged from 4-7 µm (Fig 24. B, E, C & F). On the basis of *in vitro* studies both the bacterial isolates RHS/T-382 and TRS-6 were designated as potential PGPR isolates and were taken up for all the *in vitro* and *in vivo* studies.

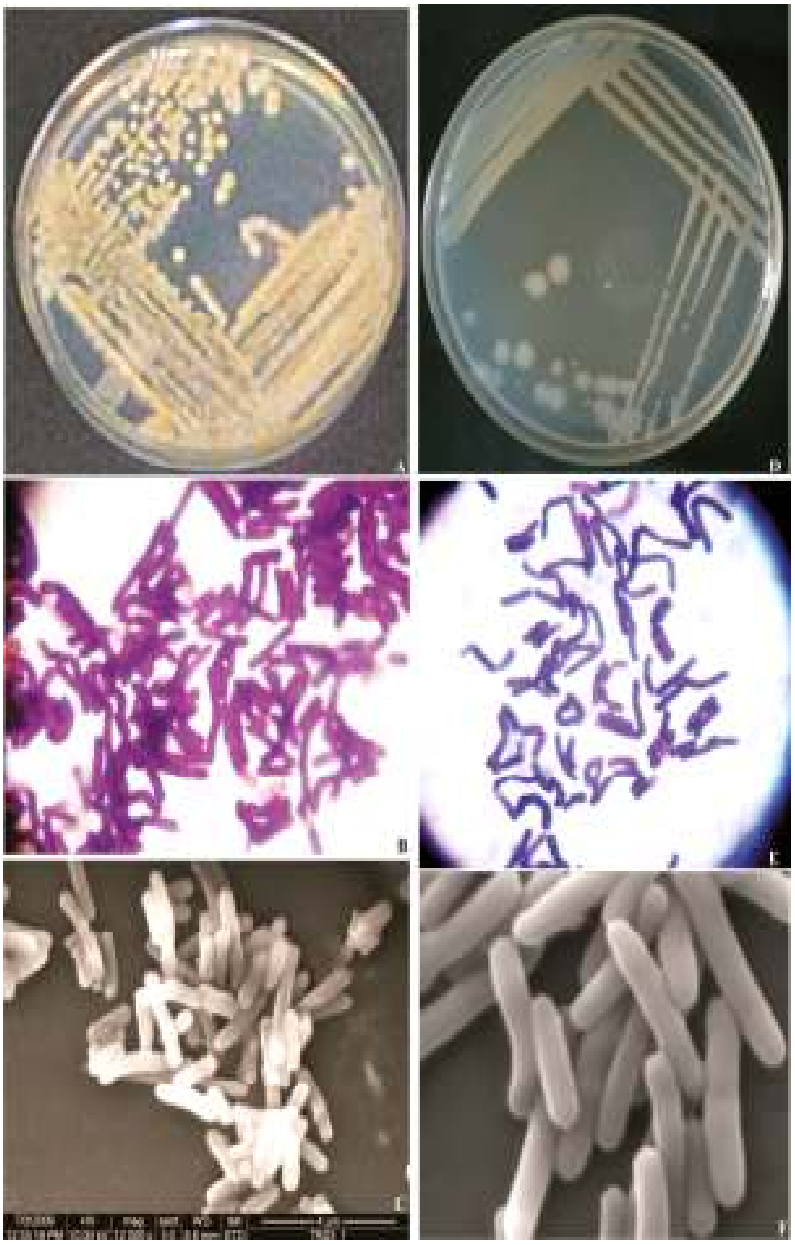

Fig. 24 Growth pattern on NA medium (A & D). Gram staining (B&E) and Scanning Electron Micrograph (C & E) of isolates RHS/T-382 (A,B,C) & TRS-6 (D,E,F)

4.6. Cultural characteristics of PGPR isolates

Cultural characters of the two potential PGPR isolates RHS/T-382 and TRS-6 obtained from tea rhizosphere were studied to determine the effect of different factors like pH, temperature, media, incubation period on overall growth of the isolates.

4.6.1. pH

Both the isolates RHS/T-382 and TRS-6 grew best at pH 6.0 and did not grow well below pH 4.0 and above 8.0 (Fig. 25A).

4.6.2. Temperature

The growth of isolates RHS/T-382 and TRS-6 were observed at different temperatures ranging from 20 to 50 ºC. NB medium was inoculated with the bacteria and flask was incubated at 20 .25.30, 35,40,45,50ºC. Bacteria grew well within these ranges of temperature but grew best at 35 ºC (Fig. 25B).

4.6.3. Media

Six different media (PDB, NB, KB, LB, NSB, and GYP) were selected to assess the growth of the PGPR and it was recorded that NB is the best medium for growth of isolates RHS/T-382 and TRS-6 (Table 11).

Table 11. Growth of PGPR isolates RHS/T-382 and TRS-6 in different media

Media	RHS/T-382 (cfu/ml)*	TRS-6 (cfu/ml)*
PDB	$3.31x10^{7}$	$3.20x10^{7}$
NB	$6.02x10^{13}$	$6.50x10^{13}$
KB	$1.90x10^{11}$	$1.60x10^{11}$
LB	$5.88x10^{8}$	$6.01x10^{8}$
NSA	$1.81x10^{6}$	$1.52x10^{6}$
GYP	$2.60x\ 10^{9}$	$2.40x10^{9}$

* Average of three replicates; incubation period 4 days.

4.6.4. Incubation period

Both the PGPR isolates RHS/T- 382 and TRS-6 were grown in NB for a period of 10 days with growth being recorded after 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 days of growth at

temperature of 35 ºC. Maximum growth was recorded after 4 days and then growth gradually decreased (Fig. 25C).

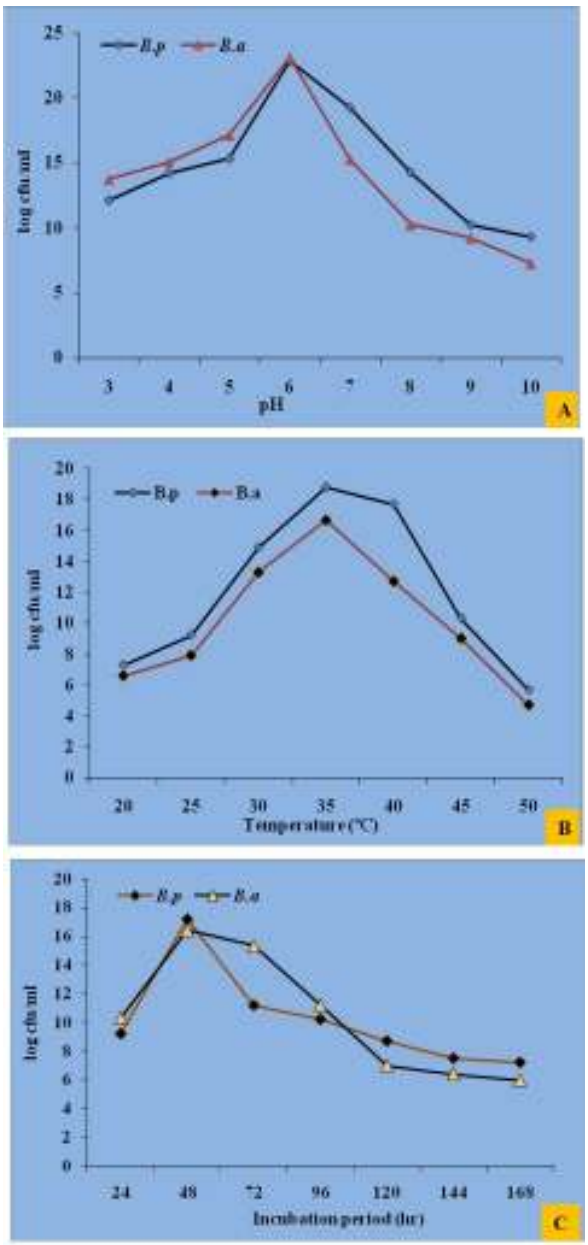

Fig. 25. Effect of pH (A), temperature (B) and incubation period (C) on growth of PGPR isolates RHS/T-382 and TRS-6

4.6.5. Antibiotic sensitivity

Antibiotic sensitivity test was performed to know the strength of isolates RHS/T-382 and TRS-6 against particular antibiotic. Antibiotics was mixed at rate of 15µg/ml in Nutrient Agar medium; Results presented in Table 10 shows that RHS/T-382 is highly sensitive to amoxicillin, streptomycin, gentamycin whereas TRS-6 is highly sensitive to Ampicillin, Chloramphenicol, Kanamycin and Tetracycline (Table 12).

Table 12. Antibiotic sensitivity of isolates RHS/T-382 and TRS-6

Antibiotics	**TRS-6**	**RHS/T-382**
Penicillin	PS	PS
Ampicillin (A^{25} mcg/disc)	HS	HS
Chloramphenicol (C^{25} mcg/disc)	HS	HS
Kanamycin (K^{30} mcg/disc)	HS	HS
Metronidazole (Mt^{5} mcg/disc)	MS	MS
Rifampicin capsule (450 mg)	MS	MS
Tetracycline capsule (500 mg)	HS	HS
Benzylpenicillin	PH	PH
Streptomycin	HS	HS

PS- partially sensitive; HS- highly sensitive; MS- moderately sensitive

4.7. 16 S rDNA sequence analysis of PGPR isolates

Genomic DNA of both the PGPR isolates RHS/T-382 and TRS-6 obtained from Tea rhizosphere were isolated and purified and suspended in 1X TE buffer until further use. Agarose gel electrophoresis of genomic DNA revealed that they were RNA free and ranged between 1.5- 1.8 Kb. Purity of DNA evaluated in terms of the ratio between absorbance of A_{260} and A_{280} showed that genomic DNA of RHS/T-382 had a ratio of 1.853 and TRS-6 had a ratio of 1.744. I TS-PCR was performed with the help of universal primer pair where a uniform product of approximately 800 bp and 1000 bp for both the PGPR isolates respectively.

4.7.1. 16S rDNA sequences and BLAST analysis

In this present investigation two bacterial isolates RHS/T-382 and TRS-6 obtained from tea rhizosphere were designated as potential PGPR. The identities of both the isolates were 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 RHS/T-382 as *Bacillus pumilus* and TRS-6 as *B. amyloliquefaciens***.** The sequences have been deposited in NCBI, GenBank database under the accession no. **JQ765580** for *B. pumilus* and **JN983127** for *B. amyloliquefaciens*. The sequence chromatograms of both the PGPR isolates have been represented in Figure 26 & 27**.**

4.7.2. Multiple sequence alignment and phylogeny

The 16S rDNA sequences of both the PGPR were successfully utilized to confirm their identities. A multiple sequence alignment of ITS gene sequences of *B. pumilus* and *B. amyloliquefaciens* was conducted. The result reveals that there were quite a number of gaps that were introduced in the multiple sequence alignment 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 & 29)**.** Phylogenetic analysis of *B. pumilus* and *B. amyloliquefaciens* was carried out with the Ex-type strain sequences obtained from NCBI GenBank Database which showed maximum homology with RHS/T-382 (Table 13 & 14). The evolutionary history was inferred using the Neighbor-Joining method (Saitou N & Nei M, 1987). The optimal tree with the sum of branch length = 0.17625589 with 725 positions in final data set and 0.18993264 with 824 positions in final dataset for *B. pumilus* and *B. amyloliquefaciens* have been shown (Figure 30 & 31). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) is shown next to the branches (Felsenstein J 1985). 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 725 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et. al.* 2007).

Table 13. Genbank Accession Numbers and Geographic locations of the Ex-Type strains of *B. pumilus* that showed homology with the isolate RHS/T- 382

Accession No.	Strain No	rDNA Sequence	Country	Organisms
AB212862	X-6-19	1501 bp	Japan	*Bacillus pumilus*
FJ573185	GESF1	1450 bp	India	*Bacillus pumilus*
EF029070	SB2	1093 bp	China	*Bacillus pumilus*
FJ544367	st9	1449 bp	China	*Bacillus pumilus*
JN642551	EN5	1471 bp	India	*Bacillus pumilus*
GU056807	SRS-3	1470 bp	India	*Bacillus pumilus*
EU596537	HN005	1457 bp	China	*Bacillus pumilus*
HQ234353	AK39885	1337 bp	India	*Bacillus pumilus*
HQ234349	AK39674	1385 bp	India	*Bacillus pumilus*
HQ234343	AK39651	1385 bp	India	*Bacillus pumilus*
EU660369	CT19	1505 bp	India	*Bacillus pumilus*
EU660366	CT19	1505 bp	India	*Bacillus pumilus*
EU660365	CT13	1511 bp	India	*Bacillus pumilus*
EU660362	CT10	1512 bp	India	*Bacillus pumilus*
EU660356	CT3	1512 bp	India	*Bacillus pumilus*
DQ118016	RGR7	1369 bp	India	*Bacillus pumilus*
GQ280116	JS-46	1413 bp	India	*Bacillus pumilus*
GQ280115	JS-45	1424 bp	India	*Bacillus pumilus*
GQ280113	JS-43	1424 bp	India	*Bacillus pumilus*
GQ280098	JS-28	1445 bp	India	*Bacillus pumilus*
DQ904611	728J	1187 bp	USA	*Bacillus pumilus*
FJ040808	HR10	1406 bp	China	*Bacillus pumilus*
EU921229	BM-F13	1414 bp	China	*Bacillus pumilus*
EU287450	GF-2	1424 bp	China	*Bacillus pumilus*
JQ765580	**RHS/T- 382**	**834 bp**	**India**	***Bacillus pumilus***

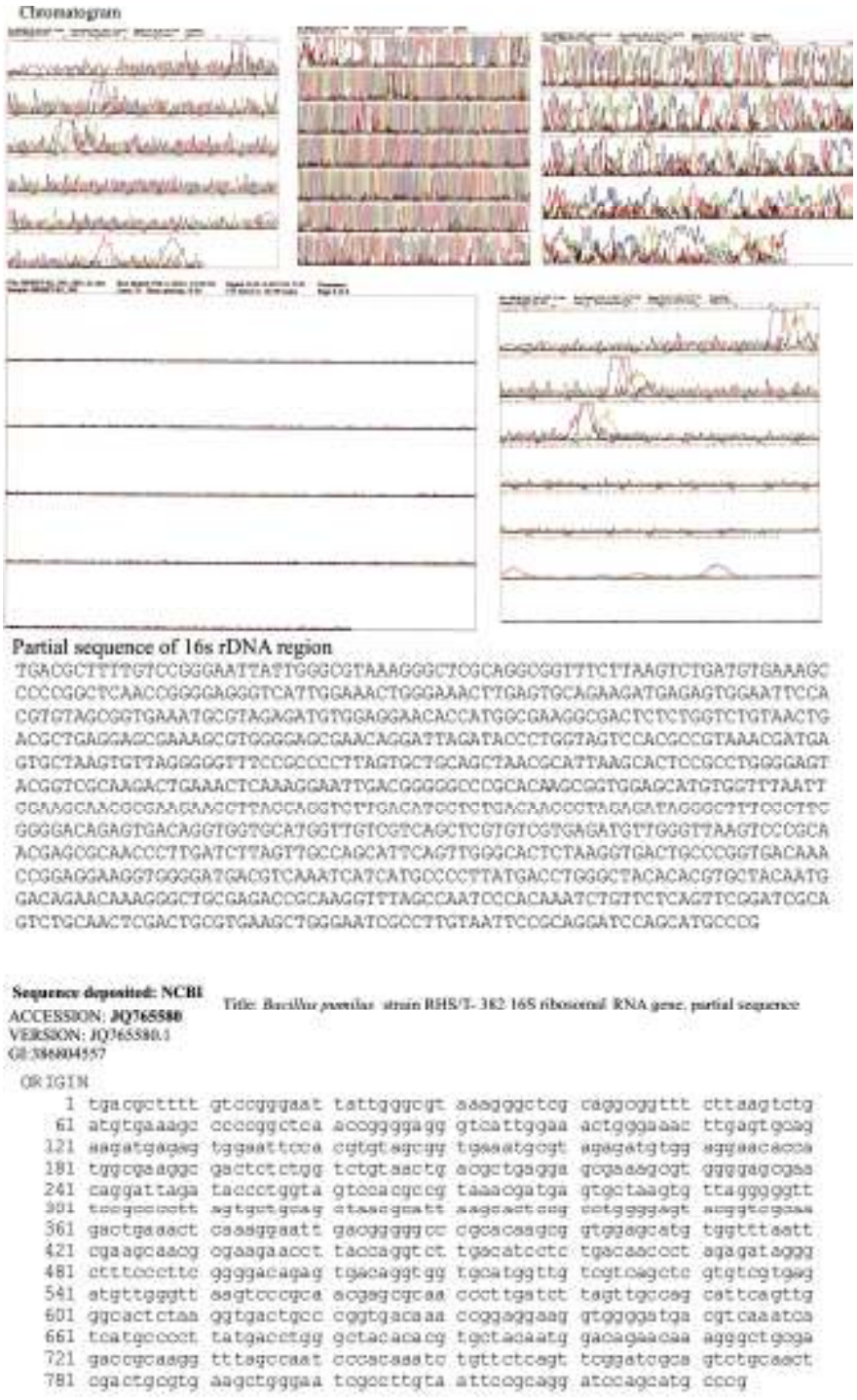

Fig. 26. Chromatogram and sequence deposition of 16S r DNA region of *Bacillus pumilus* (strain RHS/T- 382)

Chromatogram

Partial sequence of 16S rDNA region

GCGCAGGGAAGTGTTTGTGATCGCTGTGGCTCCCTGATAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAA
CCTGCCTGTAAGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATGGTTGTTTGAACCGCAGGTTCAG
ACATAAAAGGTGGCTTCGGCTACCACTTACAGATGGACCCGCGGCGCATTAGCTAGTTGGTGAGGTAACGGCTC
ACCAAGGCGACGATGCGTAGCCGACCTGAGAGGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTC
CTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATG
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TACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGG
AATTATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGG
GTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAG
AGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCGTGGG
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CTTAGTGCTGCAGCTAACGCATAAGCACTCCGCCTGGGGAGTACGGTCGCAAGACTGAAACTCAAAGGAATTG
ACGGGCGCCCGCACAAGCGGTGGAGCATGTGGTTTTAATTCGAAGCCAACCTTACCATCGTGACATCCGCTGAA
TCCTAGAGATAGGACGTCGCTCTTCGGGCAGAGTGAC

***Bacillus amyloliquefaciens* strain TRS-6 16S ribosomal RNA gene, partial sequence**

Sequence deposited: NCBI
ACCESSION: JN983127
VERSION:JN983127.1
GI: 378939972

```
   1 gcgcagggaa gtgtttgtga tcgctgtggc tccctgatag ttagcggcgg acgggtgagt
  61 aacacgtggg taacctgcct gtaagactgg gataactccg ggaaaccggg gctaataccg
 121 gatggttgtt tgaaccgcag gttcagacat aaaaggtggc ttcggctacc acttacagat
 181 ggacccgcgg cgcattagct agttggtgag gtaacggctc accaaggcga cgatgcgtag
 241 ccgacctgag agggtgatcg gccacactgg gactgagaca cggcccagac tcctacggga
 301 ggcagcagta gggaatcttc cgcaatggac gaaagtctga cggagcaacg ccgcgtgagt
 361 gatgaaggtt ttcggatcgt aaagctctgt tgttagggaa gaacaagtgc cgttcaaata
 421 gggcggcacc ttgacggtac ctaaccagaa agccacggct aactacgtgc cagcagccgc
 481 ggtaatacgt aggtggcaag cgttgtccgg aattattggg cgtaaagggc tcgcaggcgg
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 841 agtacggtcg caagactgaa actcaaagga attgacgggc gcccgcacaa gcggtggagc
 901 atgtggtttt aattcgaagc caaccttacc atcgtgacat ccgctgaatc ctagagatag
 961 gacgtcgctc ttcgggcaga gtgac
```

Fig.27. Chromatogram and sequence deposition of 16S r DNA region of *Bacillus amyloliquefaciens*

Table 14. Genbank Accession Numbers and Geographic locations of the Ex-Type strains of *B. amyloliquiquefaciens* that showed homology with the isolate RHS/T-382

Accession No.	Strain No	rDNA Sequence	Country	Organisms
JN983127	**TRS-6**	**985 bp**	**India**	***B. amyloliquefaciens***
FJ859694	BIHB 350	1512 bp	India	*B. amyloliquefaciens*
EF433406	BCRC 11601	BCRC 11601	Taiwan	*B. amyloliquefaciens*
FJ441059	sdg-26	1445 bp	China	*B. amyloliquefaciens*
EU586780	MZ-40	1498 bp	Pakistan	*B. amyloliquefaciens*
DQ993675	BCRC 17038	1538 bp	Taiwan	*B. amyloliquefaciens*
HQ283404	IPPBC_10A	1409 bp	Iran	*B. amyloliquefaciens*
HQ012267	ATCC 39374	329 bp	USA	*B. amyloliquefaciens*
HQ012266	ATCC 39374	387 bp	USA	*B. amyloliquefaciens*
HQ012265	ATCC 39320	329 bp	USA	*B. amyloliquefaciens*
HQ012264	ATCC 39320	388 bp	USA	*B. amyloliquefaciens*
DQ658169	-	540 bp	China	*B. amyloliquefaciens*
KF029598	RRLKE06	1383 bp	India	*B. amyloliquefaciens*
KC150029	anti-CA	1421 bp	China	*B. amyloliquefaciens*
JX674030	SS35	1380 bp	India	*B. amyloliquefaciens*
JQ734535	BGP20	1431 bp	China	*B. amyloliquefaciens*
HM130462	Q-426	1470 bp	China	*B. amyloliquefaciens*
JN882263	FMME044	1394 bp	China	*B. amyloliquefaciens*
KC790453	NCPSJ7	1400 bp	China	*B. amyloliquefaciens*
KC517141	SH-B74	1472 bp	China	*B. amyloliquefaciens*
KC634002	R21g9	1513 bp	China	*B. amyloliquefaciens*

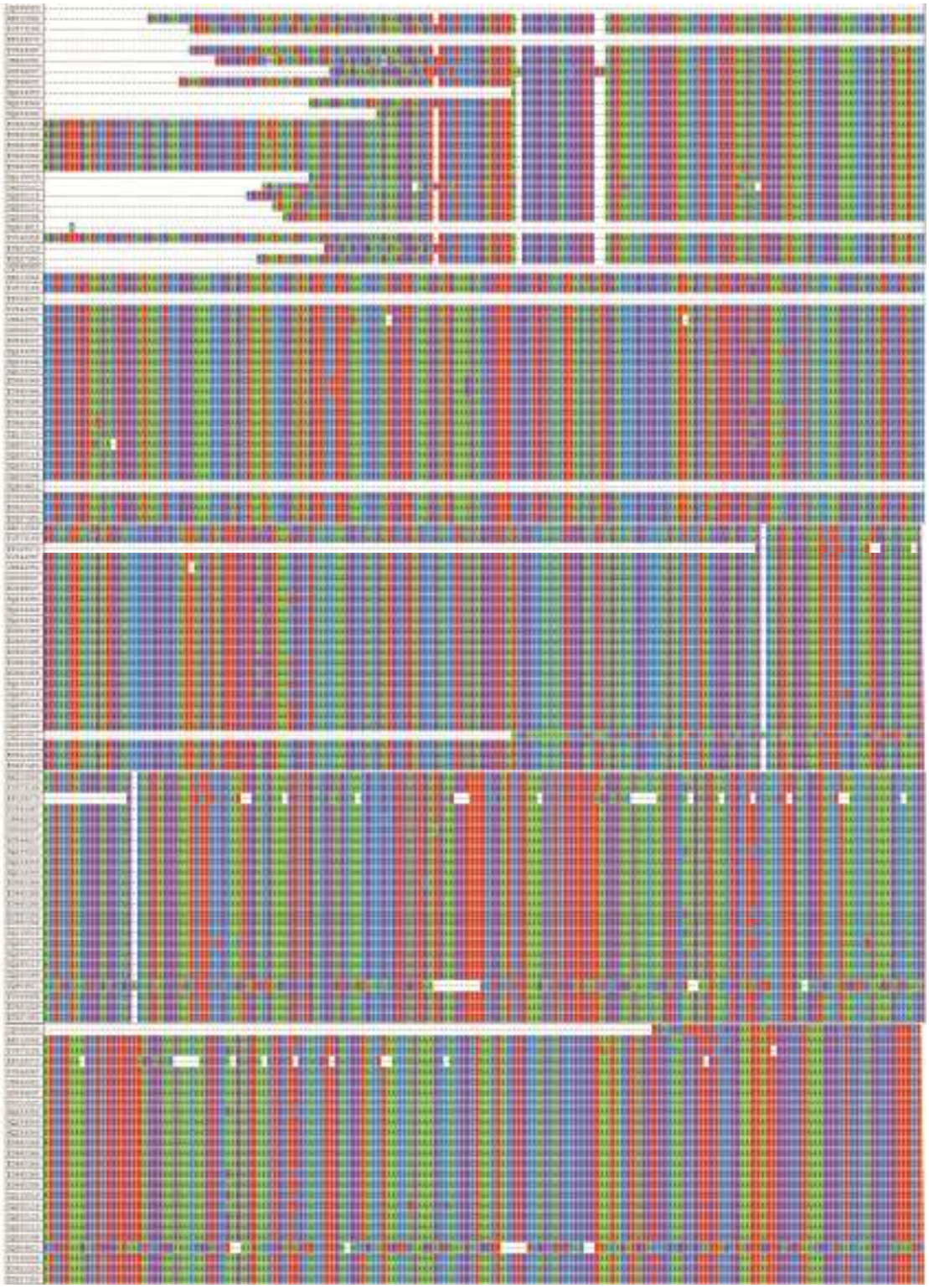


Fig. 28. 16S r DNA sequence alignments of *Bacillus pumilus* (JQ765580) with other extypes isolate. The conserved regions of the gene are demonstrated in different colour

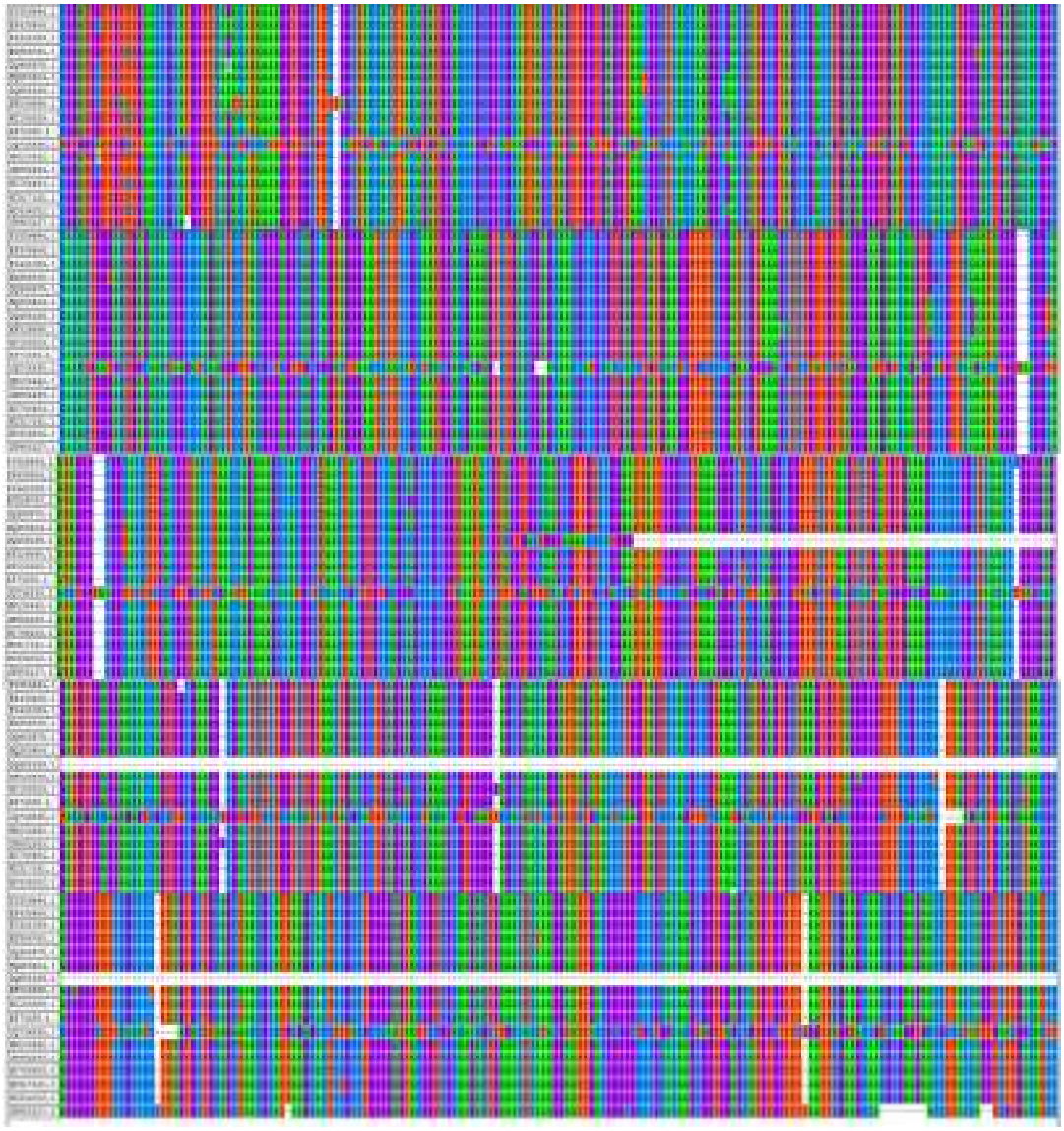


Fig. 29. 16S r DNA sequence alignments of *B. amyloliquefaciens* with other extypes isolate. The conserved regions of the gene are demonstrated in different colour

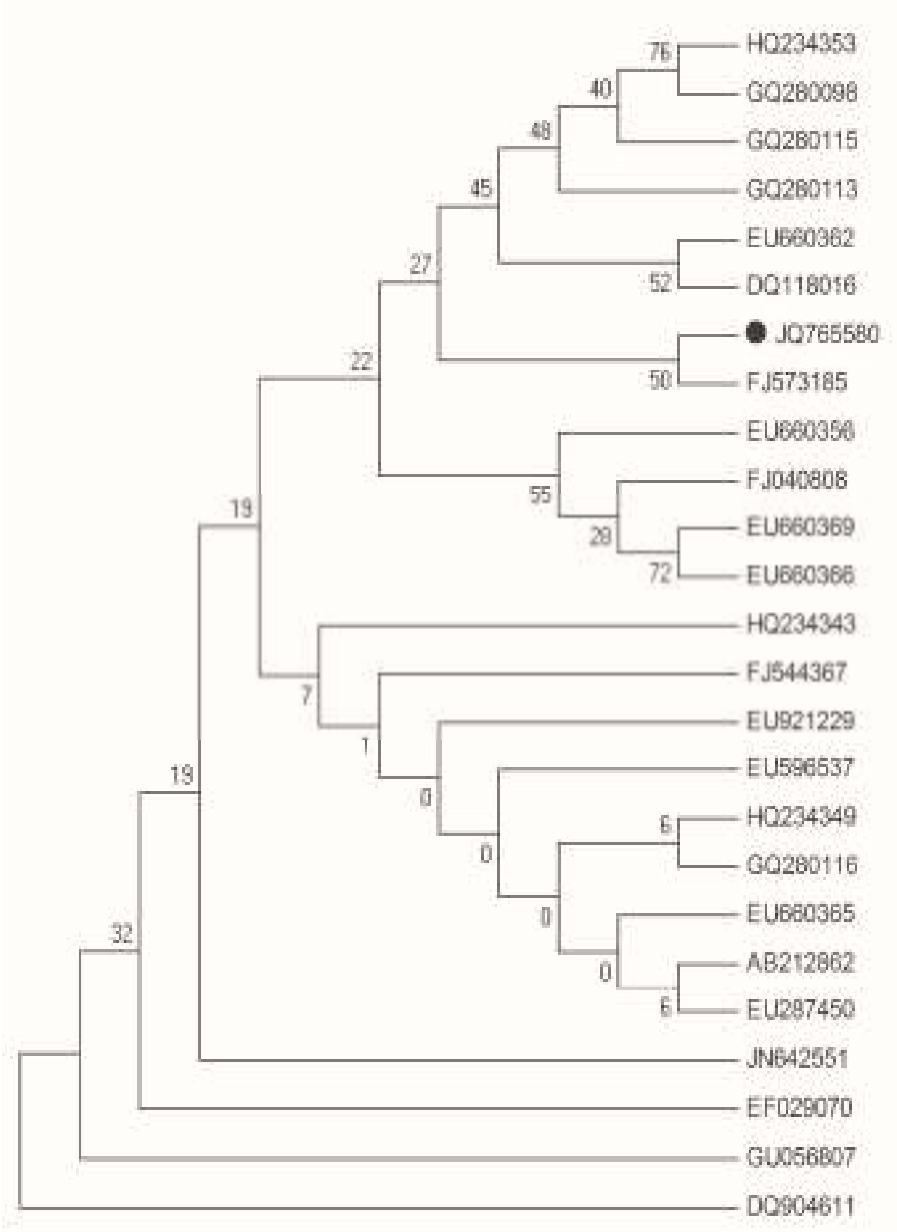


Fig.30. Phylogenetic placement of *B. pumilus* (JQ765580) with other ex-type strain sequences obtained from NCBI GenBank Database

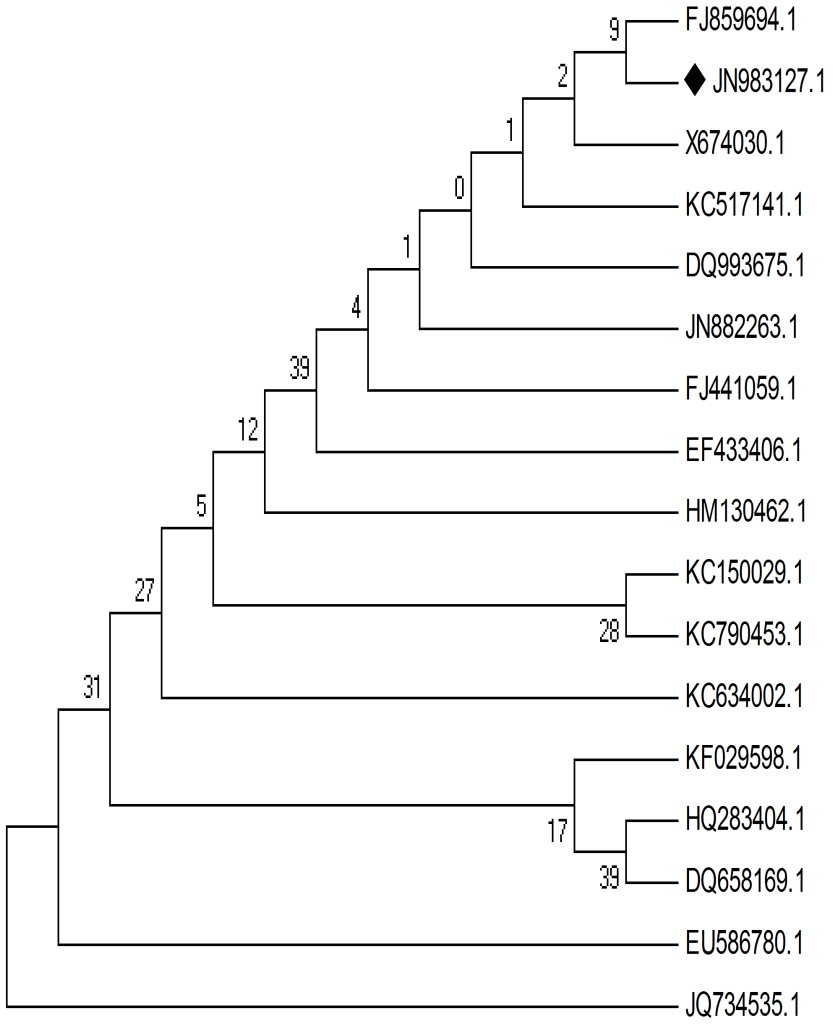


Fig. 31. Phylogenetic placement of *B. amyloliquefaciens* (JN983127) with other ex-type strain sequences obtained from NCBI GenBank Database

4.8. Morphological and Molecular characterization of tea root pathogen (*Sclerotium rolfsii*)

4.8.1. Morphological and cultural characteristics

4.8.1.1. Media

Sclerotium rolfsii was grown in different media *i.e.* Potato dextrose agar (PDA), Potato sucrose agar (PSA), Richard's Agar (RA), Carrot juice agar (CJA) Czapek-Dox agar

(CDA), and Yeast extract- dextrose agar (YDA). Results revealed that the maximum growth was recorded in PDA. Colonies of *S. rolfsii* are readily distinguished by rapidly growing silky-white hyphae that tend to aggregate into rhizomorphic cord like structure. In culture, the whole area of a Petri plate is rapidly covered with mycelium, including aerial hyphae which may cover the lid of the plate. Both in culture and in plant tissue, a fan-shaped mycelial expanse may be observed growing outward and branching acutely. At least two types of hyphae are produced. Sclerotia (0.5-2.0mm diameter) begin to develop after 6-7 days of mycelial growth. Initially a felt like white appearance then sclerotia quickly changes to a dark brown coloration (Fig. 32). Irrespective of the media used, maximum growth was observed at the temperature range lying between 25 ºC - 28ºC.

4.8.1.2. Incubation period

Table 15. Effects of incubation period on mycelial growth of *Sclerotium rolfsii*

Incubation period (Day)	Mean mycelial dry weight (mg) [a]
5	07.60 ±0.42
10	19.5± 0.80
15	37.3 ± 0.25
20	69.6± 0.40
25	77.10± 0.50
30	74.8 ± 0.51
35	66.2±0.40
40	67.2±0.37

a= Average of three replicates, ± = standard error, temp.25 ±1 ºC, pH of medium is 5

Sclerotium rolfsii was grown in PDA medium for a period of 30 days, mycelial growth was recorded after 5,10,15,20,25,30,35 and 40 days of growth and incubated at 25± 1ºC. Maximum growth was recorded after 20 days of incubation after which it declined. After 25 days of incubation the growth was negligible (Table 15).

Fig. 32. Growth of *Sclerotium rolfsii* on PDA medium. 7 and 10 days old (A&B). 10 days old culture in conical flask (C), Enlarged view of C (D).

4.8.1.3. pH

pH of the medium plays an important role in the growth of microorganism. In order to determine the effect of pH, buffer system have to be used to stabilize the pH of the media Results revealed that *Sclerotium rolfsii* grew to a lesser or greater extent over a wide range of pH (3.0-8.0), maximum growth was observed at pH 5.5 and then growth gradually declined **(Table 16).**

Table 16. Effects of different pH on mycelial growth of *Sclerotium rolfsii*

pH of medium	Mean mycelial dry weight (mg)[a]
3.0	22.50± 0.55
3.5	28.80 ± 0.48
4.0	28. 34 ± 1.20
4.5	40.12 ± 0.24
5.0	38.62 ± 2.14
5.5	53.32 ± 2.52
6.0	32.4 ± 2.41
6.5	28.10 ± 2.30
7.0	22.21 ± 1.47

[a]= average of three replicates, temperature = 25±1ºC, incubation time 25 days

4.8.1.4. Carbon sources

Like the pH of the surrounding medium the growth of fungus is greatly influenced by available nutrients. The ability of fungi to grow in different media depends on their capacity to utilize the available nutrients, basically carbohydrates. All carbohydrates are not utilized by the fungus in the same rate and so the growth rate varies with different carbon sources. In the present investigations, eight different carbon sources (dextrose, fructose, lactose, mannitol, maltose, sorbose, starch and sucrose) were tested for their effect on the growth of *Sclerotium rolfsii.* These were added separately to the basal medium. PDA medium without sugar was used as the basal medium which served as control set. Data were recorded after 20 days of incubation. Results revealed maximum growth using lactose as the carbon source while no growth was observed in sorbose which was similar to control set. Fructose and sucrose also supported comparatively good growth (Table 17).

4.8.1.5. Nitrogen sources

The availability of nitrogen for growth of the organism depends on the form in which it is supplied. Hence the most suitable medium for any particular microorganism can only be determined by testing a number of sources including both organic and inorganic. The

effect of inorganic nitrogen sources (ammonium nitrate, ammonium sulphate, calcium nitrate, potassium nitrate and sodium nitrate) as well as complex organic sources (casein hydrolysate, beef extract, peptone, urea and yeast extract) on the mycelial growth of *Sclerotium rolfsii* was tested. A basal medium without any nitrogen source was considered as control. It was revealed that maximum growth in beef extract followed by yeast extract and then in peptone. Among the inorganic sources calcium nitrate supported maximum growth. Other inorganic sources supported lower growth than organic sources, though no growth was observed in urea and insignificant growth was noted in basal medium without nitrogen (Table 18).

Table 17. Effect of different Carbon source on growth of *S. rolfsii*

Carbon sources	Mycelial dry weight (mg)[a b c]
D-Fructose	65.3±0.5
Mannitol	19.0±0.7
Sucrose	80.3±0.9
Starch	103.0±1.4
Maltose	128.0±1.4
Dextrose	236.3±1.8
Control (Without carbon)	16.3±1.6

[a] Results are on average of three replicates, [b]Incubation temperature 28°C,
[c]Incubation period- 8 days
± =Standard error

Table 18. Effect of different Nitrogen source on growth of *S. rolfsii*

Nitrogen sources	Mycelial dry weight (mg)[a b c]
Organic	
Peptone	551.3 ± 0.5
Urea	47.3±0.9
Yeast extract	2252.7±1.8
Beef extract	776±1.4
Inorganic	
Calcium nitrate	1130.0±1.4
Sodium nitrate	538.3±0.9
Ammonium sulphate	75.6±1.2
Potassium nitrate	120±0.8
Control (Richar's Agar without nitrogen)	43.3±0.9

[a] Results are on average of three replicates
[b]Incubation temperature 28°C
[c]Incubation period- 8 days
± Standard error

4.8.2. Immunodetection of *S. rolfsii*

The effectiveness of the purified antigen of *S. rolfsii* in raising PAbs was checked by homologous cross reaction following agar gel double diffusion tests. The precipitin

reaction done with PAb raised against mycelial protein yielded sharp band which was stained blue (Fig. 33A).

Dot immunobinding assay was performed using total soluble proteins of *S. rolfsii* used as homologous antigen source. Antigens were carefully spotted on nitrocellulose paper and probed with PAb of *S. rolfsii*. Results presented in Fig. 33 B, shows clear and intense color reactions. Western blot analyses using polyclonal antibody of *S. rolfsii* revealed that the PAb collected after successful immunization of the rabbits for consecutive six weeks could show different levels of homologous reactions with the antigen of *S. rolfsii*. A sharp and intense band at 35 Kda was obtained on the nitrocellulose membrane after enzymatic reaction with NBT-BCIP (Fig. 33D). Efficacy of polyclonal antibodies raised against the mycelial proteins used as antigen source was further tested with the help of indirectimmuno fluorescence of young and mature sclerotia of *S. rolfsii.* The young and old sclerotia along with the mycelia treated with PAbs and labeled with FITC showed apple green fluorescence where fluorescence was more intense on young sclerotia (Fig. 33 E-J.). Neither mycelia nor sclerotia showed any type of auto-fluorescence nor they showed any fluorescence when treated with normal serum followed by FITC.

4.8.3. 18S rDNA sequence analysis of *S. rolfsii*

Genomic DNA of *S. rolfsii* isolate - RHS/T-381 was isolated and purified and re-suspended in 1X TE buffer until further use. Agarose gel electrophoresis of genomic DNA revealed that they were RNA free was around 1.80 Kb. Purity of DNA evaluated in terms of the ratio between absorbance of A_{260} and A_{280} showed that genomic DNA of this pathogen was 1.913. ITS-PCR was performed with the help of ITS specific universal primer pair where a uniform product of 250 bp was obtained. The amplicons were sequenced and was further analyzed.

4.8.3.1. 18S rDNA sequences and BLAST analysis

The BLAST query of the 18S r DNA sequence of the isolate RHS/T-381 against GeneBank database confirmed the identity of the isolate as *Athelia rolfsii* (anamorph *Sclerotium rolfsii*). The sequences have been deposited in NCBI, GenBank database under the accession no. **JQ429785** The sequence chromatograms of both the PGPR isolates have been represented in Fig. 34.

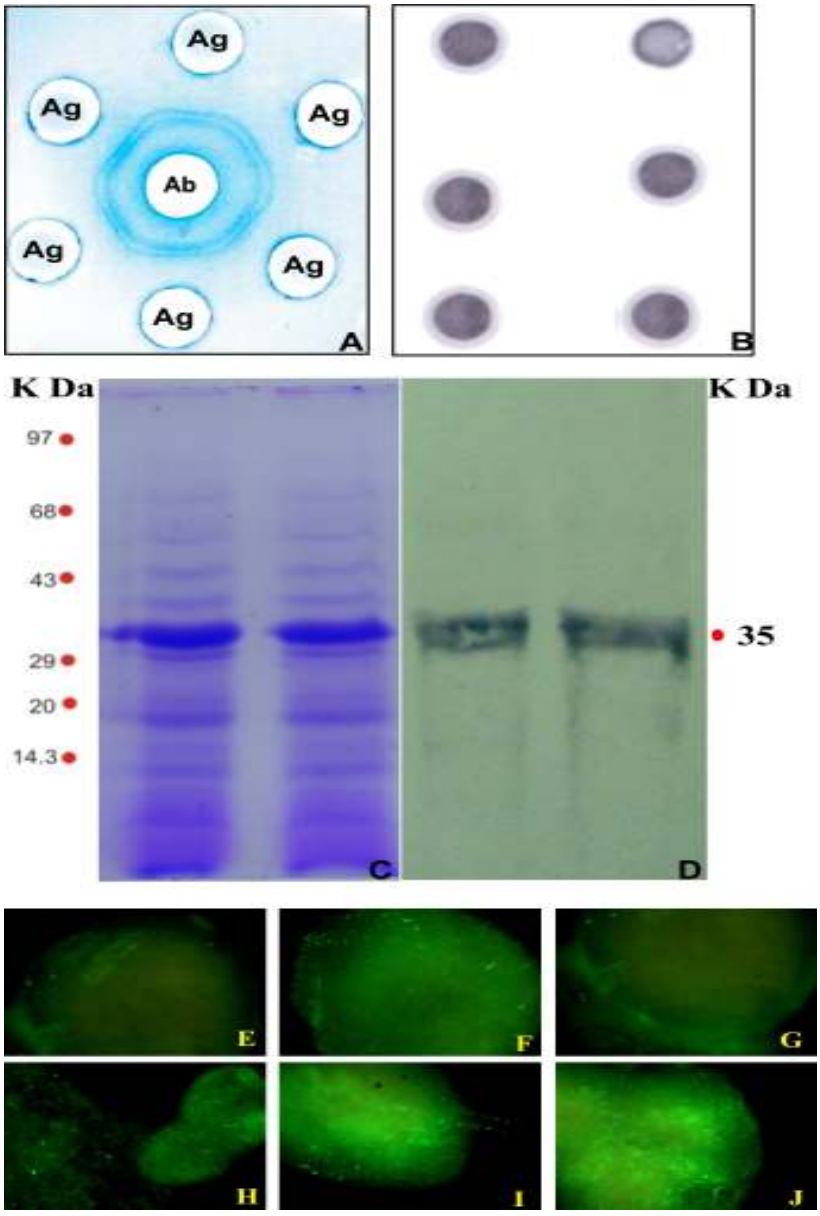


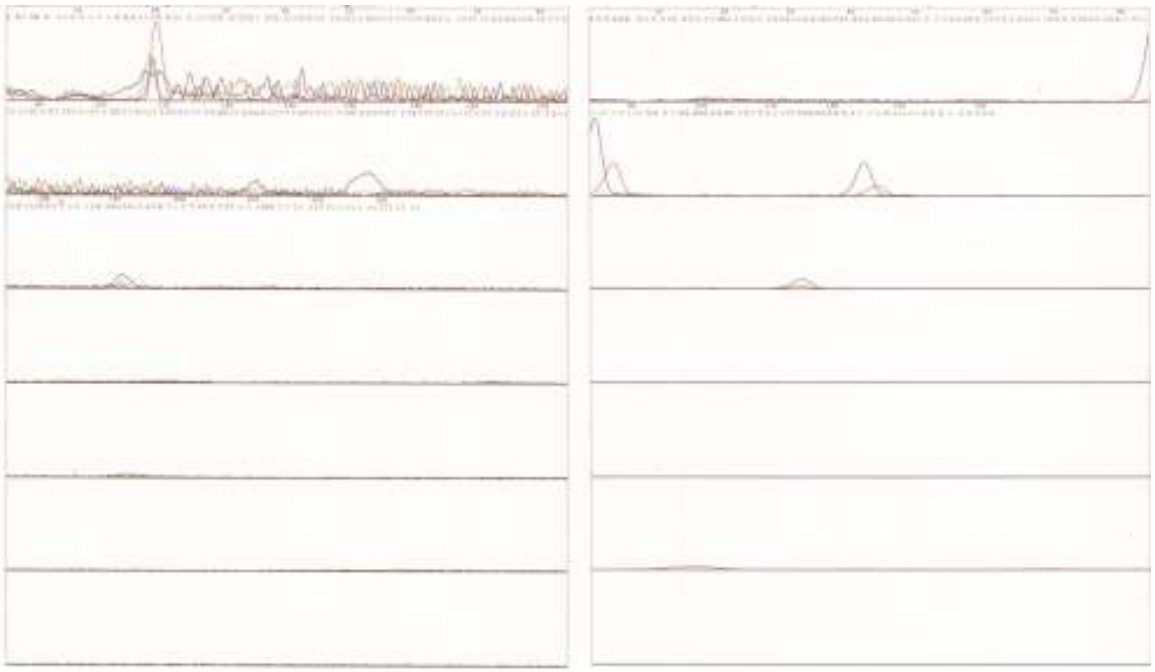
Fig.33. Serological assays of *S. rolfsii* ; Immunodiffusion (A) and Dot-blot (B) using PAb of *S. rolfsii*; SDS-PAGE (C) and Western blot (D) analysis of *S. rolfsii*; Immunofluorescence of mature (E-G) and young (H-J) sclerotia of *S. rolfsii* treated with PAbs of *S. rolfsii* and labelled with FITC

4.8.3.2. Multiple sequence alignment and phylogeny

A multiple sequence alignment of ITS gene sequences of *S. rolfsii* was conducted. The result reveals that there were quite a number of gaps that were introduced in the multiple sequence alignment 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). Phylogenitic analysis of *S. rolfsii* was carried out with the Ex-type strain sequences obtained from NCBI Genbank Database which showed maximum homology with the isolate TG1 (Table 19). The evolutionary history was inferred using the Neighbor-Joining method (Saitou N & Nei M, 1987). The optimal tree with the sum of branch length = 0.85088554 with 176 positions in final data set have been shown (Fig. 36). 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. Phylogenetic analyses were conducted in MEGA-4 (Tamura *et. al.* 2007).

Table 19. Genbank Accession Numbers and Geographic locations of the Ex-Type strains of *S. rolfsii* that showed homology with the isolate RHS/T-381

Accession No.	Strain No	rDNA Sequence	Country	Organisms
KC293992	BeanScRs1	663 bp	Italy	*Athelia rolfsii*
JF966208	KACC:45155	685 bp	Korea	*Athelia rolfsii*
JN241565	1112	646 bp	USA	*Athelia rolfsii*
JN241564	176	646 bp	USA	*Athelia rolfsii*
JN241563	AS-1	648 bp	USA	*Athelia rolfsii*
JN241562	3083	649 bp	USA	*Athelia rolfsii*
JN241561	138	649 bp	USA	*Athelia rolfsii*
JN241560	1125	649 bp	USA	*Athelia rolfsii*
JN241559	1810	647 bp	USA	*Athelia rolfsii*
JN241557	WM913	649 bp	USA	*Athelia rolfsii*
JN241556	3095	649 bp	USA	*Athelia rolfsii*
JN241555	SR2	649 bp	USA	*Athelia rolfsii*
JN241558	185	649 bp	USA	*Athelia rolfsii*
JN241554	SR1	649 bp	USA	*Athelia rolfsii*
JN241553	3082	649 bp	USA	*Athelia rolfsii*
JN241552	3087	613 b	USA	*Athelia rolfsii*
HQ420816	SR001	684 bp	Korea	*Athelia rolfsii*
HM355751	KACC42087	684 bp	Korea	*Athelia rolfsii*
FJ968783	S-07	683 bp	India	*Athelia rolfsii*
GQ148561	S-08	641 bp	India	*Athelia rolfsii*
JQ429785	**RHS/T-381**	**235 bp**	**India**	***Athelia rolfsii***



Partial sequence of 28S ribosomal RNA gene

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CGAAATAATATAAAAACAAAGGGGGGGTAAAAAGTAAAATCCCCATCCGGAAGGGGGATTCTAGCTTGTA
TGTACTACTTATAATATCATGCGCATATATTAGCCTATAAGTGCATATATGGCCATTGACTCAAATCAGT
TGTACCGTTCACTATGGTTCCCTCC

Sequence deposited: NCBI Title: Athelia rolfsii strain RHS/T-381 28S ribosomal RNA gene, partial sequence

ACCESSION:**JQ429785**
VERSION:JQ429785.1
GI:384872367

ORIGIN

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Fig.34. Chromatogram and sequence deposition of 18S r DNA of *Athelia rolfsii* (anamorph *Sclerotium rolfsii*) strain RHS/T-381

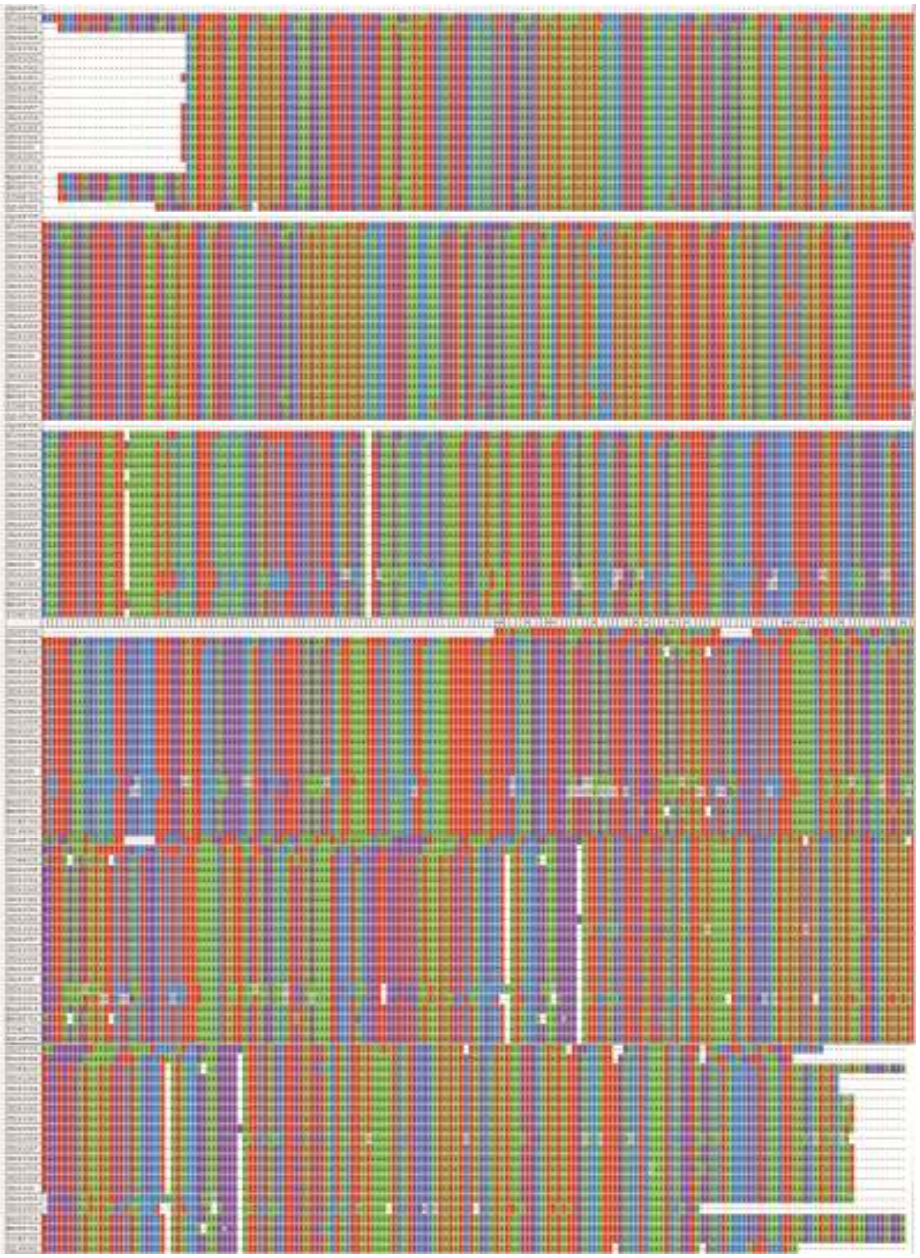

Fig.35. 18S r DNA sequence alignments of of *Athelia rolfsii (anamorph Sclerotium rolfsii)* (JQ429785) with other extypes isolate. The conserved regions of the gene are demonstrated in different colour.

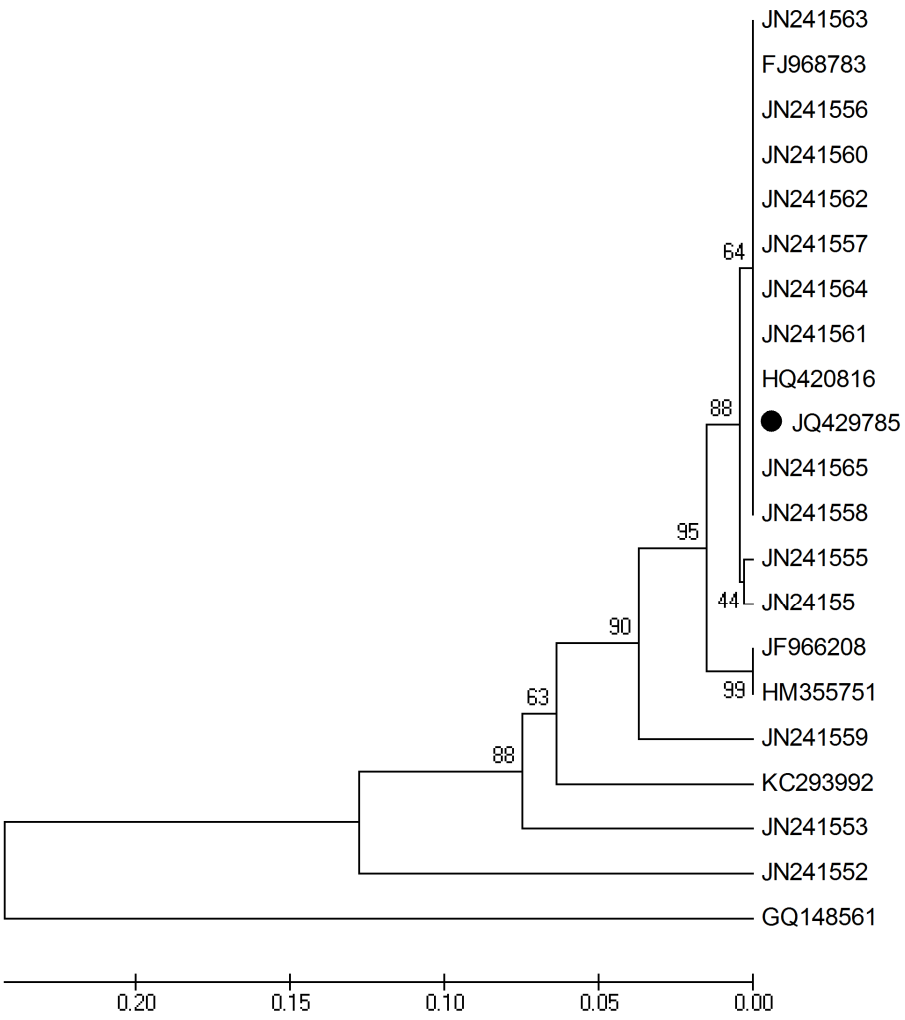

Fig.36. Phylogenetic placement of *Athelia rolfsii* (anamorph *Sclerotium rolfsii*) JQ429785 with other ex-type strains obtained from NCBI GenBank database by MEGA4 software

4.9. *In vitro* assessment for antagonistic activity of PGPR isolates against tea root pathogens

The antagonistic effect of the both the isolates *B. pumilus* RHS/T-382 and *B. amyloliquefaciens*-TRS6 which showed positive results in all the tested PGP characters were tested against tea root pathogens *viz*, *Sclerotium rolfsii, Fomes lamaoensis*, *Poria hypobrunnea* and *Spherostilbe repens*. The results of interaction between the fungal pathogens and selected PGPR isolates have been presented in Table 20 & Fig.37**.** Both the bacterial isolates were found to inhibit the test pathogens up to 84 %. Among both the isolates TRS6 was found to inhibit all the test pathogens more efficiently than RHS/T-382.

Table. 20 *In vitro* antagonistic tests of PGPR isolates against fugal test pathogens

Interacting microorganisms	Diameter of fungal colony	% inhibition
Sclerotium rolfsii	8.8±1.3	
S. rolfsii+ *B. pumilus* + (RHS/T-382)	6.2±0.16	29.54
S. rolfsii + *B. amyloliquefaciens*(TRS6)	1.4±0.29	84.09
Poria hypobrunnea	8.6±0.20	
P. hypobrunnea +*B. pumilus* (RHS/T-382)	3.8±0.09	55.81
P. hypobrunnea+ *B. amyloliquefaciens*(TRS6)	2.9±0.20	66.27
Spherostilbe repens	8.5±0.34	
S. repens + *B. pumilus* (RHS/T-382)	5.6±0.55	34.11
S. repens + *B. amyloliquefaciens*(TRS6)	3.0±0.32	64.70
Fomes lamaoensis	9.2±0.21	
F. aoensis+ *B. pumilus* (RHS/T-382)	2.1±0.22	77.17 %
F. lamaoensis+ *B. amyloliquefaciens* (TRS6)	1.5±0.09	83.69%

Values are average of three replicate experiments. ±= SE

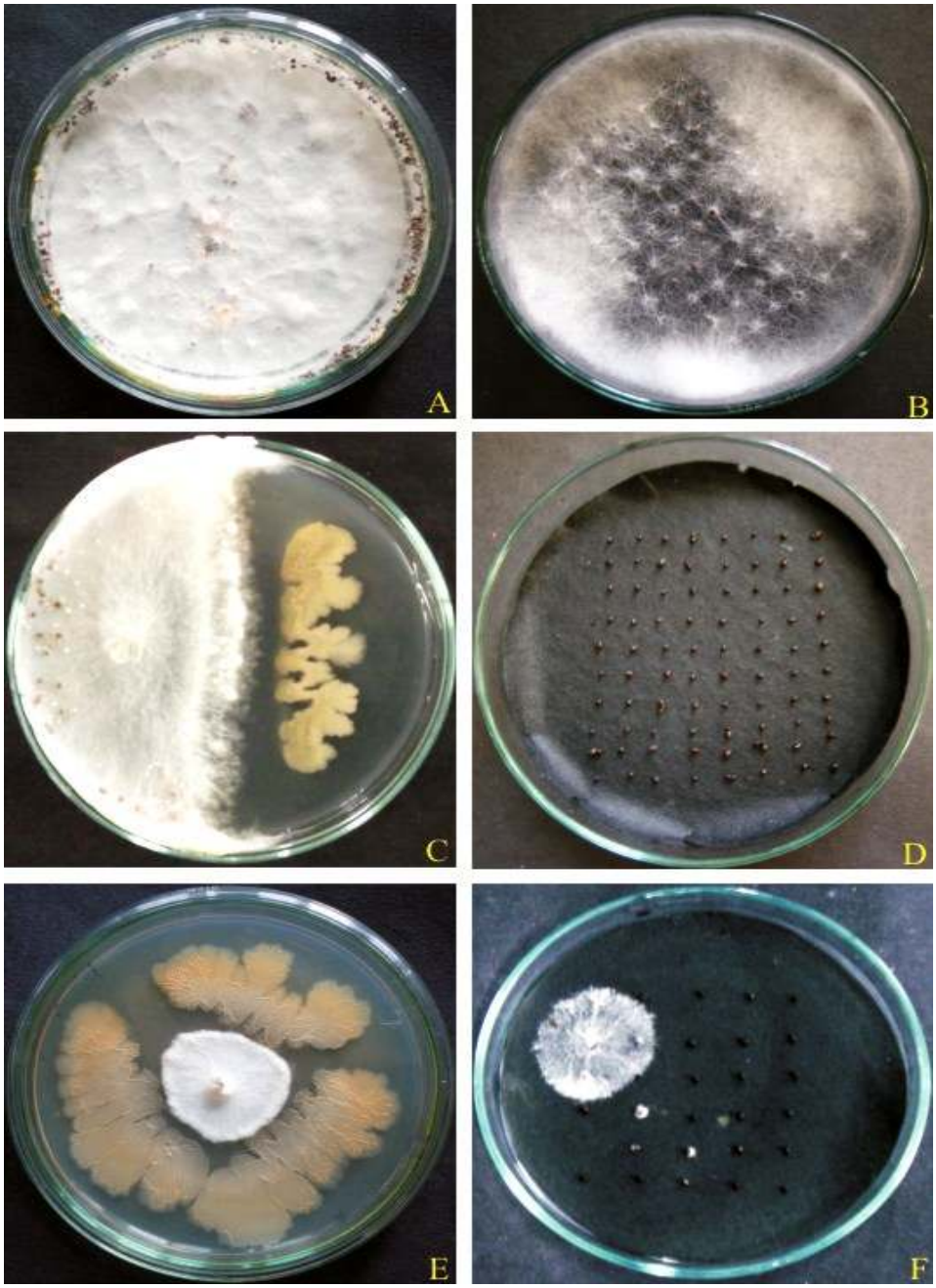

Fig.37. *In vitro* antifungal activities of isolate RHS/T-382 and TRS-6 tested against *S. rolfsii.* Inhibition of *S. rolfsii* in dual plate culture assay by RHS-382 (C) & TRS-6 (E). Inhibition of sclerotia germination by culture filtrates of RHS/T-382 (D) and TRS-6 (F). Control sets (A &B)

4.10. Incidence of sclerotial blight disease of tea

4.10.1. Disease incidence in tea gardens

Sclerotial blight caused by Sclerotium rolfsii Sacc. [telomorph: *Athelia rolfsii* (Curzi), Tu and Kimbrough = *Corticium rolfsii* (Curzi)], is a soil borne plant pathogen causing diseases on a wide range of agricultural and horticultural crops due its prolific growth rate and the production of oxalic acid and cell wall degrading enzymes. It is among one of the most aggressive root pathogens which causes considerable damage to young tea saplings in the nursery. The first visible symptom of sclerotial blight disease is observed as yellowing and wilting of lower leaves. The fungal mycelia first appeares at the base near the soil line. The pathogen then grows upwards covering the stem with a cottony white mass of mycelia. Later on, water soaked and grey lesions appear on the tea seedlings that turn brown, resulting in the death of the whole plant. A large number of small light brown, mustard like sclerotia develops in the collar zone. After the pathogen establishes itself, its subsequent advancements in production of mycelia and sclerotia is quite rapid. The infected tea seedlings ultimately topple down and die.

4.10.2. Varietal resistance in tea against *Sclerotium rolfsii*

Screening for resistance of tea varieties against *S. rolfsii* was carried out in sick plot developed specifically for this pathogen (Fig. 38)**.** Varietal resistance test of tea against ***Sclerotium rolfsii*** was carried out in ten (10) tea cultivars including five Toklai varieties (TV-25, TV-26, TV-9, TV-20, TV-18), two Teen Ali varieties (T-17 and T-78), two Upasi varieties (UP-3 and UP-26) and one Assam variety (AV-2). Three year old plant roots were inoculated with ***S. rolfsii*** and disease assessment was done on the basis of visual observation of symptoms and disease index (ranging from 1-6) was calculated after 15,30 and 45 days following inoculation as well as on the basis of histopathological studies of the infected root (Fig. 39).

Results presented in Table-21 shows that among the tested tea varieties, TV-25, TV-26, TV-9 and TV-20 were found to be less susceptible in comparison with other tea varieties. Defoliation of leaves following infection with *S. rolfsii* was evident in TV-18, T-17, AV-2, T-78, UP-3, UP-26 and TV-25 after 30 days of inoculation in relation to healthy control plant. Disease symptoms occurred in those varieties within 7 days after

transplantation following death of the plants, were selected as highly susceptible varieties, whereas the varieties showing resistant reactions were also categorized.

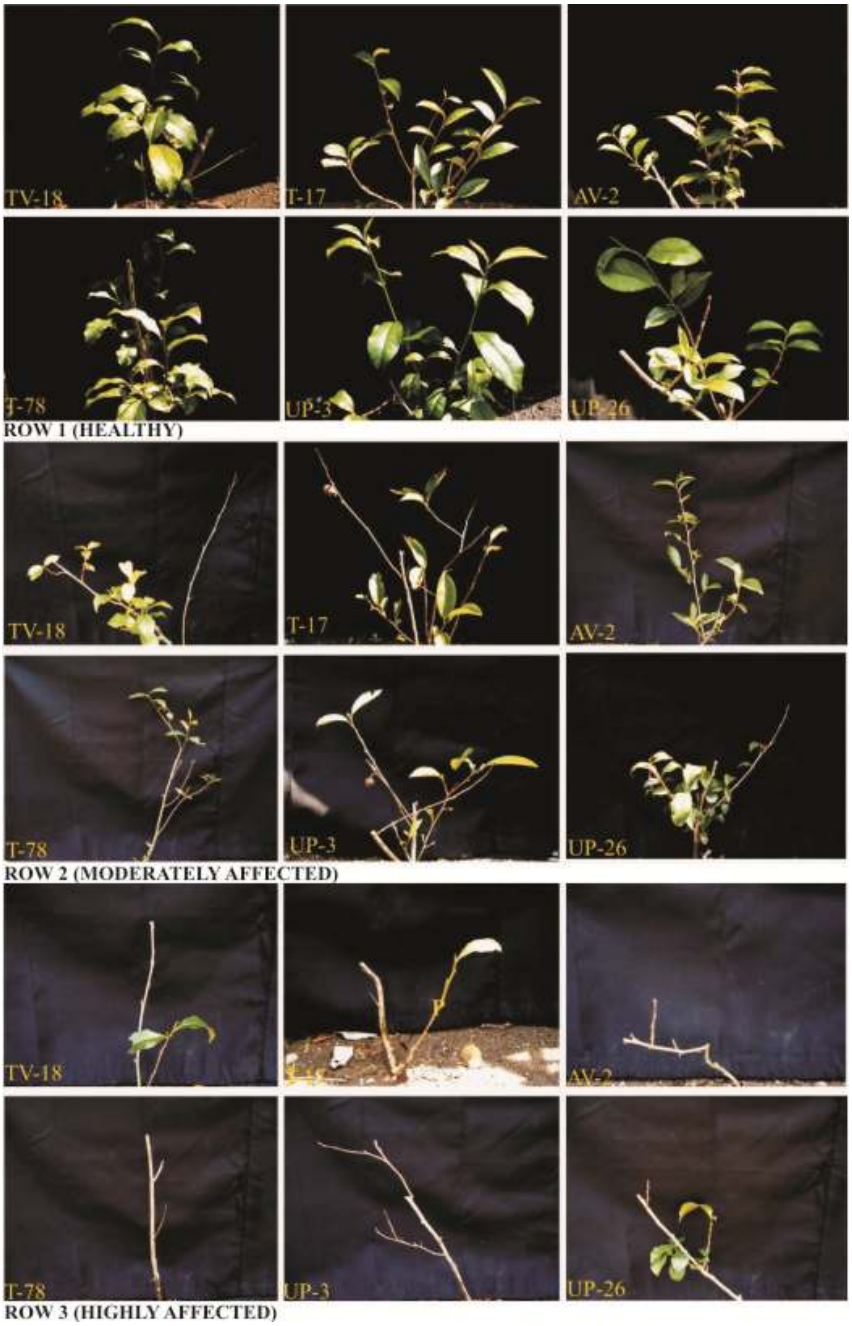

Fig. 38. Different stages of disease development in tea varieties showing disease incidence after inoculation with *S. rolfsii* in sick plots

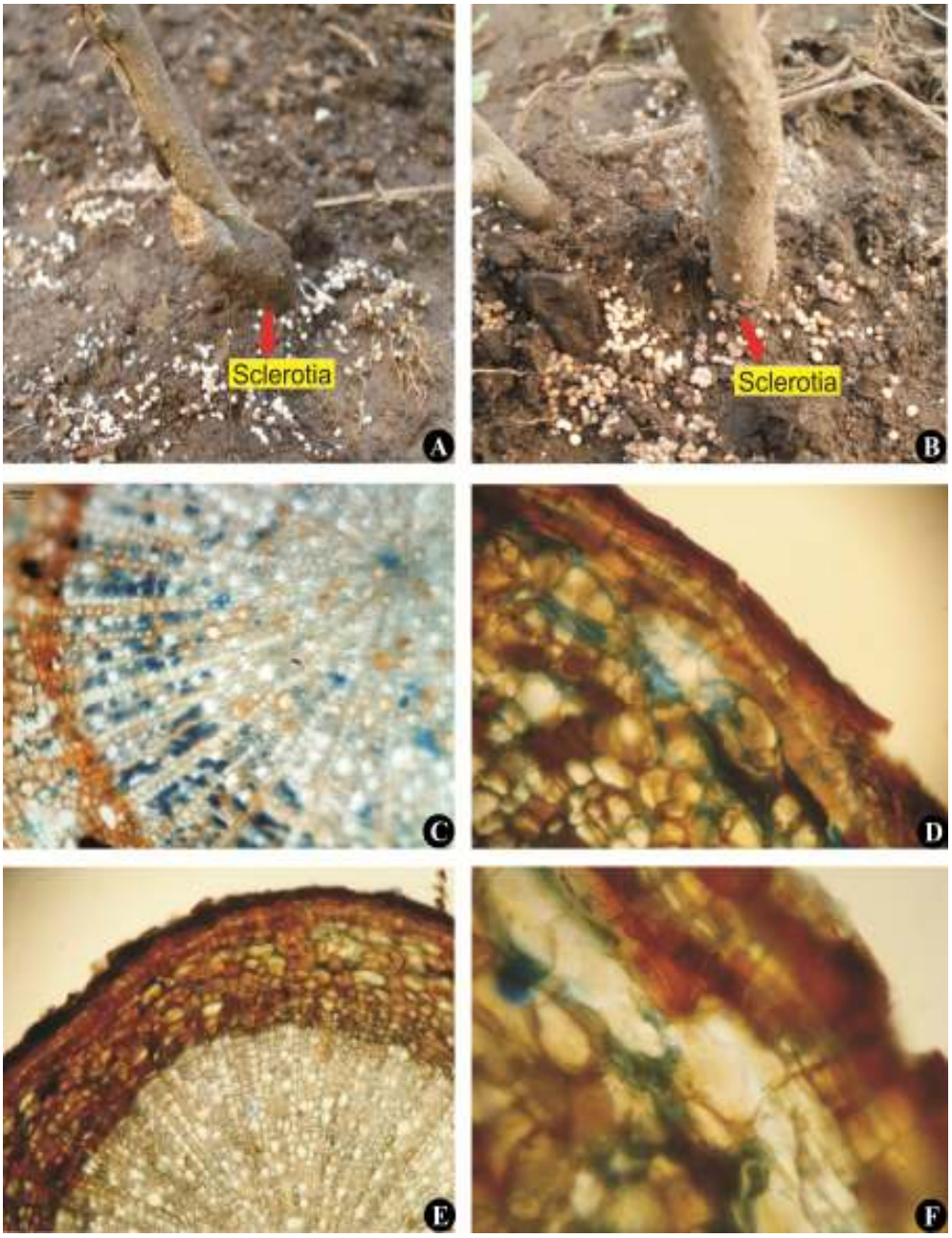

Fig. 39. Tea root rhizosphere showing development of sclerotia around the severely infected plant root and collar regions (A&B). T.S. of tea root showing affected zones and parenchyma tissue blocked by the hyphae of *S. rolfsii* and progression towards the vascular regions (C-F)

Table 21: Disease development in different tea varieties observed during varietal resistance test against *Sclerotium rolfsii* in sick plot

Tea varieties	Disease index[a b]		
	15 days	**30 days**	**45 days**
TV-25	2.64±0.01	3.02±0.03	3.6±0.04
TV-26	0.53±0.02	2.08±0.05	3.48±0.06
TV-9	1.64±0.02	3.11±0.06	3.48±0.05
TV-20	2.52±0.03	3.02±0.03	4.063±0.08
TV-18	1.67±0.03	3.53±0.09	4.06±0.08
T-17	1.65±0.02	3.46±0.04	4.09±0.08
AV-2	0.22±0.01	4.63±0.09	5.71±0.06
T-78	2.52±0.03	4.91±0.02	5.41±0.01
UP-3	0.66±0.01	3.65±0.04	4.08±0.061
UP-26	3.9±0.08	4.94±0.04	5.43±0.01

[a] Results are in average of 10 inoculated plants of each variety.
[b]Days after inoculation
± Standard error
Key to disease index :
Disease intensity was assessed as rot index on a scale of 0-6, depending on both underground and above ground symptoms as follows: Key to disease index. 0-No symptoms. 1-Small roots turn rotten; lesions appeared at the collar region. 2-Middle leaves start wilting and 10-20% of the roots turn brown.3-Leaves wilted and 20-40% roots become dry with browning of shoot.4-Extensive rotting at the collar region of root, 60-70% of the roots and leaves wilted, browning of shoot over 60%.5-80%roots affected while the root along with the leaves withered and shoot becomes brown more than 80% and 6-Whole plants die, since 100% roots were wilted.

4. 11. Biochemical changes in tea plants following inoculation with *Sclerotium rolfsii*

Artificial inoculation of tea roots with *Sclerotium rolfsii* was carried out in experimental field following sick soil technique. Seedlings of a susceptible tea variety were grown in the field and disease development was noticed. Histopathological studies revealed the establishment of pathogen in root tissues along with above ground symptom. Biochemical changes following disease development was assayed in both the untreated healthy and inoculated tea varieties.

4.11.1. Estimation of Phenol content

4.11.1.1. Total phenol

Total phenols from healthy and *Sclerotium rolfsii* inoculated tea roots of different varieties were extracted after 10 days of inoculation and estimated. Results revealed that total phenol decreased following inoculation with *Sclerotium rolfsii* in the susceptible varieties (Table 22). However there is an increase in the phenol content of resistant varieties following inoculation. Among all the varieties tested TV -20 showed maximum increase in total phenol following inoculation with the pathogen.

Table: 22 Total phenol content in healthy and *Sclerotium rolfsii* inoculated tea varieties

Tea varieties	Total phenol (mg/g tissue)*	
	Healthy	Inoculated
TV-25	4.5±0.25	7.1±0.05
TV-26	5.3±0.15	8.8±0.10
TV-9	4.6±0.40	5.0±1.21
TV-20	6.2±1.25	9.7±0.35
TV-18	4.8±0.25	6.9±0.05
T-17	6.3±0.15	9.8±0.10
AV-2	4.8±0.35	4.9±1.10
T-78	6.7±1.25	9.5±0.20
UP-3	4.5±0.25	7.1±0.05
UP-26	5.3±0.25	8.9±0.15
* Average of three replicates.± = S.E		

4.11.1.2.. Ortho- dihydroxy phenols

Ortho-dihydroxy phenols were also extracted from healthy and *Sclerotium rolfsii inoculated* tea roots of different varieties after 10 days of inoculation with the pathogen and estimated. Results revealed that ortho-dihydroxy content decreased in susceptible varieties and increase in resistant varieties following inoculation with *Sclerotium rolfsii* . Responses of TV-20 variety against the pathogen were towards increasing the levels of ortho-dihydroxy phenol (Table 23).

4.11.2. Phenylalanine ammonia lyase

Phenylalanine ammonia lyase (PAL) is the first enzyme of phenyl propanoid metabolism in higher plants and it has been suggested to play a significant role in regulating the accumulation of phenolics, and phytoalexins as well as lignin, three key factors responsible for disease resistance. In present study, PAL activity was assayed in tea roots following inoculation with *Sclerotium rolfsii*. PAL activity was assayed in each case after 7, 14 and 20 days after inoculation. Results presented in **Table 24** shows that PAL activity increased after 14 days of inoculation markedly in all the varieties except in TV-25.

Table: 23. Level of ortho-dihydroxy phenol content in healthy and *Sclerotium rolfsii* inoculated tea varieties

Tea varieties	ortho-dihydroxy phenol (mg/g tissue)*	
	Healthy	Inoculated
TV-25	1.7±0.20	2.5±2.35
TV-26	3.1±2.10	3.5±0.65
TV-9	2.1±0.35	2.8±2.34
TV-20	3.1±1.15	3.8±0.56
TV-18	1.6±0.15	2.9±2.54
T-17	3.3±1.15	3.5±0.65
AV-2	2.7±0.35	3.8±0.56
T-78	2.2±0.35	2.8±2.34
UP-3	3.4±1.15	3.5±0.65
UP-26	3.1±2.10	3.7±2.35
*= average of three replicates. .± = S.E		

Table 24 Changes in PAL activity in tea roots following inoculation with *Sclerotium rolfsii*

Tea varieties	PAL activity in tea roots (µg cinnamic acid g^{-1} m^{-1})*	
	Healthy	Inoculated
TV-25	073.4± 2.33	169.5± 2.10
TV-26	081.0±1.15	158.6± 1.00
TV-9	076.4±0.05	173.8± 0.00
TV-20	087.6±1.25	142.6±2.01
TV-18	078.4± 2.34	186.5± 2.10
T-17	085.0±1.02	154.6± 1.00
AV-2	079.4±0.03	166.8± 0.10
T-78	082.6±1.23	151.6±2.01
UP-3	089.4±0.03	158.8± 0.15
UP-26	077.6±1.22	145.6±2.05
*= average of three replicates. .± = S.E		

4.11.3. Peroxizyme

Root samples of healthy and sclerotial blight infected tea plants were collected. Peroxizyme enzyme was extracted from both healthy and infected root tissues. Native PAGE analysis of peroxidase showed the existence of three isoforms in all the varieties of tea. All the isoforms which were present in untreated control plants were also present in treated ones. However, there was clear distinction between the treated and untreated control plants as far as intensities were considered. Maximum intensity of bands was noticed in the treated plants (Fig. 40).

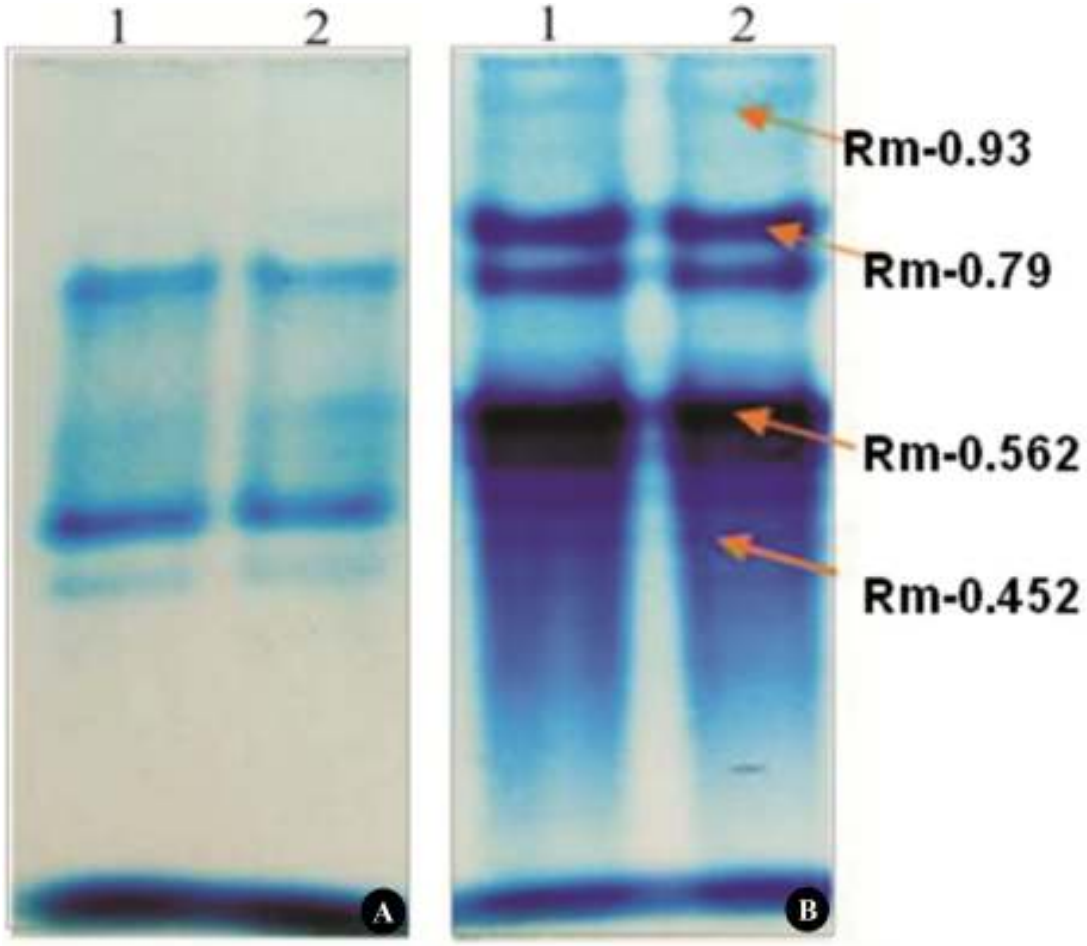

Fig.40. Native PAGE analysis of peroxidase isozyme in healthy (A-lanes 1-2) and *S. rolfsii* inoculated tea leaf (B-lanes 1-2)

4.12. AMF colonization in maize roots

4.12.1. Microscopic and histopathological observations

Single spore culture of AMF spores isolated from tea rhizosphere were maintained in laboratory conditions and then finally applied to the sterile soil of maize for mass multiplication in pots. Root colonization percentage and spore mass produced per gm soil per pot were recorded. Microscopic observations of the root inoculated with AMF showed profuse colonization especially in the feeder roots (Fig. 41, 42).

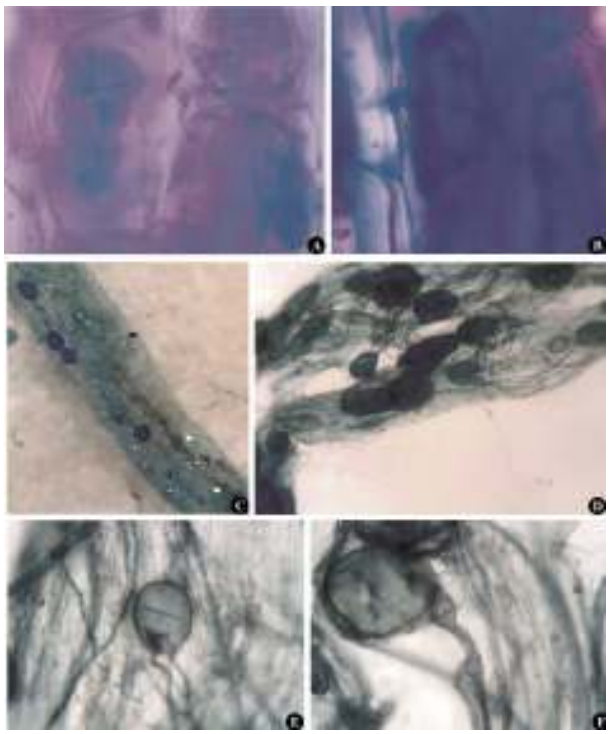

Fig. 41. Histopathological observation of maize roots following inoculation with *G. mosseae* spores showing arbuscules (A&B), vesicles (C&D) and young spores (E&F)

4.12.2. FITC labeling of maize roots

In this present study single spores of *G. mosseae* were isolated from the soils obtained from tea rhizosphere and aseptically multiplied in *Sorgum bicolor*. Finally they were mass multiplied in maize in pot conditions. Apart from histopathological observations, root colonization by *G. mosseae* was confirmed with the help of indirect immuno-fluorescent staining of maize root tissues after treating them with PAbs of *G. mosseae.* Root tissues labeled with FITC when observed under UV-microscope showed bright apple green fluorescence of the hyphae, vesicles and arbuscules within the host tissue which confirmed successful colonization by *G. mosseae* (Fig. 43).

Fig.42. Mass multiplication of AMF spores in maize in pot conditions (A), Spore mass closely adhered to maize root as seen under dissecting microscope (B) and compound microscope (C)

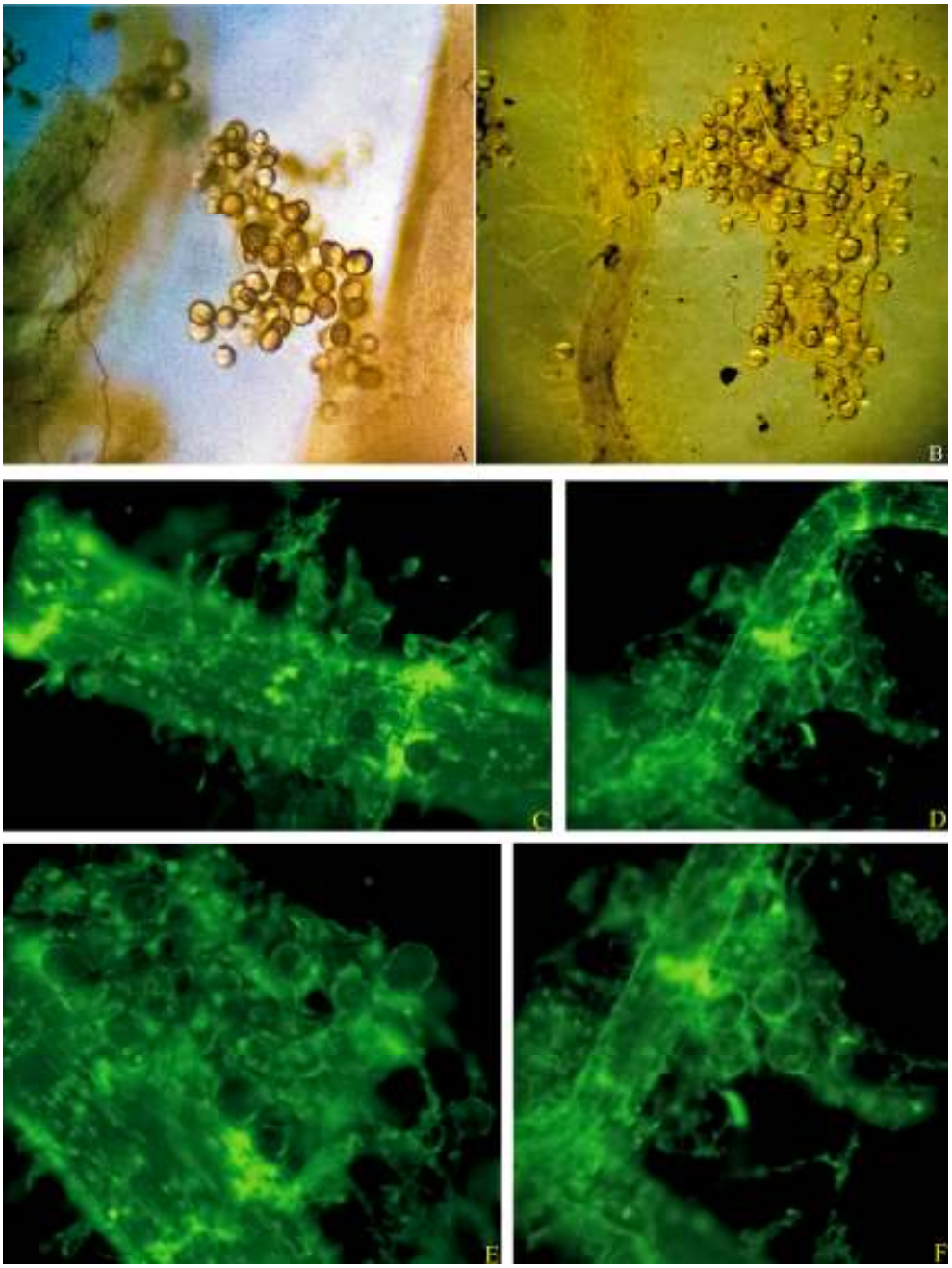

Fig.43. Spore mass closely adhered to maize root as seen under compound microscope bright field (A&B), Indirect-immunofluorescent labelling of AMF colonized maize root tissue treated with PAb of *G. mosseae* and reacted with FITC (C-F)

4.13. Effects of AMF and PGPR application on growth of tea saplings

4.13.1. *Glomus mosseae* and *Bacillus pumilus*

4.13.1.1. Growth enhancement

Four tea varieties (TV-25, TV-26, TV-9, and TV-20) were selected to test the effect of PGPR (*B. pumilus*) and AMF (*G. mosseae*) 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 that enhancement of plant growth by single as well as joint application of PGPR and AMF however the results reveals that the growth of tea saplings grown under same environmental and physical conditions were enhanced to a greater extent when both *B. pumilus* and *G. mosseae* were applied jointly. Among all the four varieties tested, maximum growth was noticed in TV 20 and TV 26 (Table 24, Fig. 44). Statistical analysis (ANOVA) revealed that the decrease in soil P and increase in root and leaf P- content were significantly enhanced when both *G. mosseae* and *B. pumilus* were applied jointly (Table 24a,b,c &d).

4.13.1.2. Total phosphate content of soil, root and leaf

The growth enhancement in tea saplings in nursery conditions was also evaluated in terms of total phosphate mobilized by *B. pumilus* and *G. mosseae*. Total residual phosphate content in soil, roots and leaves were evaluated. The overall results reveal that the total soil P-content decreased while the root and leaf phosphate increased following application of *B. pumilus* and *G. mosseae* singly or jointly which indicated efficient soil phosphate mobilization by both PGPR and AMF (Table 25). Statistical analysis (ANOVA) revealed that the decrease in soil P and increase in root and leaf P- content were significantly enhanced when both *G. mosseae* and *B. pumilus* were applied jointly (Table 25a,b,c).

4.13.1.3. Acid and alkaline phosphatase activities of soil

Alkaline phosphatase activities were found to be much more lesser than the acid phosphatase activities of the rhizosphere soil. There was no any significant difference among the varieties however, there was a significant (P=0.5) difference between the treated and control sets (Table 26)**.** Statistical analysis (ANOVA) revealed that the increase in both the acid and alkaline phosphatase activities were significantly enhanced when both *G. mosseae* and *B. pumilus* were applied jointly (Table 26a, b).

Experimental plot in field condition

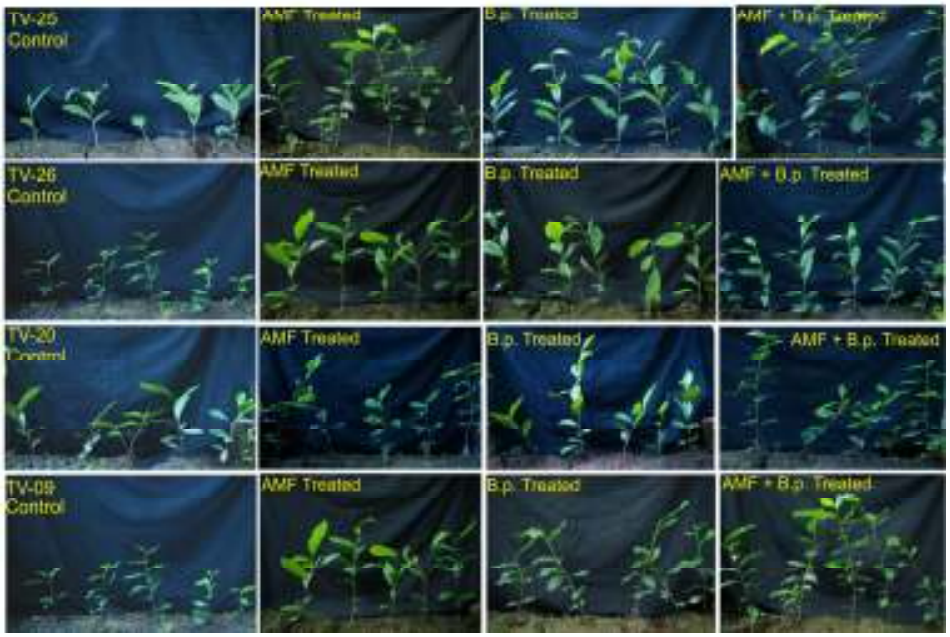

Fig. 44. Effect of *G. mosseae* and *B. pumilus* on growth of tea saplings in nursery condition

Table 24. Effect of *G. mosseae* and *B. pumilus* on growth of tea saplings in nursery conditions

Tea Varieties	Treatments	% increase in height		% increase in leaf no.	
		1 month after treatment	2 months after treatment	1 month after treatment	2 months after treatment
TV-25	Control	8.69±0.58	9.20±1.15	9.00±1.15	10.0±1.73
	G.m	14.0±0.57	15.1±0.58	13.3±0.58	14.0±1.15
	B.p	20.0±1.73	21.0±0.58	24.1±2.30	25.1±0.58
	G.m + B.p	25.0±0.58	32.0±1.44	32.0±0.63	35.0±0.80
TV-26	Control	8.00±0.57	9.10±1.73	18.1±0.83	19.2±0.28
	G.m	35.8±1.44	36.0±1.32	22.2±1.73	23.0±1.15
	B.p	21.5±0.33	24.2±0.58	26.0±0.57	27.0±0.58
	G.m + B.p	37.0±1.27	42.0±1.73	29.0±0.28	34.0±0.63
TV-09	Control	15.3±0.58	17.0±0.33	46.1±0.58	46.2±0.57
	G.m	25.8±0.57	25.8±0.58	47.6±0.69	48.0±0.58
	B.p	20.5±1.73	21.1±0.33	44.4±0.75	44.5±0.80
	G.m + B.p	28.0±1.15	29.5±1.21	49.0±0.80	51.5±1.15
TV-20	Control	15.0±0.57	15.5±0.57	15.3±1.21	16.0±1.44
	G.m	44.8±1.15	45.0±1.73	38.8±1.15	36.5±1.73
	B.p	42.5±1.15	48.0±0.57	27.7±0.57	28.2±0.33
	G.m + B.p	43.6±0.83	49.0±0.80	39.9±0.58	38.8±0.63
CD=(0.05)	Treatments	9.74	10.73	9.64	8.75
	Varieties	9.74	10.73	9.64	8.75

G.m=*Glomus mosseae*, B.p= *Bacillus pumilus*. Values are average three replicate sets; ±= Standard Error

Table 24a. ANOVA of data presented in table 24 (% increase in height, 1 month)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1091.974	3	363.9913	9.804821	0.003391	3.862548
Columns	814.1653	3	271.3884	7.310382	0.008727	3.862548
Error	334.1133	9	37.1237			
Total	2240.252	15				

Table 24b. ANOVA of data presented in table 24 (% increase in height, 2 month)

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	1367.732	3	455.9106	10.11834	0.00305	3.862548
Columns	900.6819	3	300.2273	6.663152	0.01156	3.862548
Error	405.5206	9	45.05785			
Total	2673.934	15				

Table 24c. ANOVA of data presented in table 24(% increaseleaf number,1 month)						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	473.2869	3	157.7623	4.334981	0.037723	3.862548
Columns	1711.097	3	570.3656	15.67247	0.000644	3.862548
Error	327.5356	9	36.39285			
Total	2511.919	15				

Table 24d. ANOVA of data presented in table 24 (% increaseleaf number,1 month)						
Source of Variation	***SS***	***df***	***MS***	***F***	***P-value***	***F crit***
Rows	**578.8725**	**3**	**192.9575**	**6.436386**	**0.01281**	**3.862548**
Columns	**1606.773**	**3**	**535.5908**	**17.86543**	**0.000394**	**3.862548**
Error	**269.8125**	**9**	**29.97917**			
Total	**2455.458**	**15**				

Table 25. Effect of *G. mosseae* and *B. pumilus* on Total Phosphate content of tea saplings in nursery condition

Tea varieties	Treatments	Total phosphate content (µg/g tissue)		
		Soil	Root	Leaf
TV-25	Control	39.64±0.75	35.0±1.3	179.0±1.2
	B.pumilus	29.02±1.15	67.0±1.1	267.7±1.8
	Glomus mosseae	28.42±1.73	75.0±1.8	277.0±1.4
	Glomus mosseae + *B.pumilus*	27.00±0.98	76.0±1.9	297.0±1.3
TV-26	Control	26.18±0.93	39.8±2.0	176.0±1.5
	B.pumilus	18.55±0.63	65.2±1.7	277.2±1.4
	Glomus mosseae	19.45±0.33	77.0±1.8	279.8±1.8
	Glomus mosseae + *B.pumilus*	17.70±0.86	81.0±1.5	281.0±2.4
TV-9	Control	92.76±1.73	41.8±1.2	185.0±1.7
	B.pumilus	72.11±1.15	66.0±1.6	265.7±1.6
	Glomus mosseae	72.41±1.90	69.0±1.3	272.2±1.1
	Glomus mosseae + *B.pumilus*	70.11±1.32	71.2±1.8	278.0±1.3
TV-20	Control	46.38±0.92	32.0±1.1	178.5±1.7
	B.pumilus	32.40±0.75	69.0±1.7	273.8±1.1
	Glomus mosseae	31.42±0.80	77.5±1.5	269.0±1.1
	Glomus mosseae + *B.pumilus*	30.12±1.15	78.0±1.9	272.5±1.7
CD (P=0.05)	Treatments	4.66	6.18	10.91
	Varieties	4.66	6.18	10.91

Values are average three replicate sets; ±= Standard Error

Table 25a. ANOVA of the data presented in table 25 Soil Phosphate content- µg/g tissue						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	583.3514188	3	194.4505	22.88141	0.000151	3.862548
Columns	7364.344519	3	2454.782	288.8595	2.92E-09	3.862548
Error	76.48365625	9	8.498184			
Total	8024.179594	15				

Table 25b. ANOVA of the data presented in table 25 Root Phosphate content- µg/g tissue						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	3995.857	3	1331.952	89.06691	5.19E-07	3.862548
Columns	29.79688	3	9.932292	0.664167	0.594727	3.862548
Error	134.5906	9	14.95451			
Total	4160.244	15				

Table 25c. ANOVA of the data presented in table 25 Leaf Phosphate content- µg/g tissue						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	28066.44	3	9355.481	200.9231	1.46E-08	3.862548
Columns	111.9125	3	37.30417	0.801163	0.523899	3.862548
Error	419.0625	9	46.5625			
Total	28597.42	15				

Table 26. Acid and Alkaline Phosphatase activities (µg pNPP/g/h)			
Tea varieties	**Treatments**	**Acid Phosphatase**	**Alkaline phosphatase**
TV-25	Control	61.0±1.15	19.0±1.15
	B.pumilus	74.3±0.98	26.5±1.73
	Glomus mosseae	77.2±1.73	29.0±0.58
	Glomus mosseae + *B.pumilus*	88.5±1.15	37.0±0.92
TV-26	Control	59.0±0.57	15.5±1.73
	B.pumilus	76.5±0.63	29.4±0.57
	Glomus mosseae	79.0±0.69	32.6±1.15
	Glomus mosseae + *B.pumilus*	92.5±0.57	36.5±1.32
TV-9	Control	63.5±1.73	17.0±1.09
	B.pumilus	88.5±1.15	29.0±0.75
	Glomus mosseae	83.0±0.58	21.5±0.33
	Glomus mosseae + *B.pumilus*	95.0±1.73	38.0±1.21
TV-20	Control	61.0±1.32	15.5±0.92
	B.pumilus	86.0±0.92	28.0±0.58
	Glomus mosseae	81.0±0.57	21.5±1.15
	Glomus mosseae + *B.pumilus*	94.3±0.69	35.5±0.57
CD (P=0.05)	Treatments	4.32	4.82
	Varieties	4.32	4.82

Table 26a. ANOVA of the data presented in table 26, Acid phosphatase						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	2040.362	3	680.1206	92.90472	4.32E-07	3.862548
Columns	134.5669	3	44.85563	6.127294	0.014792	3.862548
Error	65.88562	9	7.320625			
Total	2240.814	15				

Table 26b. ANOVA of the data presented in table 26, Alkaline phosphatase						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	809.3769	3	269.7923	29.68171	5.35E-05	3.862548
Columns	27.67188	3	9.223958	1.014791	0.430436	3.862548
Error	81.80563	9	9.089514			
Total	918.8544	15				

4.13.1.3. N, P and K contents of soil

Among all the tested tea varieties, effect of both *G. mosseae* ad *B. pumilus* was maximum in the variety TV-20. The effect of AMF and PGPR inoculation in this variety was measured in terms of availability of chief macro nutrients like Nitrogen (N), Phosphorus (P) as well as Potassium (K) in the rhizosphere soil even after three months of inoculation. Oxidizable organic carbon percentage (OC %) and Moisture percentage were also evaluated. Results presented in Table 27 suggest that there was a significant increase in N, P & K concentration of the soil of TV-20 in comparison to the control. It was observed that the OC% was much higher in soil treated only with *G. mosseae* followed by joint application and *B. pumilus*.

4.13.1.4. Effect of *G. mosseae* and *B. pumilus* on tea catechins

Catechins were analysed in HPLC from leaves of variety TV-20 that showed maximum response towards AMF and PGPR whose rhizosphere was soil drenched with *Bacillus pumilus* and roots were inoculated with spores of *G. mosseae*. Analysis revealed that a few isomers were observed by the treatments, a few new ones developed and few were lost. Few were lost or there was suppression of few isomers by the treatment of *B. pumilus* and AMF alone in comparison to healthy plants. In TV-20 variety, in untreated control two isomers- gallo catechin (GC) and gallo catechin gallate (GCG) with retention times of 4.59 min and 13.20 min, whereas, in *B.pumilus* treatment, one isomer-gallocatechin (GC) with retention time of 4.59 min were detected. However, no major loss of isomers were noted due to treatments indicating that flavor components were not lost (Fig. 45; Tables 28-31).

Table 27. N, P and K contents of soil following treatment with AMF and PGPR

Variety : TV-20	pH	OC % (oxidizable organic carbon)	Total N kg/ha	N kg/ha	P kg/ha	K kg/ha	Moisture % (Dry wt, basis)
Untreated Healthy	6.03	3.55	1075.2	232.06	38.83	163.11	27.02
AMF	5.51	3.86	1008	200.70	32.11	108.74	26.15
BP	5.5	3.64	1008	181.89	32.41	108.74	25.51
AMF/BP	5.87	3.74	907.2	206.98	32.41	152.23	26.98

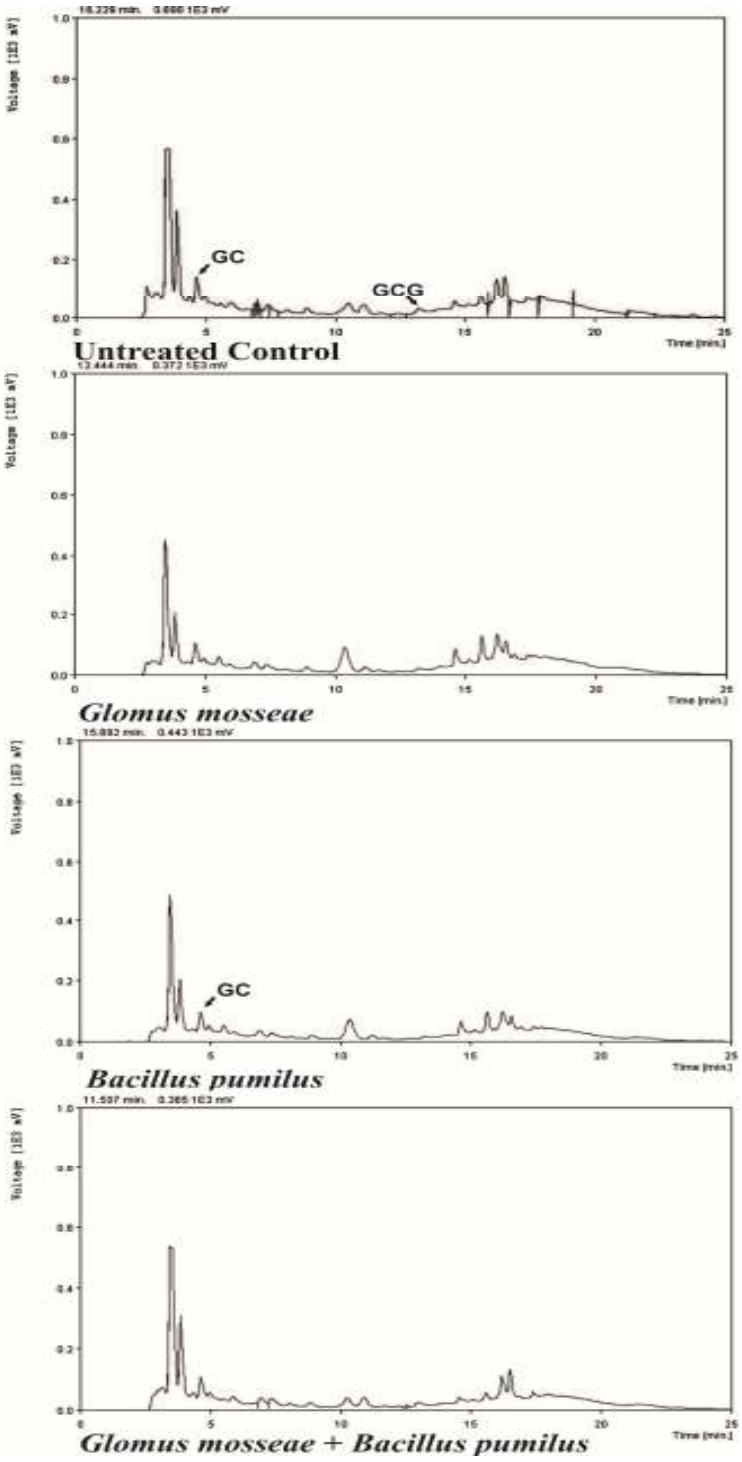

Fig. 45. HPLC profiles of catechins obtained from tea leaves (Variety- TV-20) showing different peaks of gallo catechin (GC) and gallo catechin gallate (GCG) in control, AMF and PGPR treated plants

Table 28. Peak result of HPLC analysis of catechin extracts from leaves of untreated control (cv. TV20)		
Peak no	**Retn time (min)**	**Height(mV)**
1	2.52	0.09
2	2.70	0.06
3	3.00	0.55
4	3.52	0.35
5	4.59	0.145
6	6.20	0.035
7	8.50	0.025
8	10.56	0.050
9	11.20	0.049
10	13.20	0.035
11	14.80	0.065
12	15.50	0.070
13	16.10	0.135
14	16.30	0.145

Table 29: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Bacillus pumilus* (cv. TV20)		
Peak no	**Retn time (min)**	**Height(mV)**
1	2.71	0.049
2	3.30	0.470
3	3.70	0.180
4	4.59	0.080
5	5.80	0.050
6	7.32	0.030
7	10.31	0.070
8	11.50	0.021
9	14.81	0.060
10	15.50	0.081
11	16.10	0.070
12	16.40	0.079

Table 30. Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with AMF (cv. TV20)		
Peak no	**Retn time (min)**	**Height(mV)**
1	2.90	0.050
2	3.55	0.450
3	3.75	0.180
4	4.70	0.080
5	5.90	0.065
6	7.30	0.049
7	7.90	0.030
8	8.76	0.022
9	10.50	0.10
10	11.25	0.095
11	14.80	0.12
12	15.81	0.135
13	16.25	0.136
14	16.50	0.11

Table 31. Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with AMF and *Bacillus pumilus* (cv. TV20)		
Peak no	**Retn time (min)**	**Height(mV)**
1	3.00	0.065
2	3.30	0.520
3	3.75	0.30
4	4.75	0.10
5	7.10	0.040
6	7.50	0.039
7	10.20	0.049
8	11.25	0.050
9	15.50	0.065
10	16.25	0.10
11	16.60	0.13

4.13.2. *Glomus fasciculatum and Bacillus amyloliquefaciens*

4.13.2.1. Growth enhancement

In another set of experiments with AMF and PGPR growth promotion of tea saplings by another dominant AMF of tea, *Glomus fasciculatum* and PGPR- *B. amyloliquefacien**s*** was tested in six tea varieties (TV-18, T-17, AV-2, T-78, UP-3 and UP-26) in pot conditions and growth enhancement was evaluated in terms of percent increase in height and leaf number over similar increase in control plants. A significant increase in growth and number of leaves in all the tea varieties treated with *G. fasciculatum* and B. *amyloliquefaciens* was observed. In single treatments *B. amyloliquefaciens,* showed better growth than *G. fasciculatum.* However growth enhancement was significantly higher when both the AMF and PGPR were applied jointly (Fig. 46).

4.13.2.2. Total phosphate content of soil, root and leaf

The growth enhancement in tea saplings in pot conditions was also evaluated in terms of total phosphate mobilized by *Glomus fasciculatum* and *B. amyloliquefacien**s*** in pot conditions. Total residual phosphate content in soil, roots and leaves were evaluated. The overall resuts reveal that the total soil P-content decreased whilke the root and leaf phosphate increased following application of *B. pumilus* and *G. mosseae* singly or jointly which indicated efficient soil phosphate mobilization by both PGPR and AMF (Table 32). Statistical analysis (ANOVA) revealed that the decrease in soil P and increase in root and leaf P- content were significantly enhanced when both *Glomus fasciculatum* and *B. amyloliquefacien**s*** were applied jointly (Table 32a,b,c).

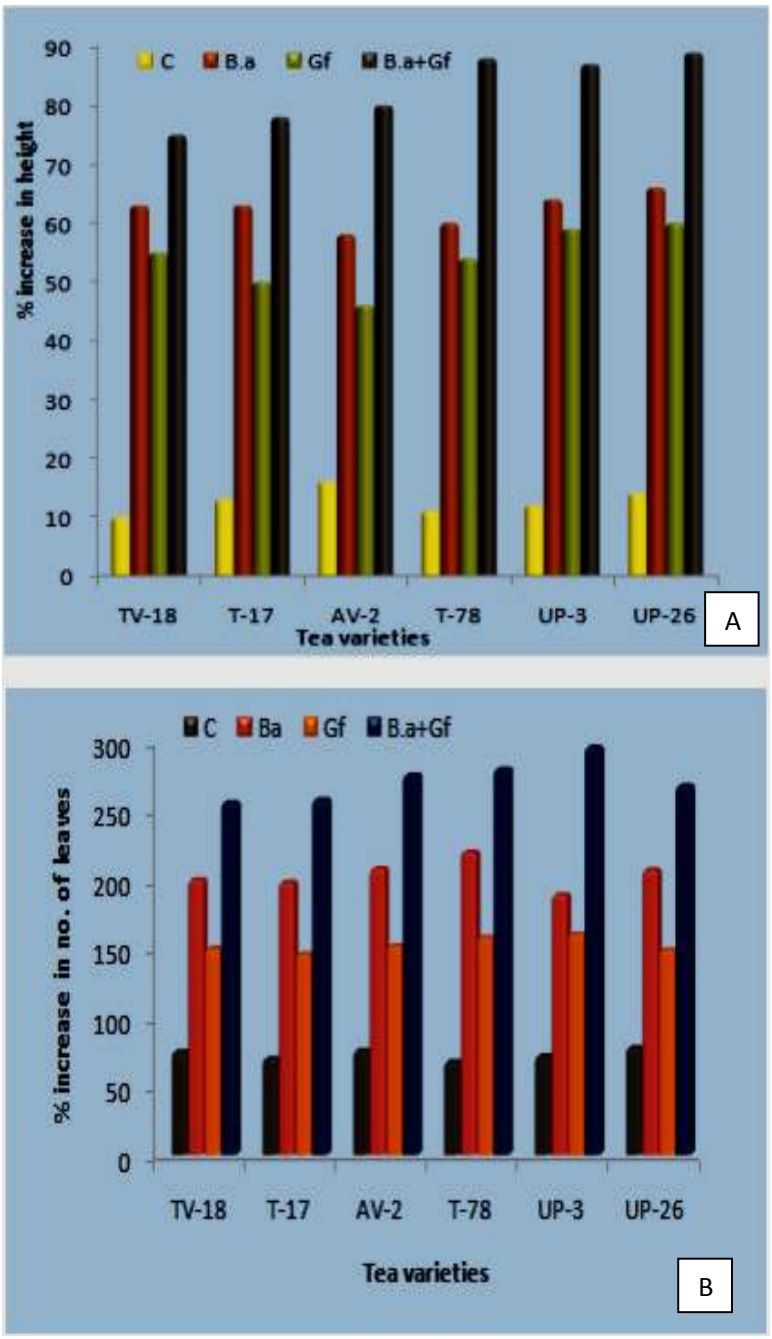

Fig 46. Effect of *Glomus fasciculatum* and *B. amyloliquefaciens* on growth of tea saplings in pot condition measured in terms of percent increase in height (A) and leaves (B)

Table 32. Effect of *G. fasciculatum* and *B. amyloliquefaciens* on Total Phosphate content of tea saplings in pot condition				
Tea varieties	**Treatment**	**Total phosphate content (µg/g tissue)**		
		Soil	**Root**	**Leaf**
TV-18	Control	41.0	115.0	202.0
	G. fasciculatum	26.5	210.0	271.0
	B. amyloliquefaciens	28.5	213.0	268.0
	B.amyloliquefaciens +G. fasciculatum	24.0	216.0	269.0
T-17	Control	39.5	110.0	199.0
	G. fasciculatum	23.5	215.5	270.0
	B. amyloliquefaciens	25.0	217.0	266.0
	B.amyloliquefaciens +G. fasciculatum	22.0	221.0	273.0
T-78	Control	43.0	109.0	192.0
	G. fasciculatum	31.0	218.0	273.0
	B. amyloliquefaciens	32.0	219.0	265.0
	B.amyloliquefaciens +G. fasciculatum	30.0	221.0	279.0
UP-3	Control	45.5	108.0	187.0
	G. fasciculatum	32.0	212.0	271.0
	B. amyloliquefaciens	31.0	219.0	267.0
	B.amyloliquefaciens +G. fasciculatum	28.5	227.0	278.0
UP-20	Control	42.5	115.0	189.0
	G. fasciculatum	33.0	223.0	268.0
	B. amyloliquefaciens	31.0	226.0	269.0
	B.amyloliquefaciens +G. fasciculatum	25.5	228.0	272.0
AV-2	Control	43.0	117.0	195.0
	G. fasciculatum	34.0	226.5	287.5
	B. amyloliquefaciens	31.0	228.5	281.0
	B.amyloliquefaciens +G. fasciculatum	29.0	235.0	295.0
CD (P=0.05)	(Treatments)	1.80	3.99	6.47
	(Varieties)	2.20	4.89	7.93

Table 32a. ANOVA of data presented in table 32(Root phosphate content- µg/g tissue)						
ANOVA						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	53158.11	3	17719.37	1681.443	3.55E-19	3.287382
Columns	507.8021	5	101.5604	9.637364	0.000283	2.901295
Error	158.0729	15	10.53819			
Total	53823.99	23				

Table 32c. ANOVA of data presented in table 32 (Leaf phosphate content- µg/g tissue)						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	28629.61	3	9543.205	344.4596	4.73E-14	3.287382
Columns	608.3021	5	121.6604	4.391302	0.011583	2.901295
Error	415.5729	15	27.70486			
Total	29653.49	23				

Table 32b. ANOVA of data presented in table 32 (Soil phosphate content- µg/g tissue)						
Source of Variation	SS	df	MS	F	P-value	F crit
Rows	886.25	3	295.4167	137.4031	3.93E-11	3.287382
Columns	156.8333	5	31.36667	14.58915	2.64E-05	2.901295
Error	32.25	15	2.15			
Total	1075.333	23				

4.14. Management of seedling blight of tea by AMF and PGPR

4.14.1. Glass house condition

4.14.1.1. Disease development

In the first set of trial, *G. fasciculatum* and *B. amyloliquefaciens* were tested for their effect in inhibiting sclerotial blight of tea saplings in pot conditions in glass house when applied singly and in combination. The experiment was set in complete random block design and both AMF and PGPR were inoculated to the rhizosphere of three year old tea plants prior to pathogen challenge. Under pot condition, *G. fasciculatum* and *B. amyloliquefaciens* alone could effectively reduce disease incidence. However combined

inoculation with *G. fasciculatum* and *B. amyloliquefaciens* showed better results (Table 33).

4.14.1.2. Biochemical Changes associated with induction of resistance in tea plants

Application of *G. fasciculatum* and *B. amyloliquefaciens* prior to pathogen challenge in tea saplings in nursery conditions was found to elicit a series of biochemical responses in tea plants. Experiments were conducted to assess the effect of single as well as combined application of *G. fasciculatum* and *B. amyloliquefaciens* on biochemical components of tea leaves. Multifold increase in activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in roots as well as leaf of tea plants was observed on application of AMF and PGPR to soil followed by inoculation with *S. rolfsii***.** Overall results show that the defense enzyme activities were higher in leaves than the roots. (Fig.47). Apart from this the total phenol content in the roots and leaves were also evaluated which was found to be higher in the leaves than the roots (Fig. 48).

Table. 33. Disease index of sclerotial blight incidence of tea saplings following AMF and PGPR treatment and pathogen challenge in pot condition

Tea varieties	Disease index (45 days after inoculation) Pre treated with*			
	None	*G.f*	*B.a*	*G.f +B.a*
TV-18	4.1	1.3	3.2	1.9
T-17	5.9	1.9	4.4	2.7
AV-2	3.5	1.1	3.0	1.6
T-78	4.0	2.0	2.9	2.1
UP-3	4.0	1.9	2.8	2.2
UP-26	5.4	2.4	3.4	2.5
C.D.(P=0.05) (Treatments)				0.486
(Varieties)				0.595

10 pots/treatment;**B.a = Bacillus amyloliquefaciens*; *G.f= Glomus fasciculatum*. Disease Index computed on a scale of 0-6 on the basis of underground and above ground symptoms. Disease intensity was assessed as rot index on a scale of 0-6, depending on both underground and above ground symptoms as follows: 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.

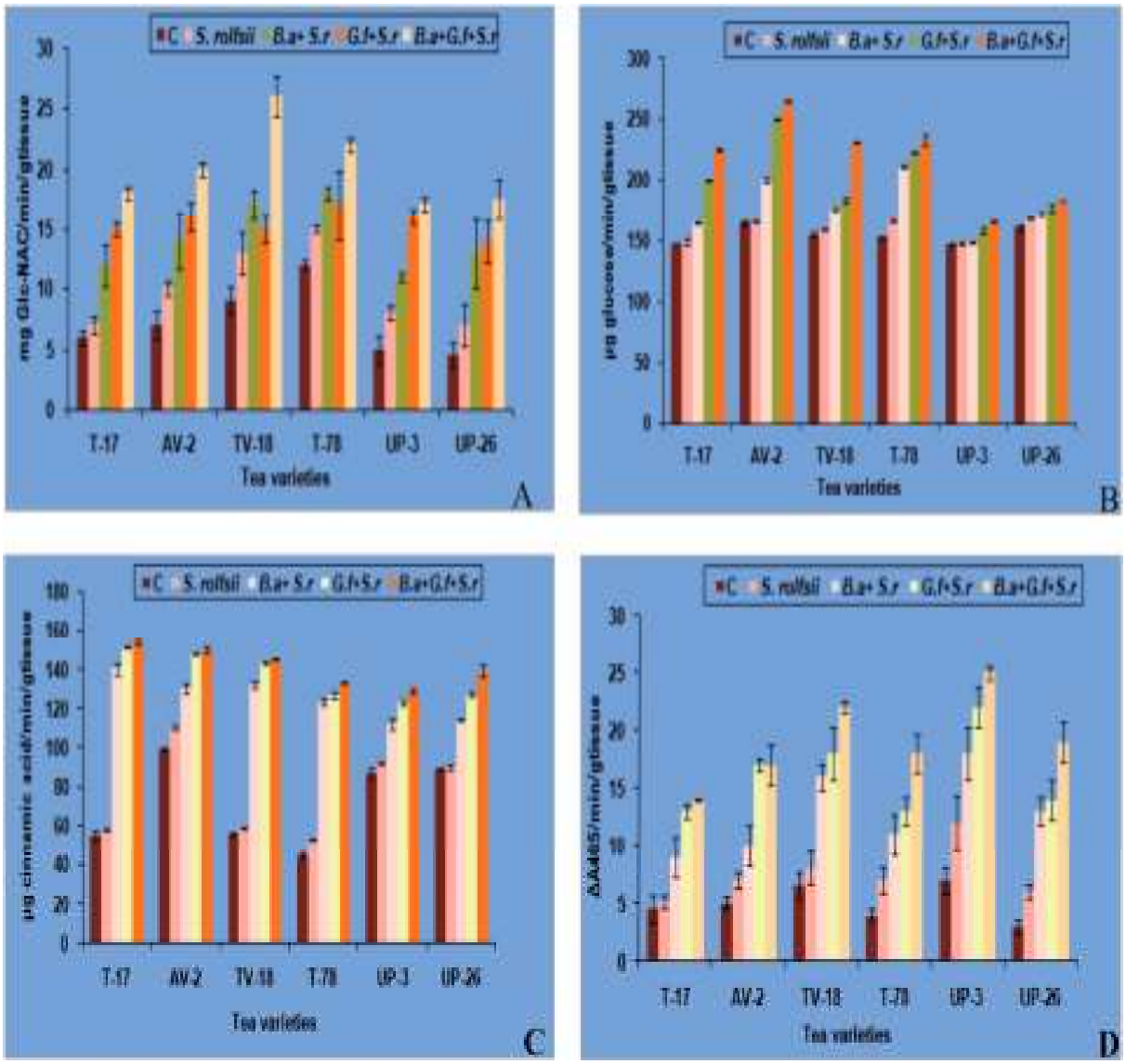

Fig. 47. Chitinase (A), Glucanase (B), Peroxidase (C) and PAL (D) activities in AMF and PGPR treated tea varieties following inoculation with *S. rolfsii*

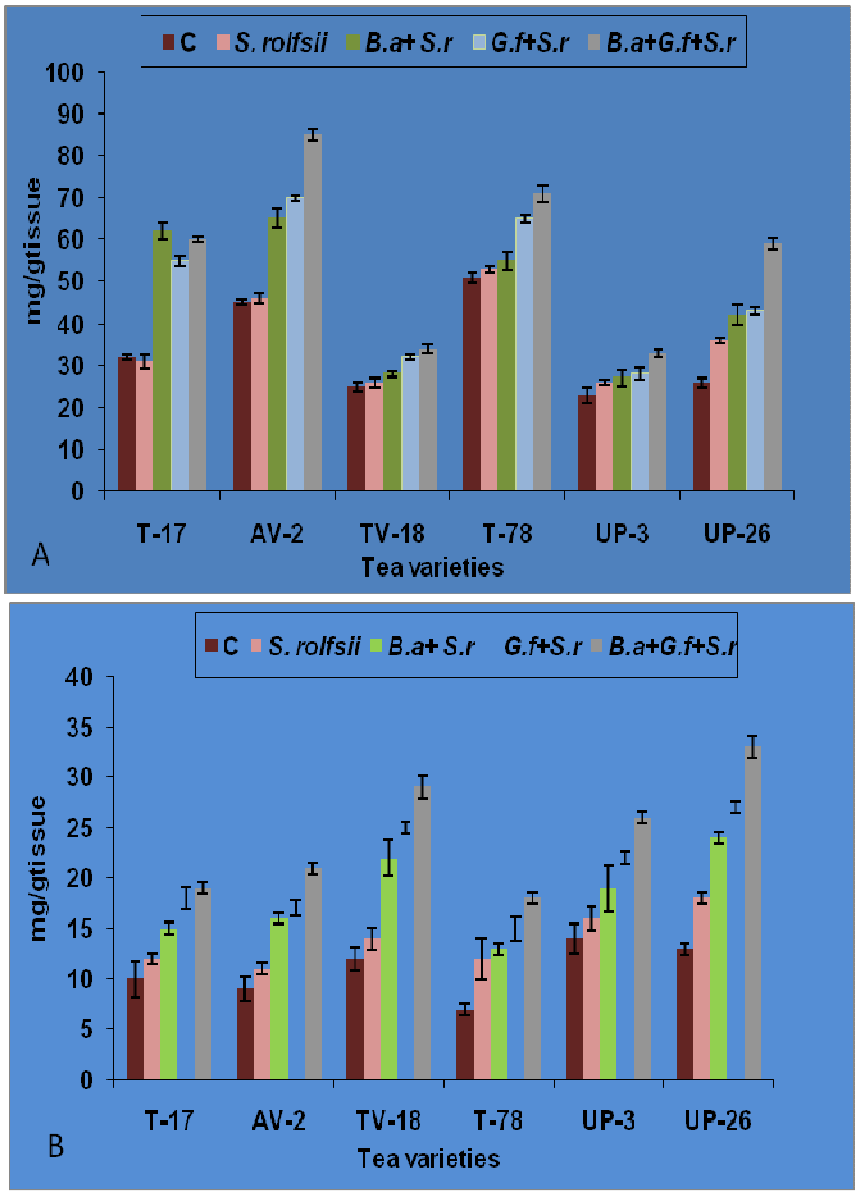

Fig.48. Total phenol (A) and Orthodihydroxy phenol (B) content in AMF and PGPR treated tea varieties following inoculation with *S. rolfsii*

4.14.2. Nursery condition

4.14.2.1. Disease development

Effects of AMF and PGPR in management of sclerotial blight disease of tea were tested in pot as well as nursery conditions. In the first set of trial *Glomus mosseae* and *Bacillus pumilus* were tested for their effect in inhibiting sclerotial blight of tea saplings in nursery conditions when applied singly and in combination. Under nursery condition, *G. mosseae* and *B. pumilus* alone could effectively reduce disease incidence. However combined inoculation with *G. mosseae* and *B. pumilus* showed 20% disease incidence recorded after 45 days of pathogen challenge (Table 34. Fig. 49).

Table. 34. Disease index of sclerotial blight incidence of tea saplings following AMF and PGPR treatment and pathogen challenge in nursery condition

Tea varieties	**Disease index (45 days after inoculation) Pre treated with***			
	Sclerotium rolfsii	***S. rolfsii* + *G. mosseae***	***S. rolfsii* + *B. pumilus***	***S. rolfsii*+ *G. mosseae*+ *B. pumilus***
TV-25	4.18±0.83	2.15±0.55	1.60±0.47	1.30±0.36
TV-26	5.90±0.77	2.18±0.58	1.85±0.38	1.25±0.32
TV-9	4.55±0.72	2.50±0.43	1.75±0.33	1.15±0.43
TV-20	5.25±0.65	1.80±0.47	1.50±0.44	1.20±0.37

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

4.14.2.2. Biochemical Changes associated with induction of resistance in tea plants

Experiments were conducted to assess the effect of single as well as combined application of *G. mosseae* and *B. pumilus* on biochemical components of tea leaves. Activities of some of the enzymes which are involved in phenol metabolism as well as in defense-i.e., peroxidase, phenylalanine ammonia lyase, chitinase, β-1, 3 glucanase were also determined. Polyphenols are major constituents of tea leaves and hence phenol contents were determined. Multifold increase in activities of chitinase, β-1, 3-glucanase, peroxidase and phenylalanine ammonia lyase in roots were observed on application of

AMF and PGPR to soil followed by inoculation with *S. rolfsii* which was significantly higher in those plants which were jointly inoculated with AMF and PGPR (Table 35)**.** Apart from this the total phenol content in the roots and leaves were also evaluated and was found to be higher in plants treated jointly with *G. mosseae* and *B. pumilus* (Table 36)**.** Similarly, catechins were also analysed from leaves of tea plants treated with *S. rolfsii*, *S. rolfsii* + *B. pumilus*, *S. rolfsii* + *G. mosseae* and *S. rolfsii*+ *B. pumilus*+ *G. mosseae*. There was suppression of few isomers in joint treatments in comparison to individual treatment by *S. rolfsii*. In *S. rolfsii* treatment, gallo-catechin (GC) with retention time of 4.60 was predicted as one isomer of catechins. However, no major losses of isomers were noted due to treatments indicating that flavour components were not lost (Table 37-40).

Table. 35. Changes in the level of defense enzyme activities in tea root grown in soil amended with AMF and PGPR and inoculation with *S. rolfsii*

Tea Varieties	Treatment	Enzyme activities			
		POX	PAL	CHT	GLU
TV-25	Control	3.8	62.0	12.5	320
	S. rolfsii	4.5	89.0	18.5	423
	G. mosseae + *S. rolfsii*	6.5	96.0	20.1	471
	B. pumilus + *S. rolfsii*	8.5	146.0	26.0	535
	G.moseeae +*B. pumilus* + *S. rolfsii*	9.0	152.0	29.0	546
TV-26	Control	3.3	65.0	16.4	330
	S. rolfsii	4.6	75.0	17.8	482
	G. mosseae + *S. rolfsii*	5.0	88.0	22.5	549
	B. pumilus + *S. rolfsii*	6.7	132.0	31.5	614
	G.moseeae +*B. pumilus* + *S. rolfsii*	8.9	149.0	34.0	619
TV-9	Control	4.5	84.0	18.3	450
	S. rolfsii	5.6	97.0	25.5	460
	G. mosseae + *S. rolfsii*	6.7	119.0	31.5	512
	B. pumilus + *S. rolfsii*	7.9	133.0	35.5	532
	G.moseeae +*B. pumilus* + *S. rolfsii*	8.7	151.0	39.0	565
TV-20	Control	3.5	78.0	17.5	420
	S. rolfsii	6.6	98.0	27.5	470
	G. mosseae + *S. rolfsii*	7.7	131.0	27.0	522
	B. pumilus + *S. rolfsii*	8.0	142.0	33.3	537
	G.moseeae +*B. pumilus* + *S. rolfsii*	9.0	154.0	39.8	543
CD (P=0.05)	Treatments	0.983	13.80	2.90	56.92
	Varieties	0.879	12.31	2.60	50.91

POX activity assayed as Δ A 465 min^{-1} g $tissue^{-1}$; PAL activity assayed as µg N-Acetyl glucosamine released by enzyme from 1 g tissue min^{-1} , Chitinase activity assayed as mg Glc-Nac / gm tissue/min and ß 1,3- GLU activity assayed as µg glucose released by enzyme from 1g tissue min^{-1}

Table 35a. ANOVA of data presented in table 34(POX activity)						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	64.72	4	16.18	39.68929	7.92E-07	3.259167
Columns	4.378	3	1.459333	3.579722	0.046798	3.490295
Error	4.892	12	0.407667			
Total	73.99	19				

Table 35b. ANOVA of data presented in table 34(PAL activity)						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	17346.7	4	4336.675	53.97791	1.42E-07	3.259167
Columns	1050.15	3	350.05	4.357017	0.027047	3.490295
Error	964.1	12	80.34167			
Total	19360.95	19				

Table 35c. ANOVA of data presented in table 34(CHT activity)						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	923.268	4	230.817	64.72715	5.09E-08	3.259167
Columns	249.908	3	83.30267	23.36025	2.68E-05	3.490295
Error	42.792	12	3.566			
Total	1215.968	19				

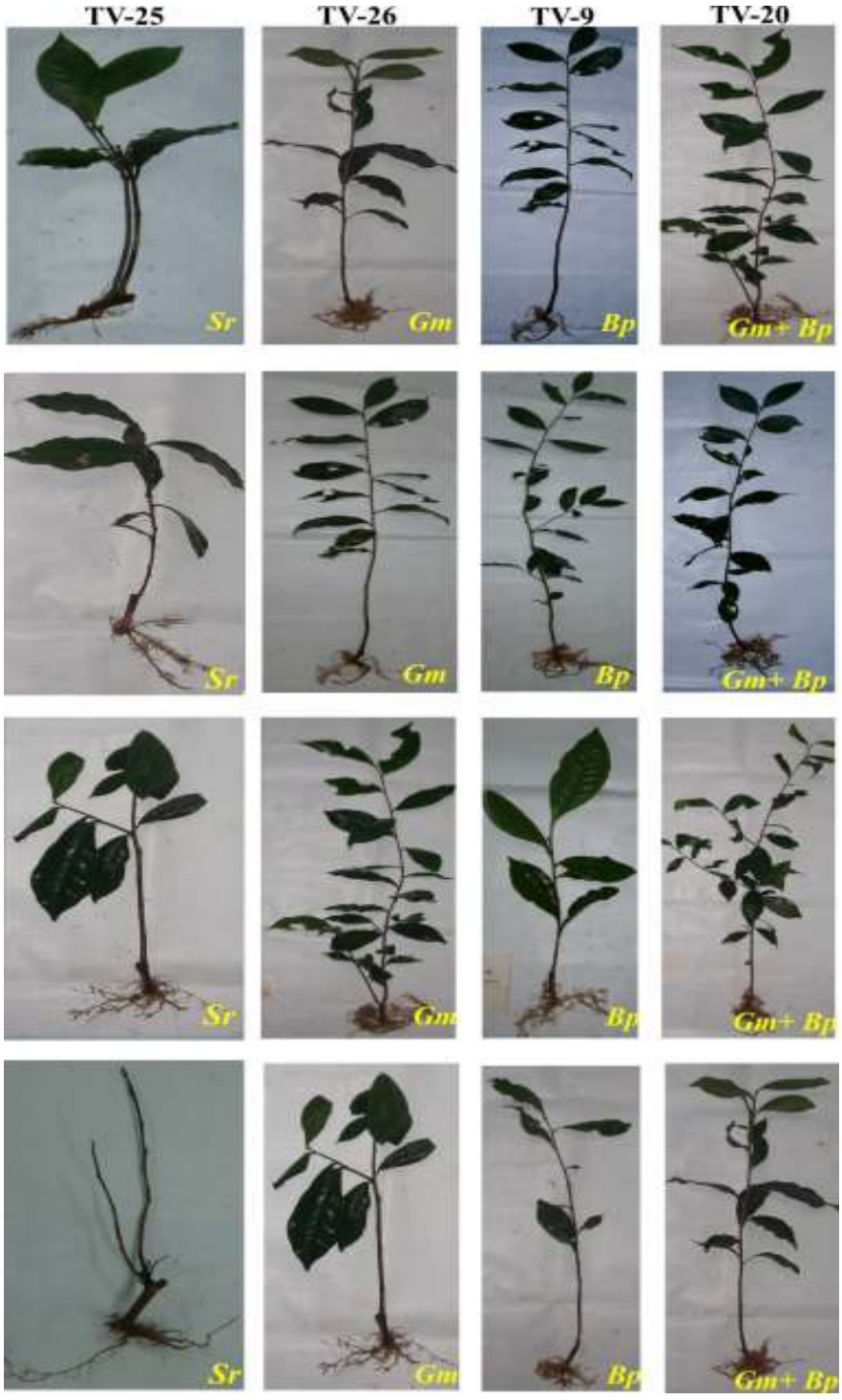

Fig 49. Effect of *Glomus mosseae* and *B. pumilus* on sclerotial blight disease development of four different varieties of tea saplings in nursery conditions.

Table. 36. Total Phenol content in tea root grown in soil amended with AMF and PGPR and inoculation with *S. rolfsii*

Tea Varieties	Treatment	Total phenol content	
		Root	Leaf
TV-25	Control	5.32±1.77	0.57±0.055
	S. rolfsii	7.88±1.16	0.96±0.038
	G. mosseae + *S. rolfsii*	10.1±0.93	1.44±0.073
	B. pumilus + *S. rolfsii*	10.7±0.92	1.46±0.074
	G.moseeae +*B. pumilus* + *S. rolfsii*	11.4±1.33	1.88±0.061
TV-26	Control	6.15±0.43	0.52±0.044
	S. rolfsii	8.12±0.45	0.98±0.045
	G. mosseae + *S. rolfsii*	9.90±0.33	1.23±0.042
	B. pumilus + *S. rolfsii*	10.8±0.32	1.38±0.043
	G.moseeae +*B. pumilus* + *S. rolfsii*	12.5±0.46	1.78±0.036
TV-9	Control	6.33±0.73	0.56±0.077
	S. rolfsii	7.23±0.88	0.88±0.082
	G. mosseae + *S. rolfsii*	8.89±0.74	1.43±0.054
	B. pumilus + *S. rolfsii*	10.3±0.76	1.54±0.055
	G.moseeae +*B. pumilus* + *S. rolfsii*	12.5±0.88	1.77±0.059
TV-20	Control	4.58±0.88	0.43±0.063
	S. rolfsii	6.27±0.76	1.14±0.078
	G. mosseae + *S. rolfsii*	8.43±0.93	1.28±0.077
	B. pumilus + *S. rolfsii*	10.1±0.94	1.46±0.072
	G.moseeae +*B. pumilus* + *S. rolfsii*	13.2±0.73	1.84±0.064
CD (P=0.05)	Treatments Varieties	1.04 0.93	0.128 0.115

Total phenol content assayed as mg/g tissue; Average of three replicate experiments; ±= SE.

Table 36a. ANOVA of data presented in table 34(Total phenol content in root)						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	112.2922	4	28.07305	61.20978	6.99E-08	3.259167
Columns	2.41146	3	0.80382	1.752629	0.209585	3.490295
Error	5.50364	12	0.458637			
Total	120.2073	19				

Table 36b. ANOVA of data presented in table 34(Total phenol content in leaf)						
Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Rows	3.89168	4	0.97292	139.3203	6.03E-10	3.259167
Columns	0.018575	3	0.006192	0.886635	0.475721	3.490295
Error	0.0838	12	0.006983			
Total	3.994055	19				

Table 37. Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *S. rolfsii* (cv. TV20)

Peak no	Retn time (min)	Height(mV)
1	2.60	0.020
2	3.50	0.230
3	3.75	0.070
4	4.60	0.045
5	16.25	0.050

Table 38. Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Bacillus pumilus* and *S. rolfsii* (cv. TV 20)

Peak no	Retn time (min)	Height(mV)
1	2.70	0.020
2	3.30	0.050
3	3.75	0.10
4	4.82	0.039
5	16.00	0.040

Table 39. Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *G. mosseae* and *S. rolfsii* (cv. TV 20)

Peak no	Retn time (min)	Height(mV)
1	3.50	0.080
2	3.75	0.049
3	4.70	0.020
4	16.00	0.022

Table 40.Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with AMF, *Bacillus pumilus* and *S. rolfsii* (cv. TV 20)

Peak no	Retn time (min)	Height(mV)
1	3.52	0.051
2	3.75	0.030
3	4.70	0.010
4	16.10	0.035

4.15. Cellular location of chitinase in tea leaf tissues following induction of resistance by AMF and PGPR

Application of AMF and PGPR in the rhizosphere of tea saplings prior to pathogen challenge could successfully reduce sclerotial blight incidence. 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.

Apart from assessment of defense enzymes, an attempt was also made to determine cellular localization of chitinase in tea leaf tissues following indirect immunofluorescence test using FITC binding and treatment with PAb raised against chitinase. For this most responsive tea variety, TV-20 was selected. Leaf sections from untreated control, untreated pathogen (S. *rolfsii*) inoculated, *B. pumilus* treated, *G. mosseae* inoculated, *G. mosseae* and *B. pumilus* treated and *S. rolfsii* inoculated plants were taken. Immunolocalization of chitinase in treated as well as pathogen inoculated tea leaves were observed using FITC labeling and treatment with PAb of chitinase.. Joint inoculation with *G. mosseae and* treated with *B. pumilus* following challenge inoculation with the pathogen (*S. rolfsii*) showed maximum bright apple green fluorescence in the mesophyll tissues (Fig. 50).

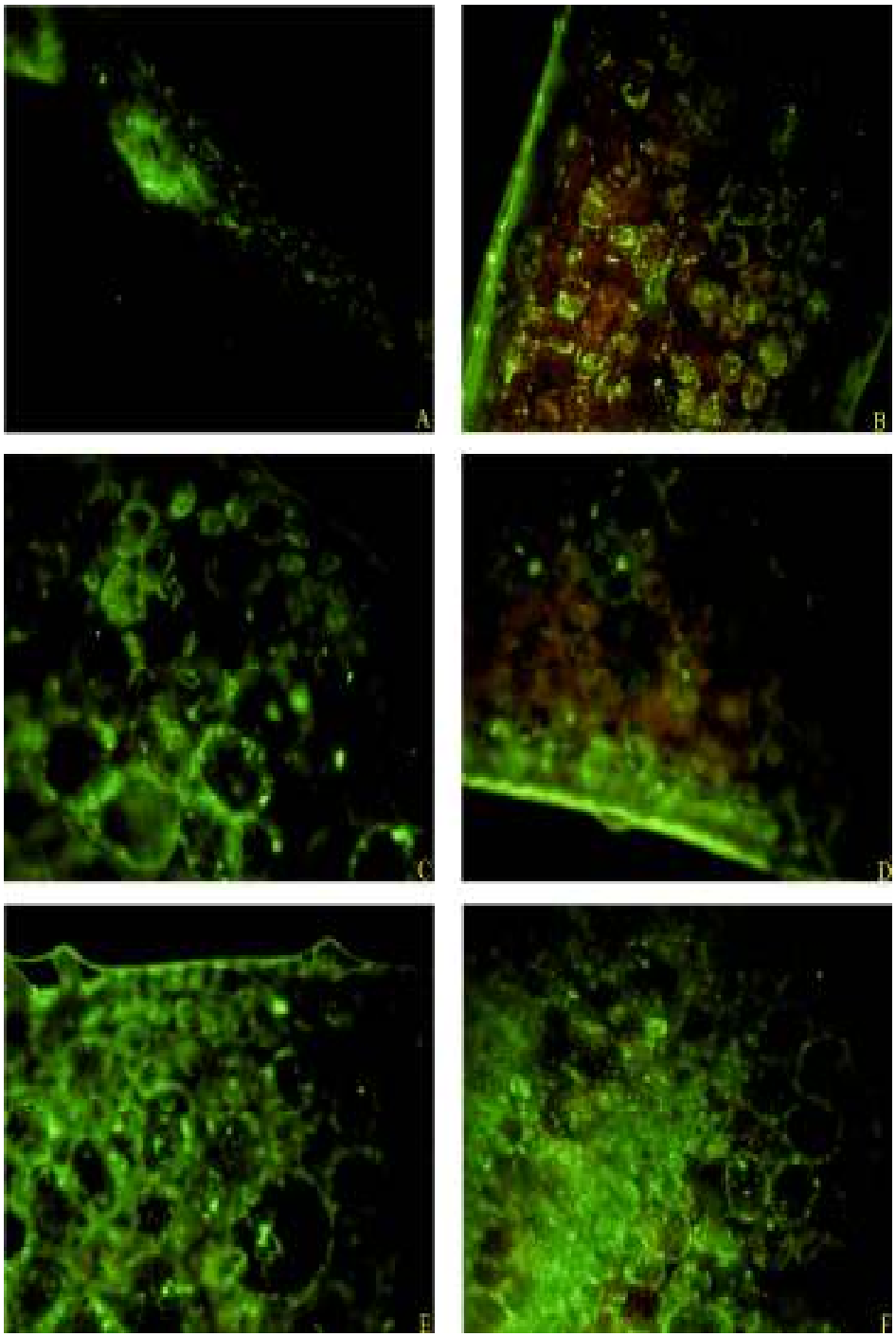

Fig 50. FITC labelling of tea leaf tissues with PAb of chitinase enzyme following treatment with *G. mosseae* and *B. pumilus* and inoculation with *S. rolfsii*. Untreated control (A), *S. rolfsii* inoculated (B&D), *B. pumilus* treated (C), *G. mosseae* inoculated (E), *G. mosseae* and *B. pumilus* treated and *S. rolfsii* inoculated.

4.16. Bacteria associated with spores of the AMF (*Glomus mosseae*) and its effect on spore germination

Glomus mosseae was found as one of the dominant spores associated with tea roots in the present investigation. Mass multiplication of *G. mosseae* and their field application alone and in combination with potential plant growth promoting rhizobacterium (*Bacillus pumilus*) have clearly indicated their effect in the improvement of health status of tea plants as well as induction of resistance towards the pathogen (*Sclerotium rolfsii*).

An optimal root colonization of tea plants in agricultural fields depends not only on the presence of extraradical hyphae or mycorrhizal root debris but, mainly, on the survival and well-timed germination of AMF spores in the soil. This process can be altered by various abiotic and biotic factors, in particular by the association with soil organisms. Indeed, some bacterial populations, called mycorrhiza helper bacteria, have beneficial effects on AMF growth not only by improving mycorrhizal root colonization and stimulating extraradical hyphal growth but also by facilitating AMF spore germination.

Spores of the arbuscular mycorrhizal fungus – *Glomus mosseae* were harvested from tea rhizosphere followed by single-spore-derived cultures with *Sorghum bicolor* as host plant. Scanning electron microscopy of *G. mosseae* spores revealed that the thin outer hyaline wall layer gradually degraded and replaced by mucilaginous products. The rough surface composed of the degraded and mucilated outer hyaline layer was present to different extents depending on the spore maturity stage. Out of 20 light-coloured spores observed, only 5 had a smooth surface, 11 were covered with roughness of up to half of the visible surface by SEM and the surfaces of 4 were entirely rough. Bacterial cells of different sizes were present either in the sloughing hyaline layer or on the surface of the second, laminated wall layer. Decaying material complicated the observation of bacterial cells because they appeared to be covered with their own mucilage. The spore surface also contained many holes, possibly corresponding to lysis zones **(**Fig 51 A&B**).** A total of three bacteria (T/GL/1, T/GL/2 and T/GL/3) were successfully isolated from *Glomus mosseae* spore originally obtained from tea root rhizosphere. All the three bacteria isolated were rod shaped and gram positive. Biochemical characterizations of all the bacterial isolates have been presented in Table 42**.** The effect of the isolate T/GL/1 was studied *in vitro* to evaluate the induction of germination of *G. mosseae*. Spores of *G. mosseae* and bacterial isolate T/GL/1 was kept in two sides of the petriplate with water agar media where there was no physical contact with each other. It was found that hyphal

length increased significantly in *G. mosseae* when the bacterial isolate were present than that of non bacterial control (Table 41, Fig. 52)

Table 41. Germination percentage of *G. mosseae* in presence of bacterial isolate T/GL/1

Substrate	Germination (%)	Length of germ tube (mm)
Water agar (non bacterial control)	13	2.62
Water agar and bacterial isolate T/GL/1	85	75.65

Table 42. Morphology and Biochemical characterization of Mycorrhizal Helper Bacteria (MHB) isolated from *Glomus mosseae*

Host	Code	Shape	Gram reaction	H_2S production	Phosphate solubilization	Starch hydrolysis	Casein hydrolysis	Chitin degrading	Siderophore production	Catalase production	Protease production	Urase production	Cellulase Production	Indolae Production	Identification
G. mosseae (obtained from tea rhizosphere)	**T/GL/1**	**Rod**	+	-	+	+	+	+	_	+	+	+	+	-	***Bacillus flexus***
	T/GL/2	Rod	+	-	+	+	+	+	+	+	+	+	+	-	*Bacillus* sp.
	T/GL/3	Rod	+	-	+	-	+	+	-	-	+	+	+	-	*Bacillus* sp.

4.16.1. 16 S rDNA sequence analysis of Mycorrhizal Helper Bacteria (MHB)

Genomic DNA was isolated from Mycorrhiza helper bacteria (isolate T/GL/1) purified and resuspended in 1X TE buffer until further use. Agarose gel electrophoresis of genomic DNA revealed that they were RNA free was around 1.67Kb. Purity of DNA evaluated in terms of the ratio between absorbance of A_{260} and A_{280} showed that genomic DNA of this MHB isolate was 1.816. ITS-PCR was performed with the help of universal primer pair where a uniform product of 1400 bp was obtained.

4.16.1.1. 16S rDNA sequences and BLAST analysis

The BLAST query of the 16S r DNA sequence of the isolate T/GL/1 designated as MHB against GenBank database confirmed the identity of the isolate as *Bacillus flexus*. The sequences have been deposited in NCBI, GenBank database under the accession no. KF552068. The sequence chromatograms of both the PGPR isolates have been represented in Fig. 53.

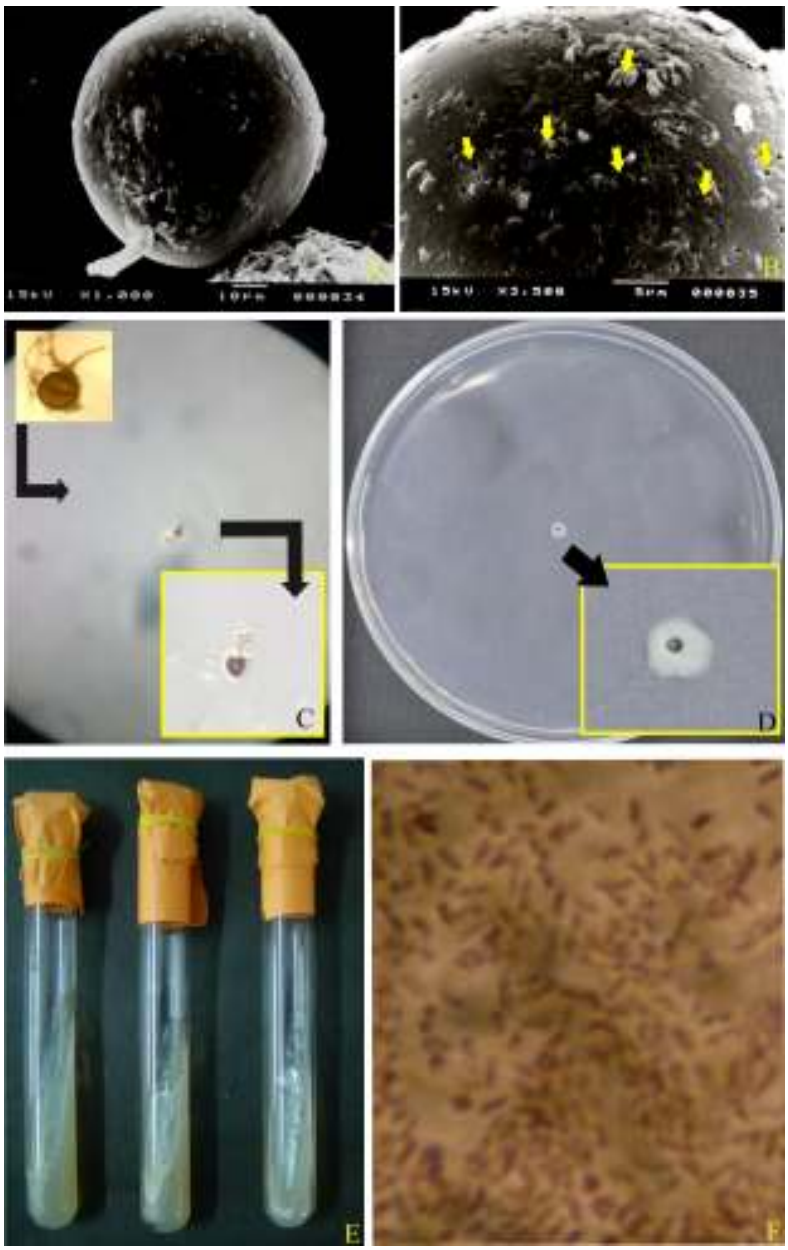

Fig. 51. SEM micrographs of *G. mosseae* ahowing characteristic hyphal attachment (A) and spore wall structures showing pits and bacteria like organisms (B). Isolation of MHB on agar medium (C&D). Pure culture maintained on nutrient agar slants (E). Gram staining and microscopic characters of MHB isolated from *G. mosseae* (F)

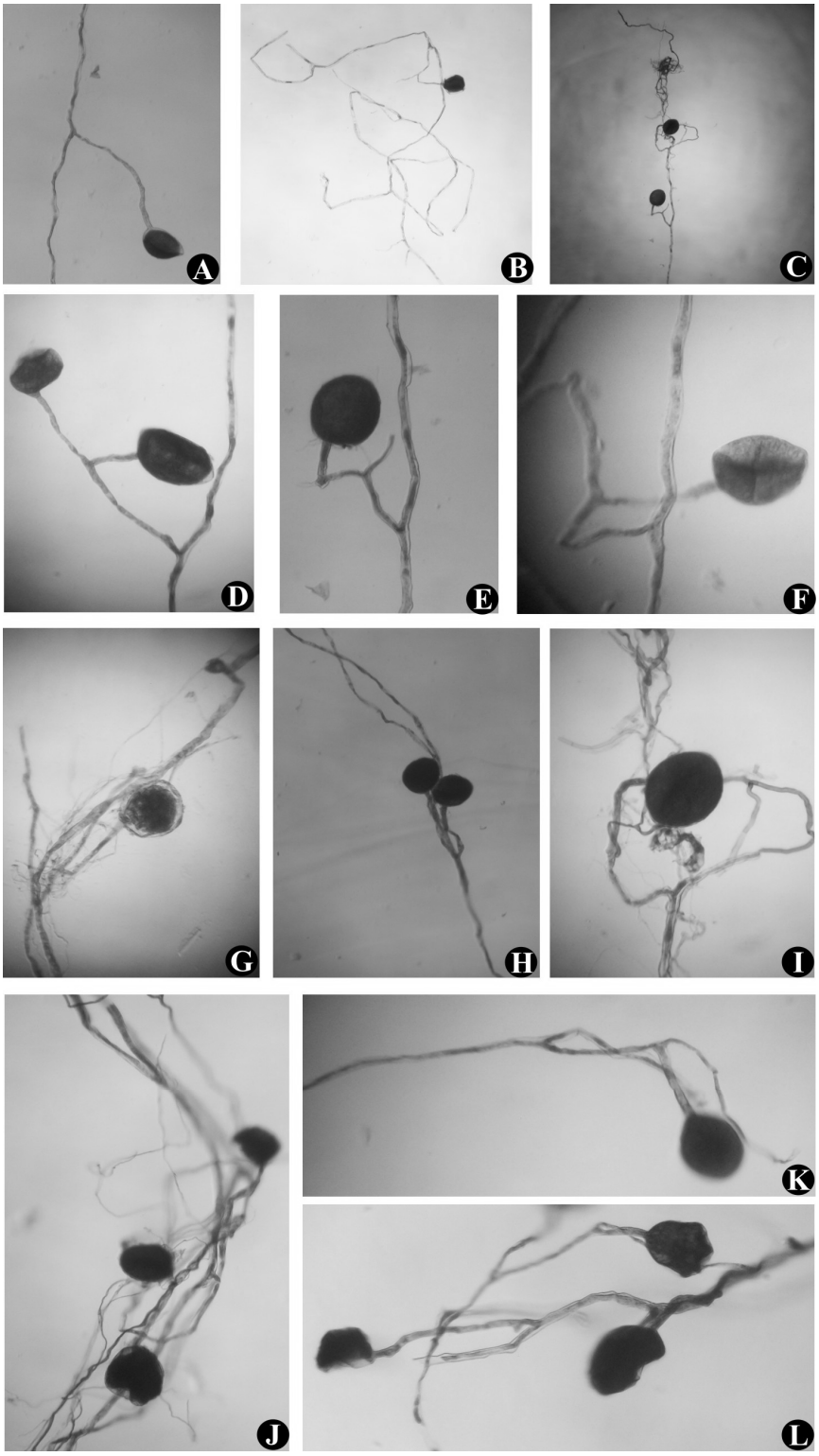

Fig. 52. *In vitro* test of bacterial isolate (T/GL/1) on germination of *Glomus mosseae* spores in water agar media. Untreated control (A), T/GL/1 treated (B-L) spore germination following 10 days (B-F) and 18 days (G-L) of incubation.

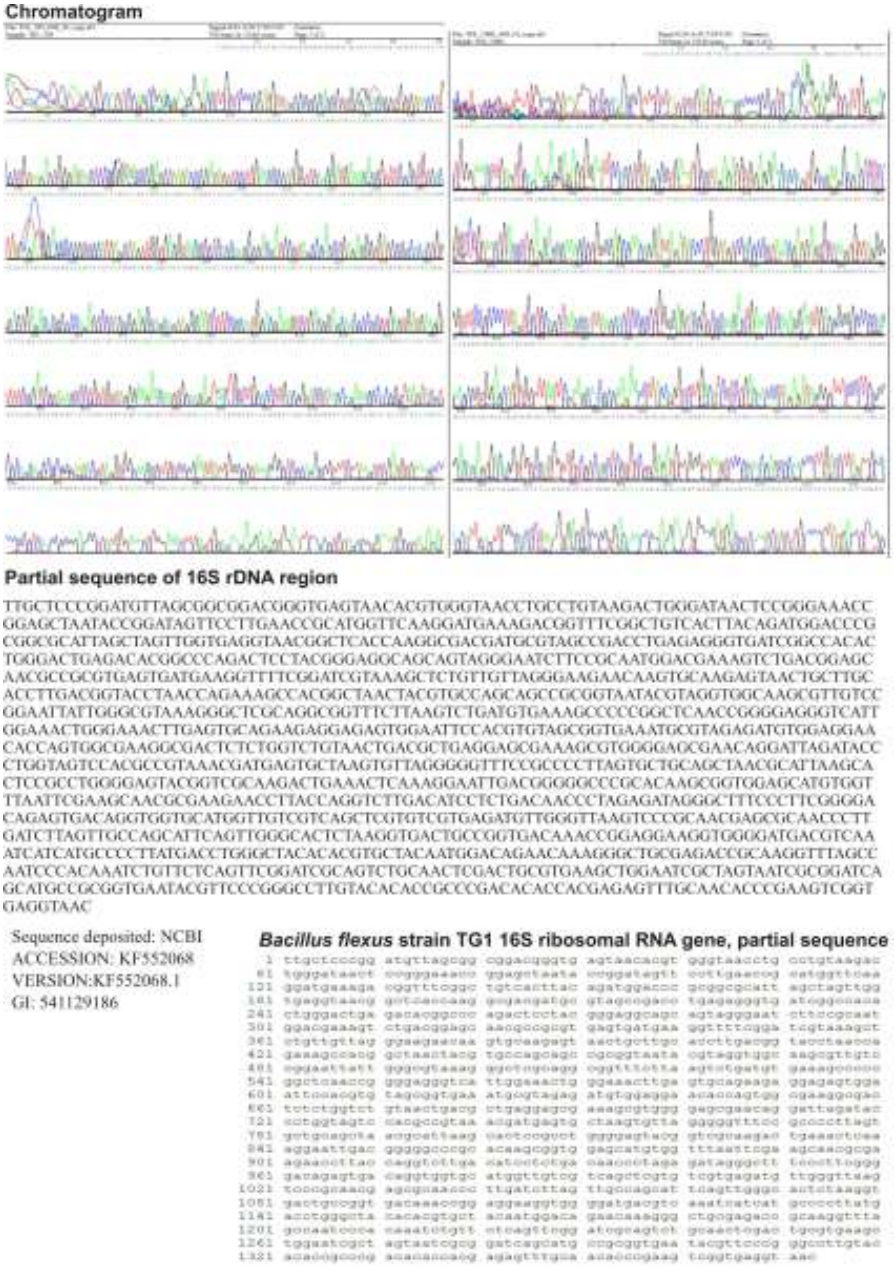

Fig. 53. Chromatogram and sequence deposition of 16S r DNA region of *Bacillus flexus* KF552068

4.16.1.2. Multiple sequence alignment and phylogeny

A multiple sequence alignment of ITS gene sequences of *B. flexus* was conducted. The result reveals that there were quite a number of gaps that were introduced in the multiple sequence alignment 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.54). Phylogenetic analysis of *B. flexus* was carried out with the Ex-type strain sequences obtained from NCBI GenBank Database which showed maximum homology with the isolate T/GL/1 (Table. 43).The evolutionary history was inferred using the Neighbor-Joining method (Saitou N &Nei M, 1987). The optimal tree with the sum of branch length = 0.04924887 with 964 positions in final data set have been shown (Fig. 55).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 J 1985). 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 725 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 (Tamura *et. al.* 2007).

Table 43.Genbank Accession Numbers and Geographic locations of the Ex-Type strains of *B. flexus* that showed homology with the isolate T/GL/1

Accession No.	Strain No	rDNA Sequence	Country	Organisms
KF552068	**T/GL/1**	**1373 bp**	**India**	***Bacillus flexus***
GU297607	AUCAB22	1535 bp	India	*Bacillus flexus*
KF000098	BLY01	1471 bp	India	*Bacillus flexus*
KC951110	LAM-CQ-3	1443 bp	China	*Bacillus flexus*
KC608047	GS5	986 bp	India	*Bacillus flexus*
GU112451	SSZ01	307 bp	Saudi Arabia	*Bacillus flexus*
AB686280	INSPDST8	930 bp	India	*Bacillus flexus*
HQ379854	IGCAR-01/10	1431 bp	India	*Bacillus flexus*
HM595743	NJY4	536 bp	Mexico	*Bacillus flexus*
HM595742	NJY2	536 bp	Mexico	*Bacillus flexus*
JF831121	KRG	1304 bp	India	*Bacillus flexus*

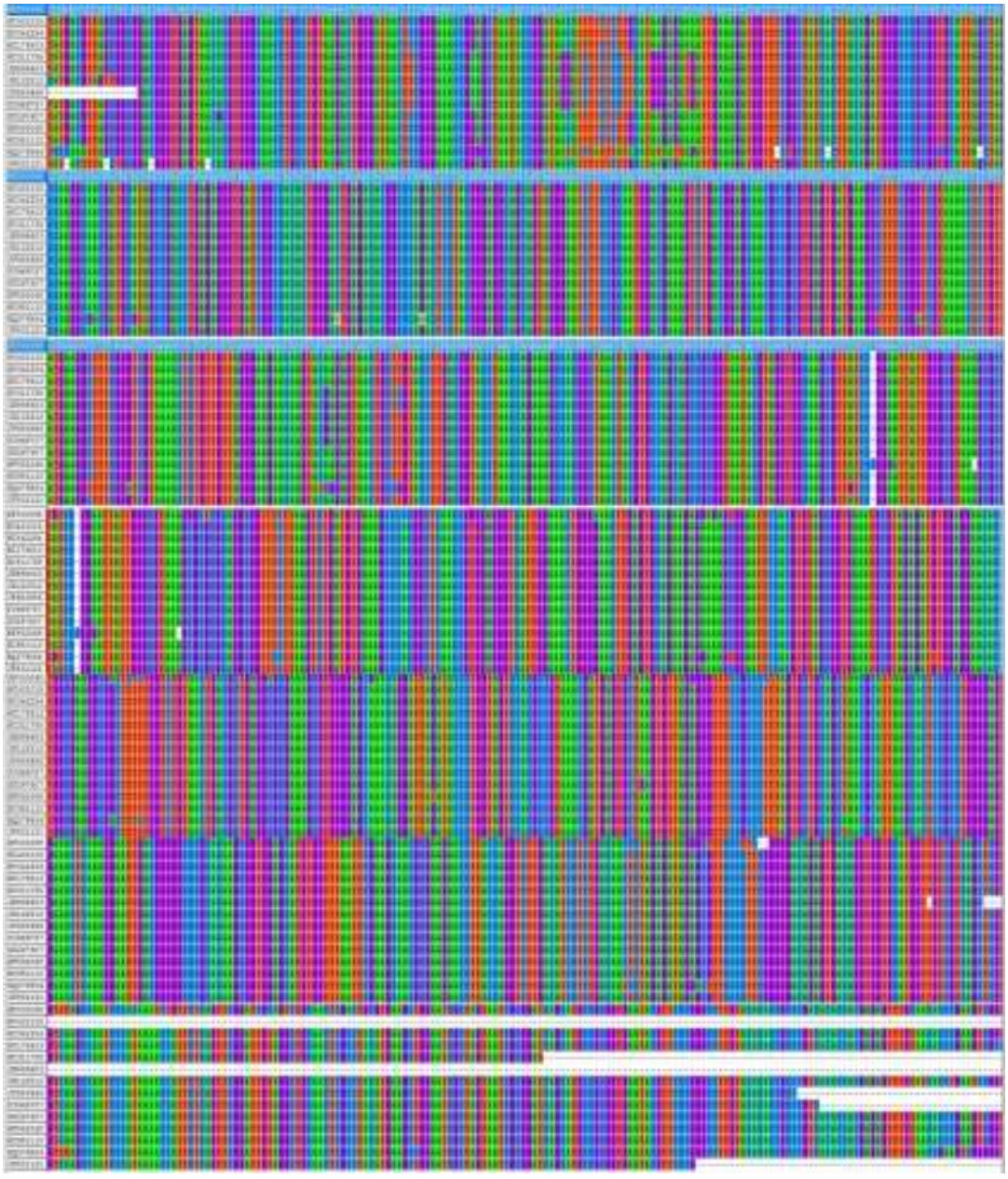

Fig. 54. 16S r DNA sequence alignments of *B. flexus* with other extypes isolate. The conserved regions of the gene are demonstrated in different colour

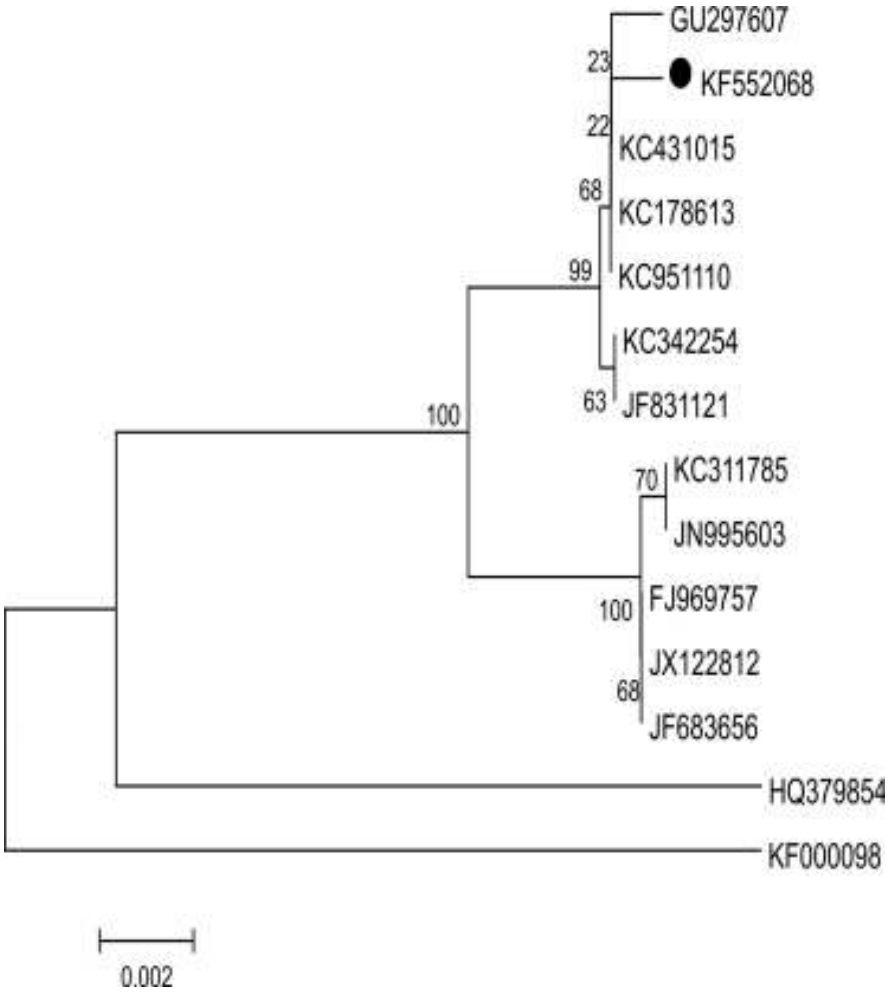

Fig. 55. Phylogenetic placement of *B. flexus* (KF552068) with other ex-type strain sequences obtained from NCBI GenBank Database on the basis of 16S rDNA sequences

4.17. Serological detection of pathogen (*Sclerotium rolfsii*) and AMF (*Glomus mosseae*) in soil and tea root tissue using indirect immunofluorescence following induction of resistance

Fluorescent antibody labelling with FITC is known to be one of the best techniques to detect a number of microorganisms in a given sample. In the present study polyclonal antibody raised against *S. rolfsii* and *G. mosseae* were used to detect their presence as well as their nature of colonization in the host root tissues with the help of two fluorescent dyes FITC.

Tea roots following colonization with AMF alone or in combination with PGPR could induced resistance against the pathogen. This was evident in the previous experiments when the treated tea leaves showed bright apple green fluorescence in mesophyll tissues when these were labelled with FITC and reacted with PAb of chitinase. The present investigation was designed to locate the pathogen in the rhizosphere as well as presence of AMF in rhizoplane as well as their cellular localization in root tissues using PAbs of the pathogen (*S. rolfsii*) and AMF (*G. mosseae*). In untreated inoculated tea plants, where disease was prevalent, sclerotial population was also evident when soil samples were examined by indirect immunofluorescence test using PAb of *S. rolfsii* (Fig. 56).

However, in the AMF treated tea plants, when PAb of *G. mosseae* was used in indirect immunofluorescence test, bright apple green fluorescence developed which was distributed throughout the spore wall. The green fluorescent was also prominent in the subtending hyphae (Fig. 57). 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 (Fig. 58). Similarly feeder roots of tea were also treated with PAbs of *G. mosseae* and labelled with FITC to specifically locate the hyphal proliferation within the tissues. Observations of treated root tissues under UV- microscope showed bright apple green fluorescence of the hyphae, vesicles and arbuscules within the host tissue. Flourescence was more prominent towards the cell wall in most of the tissues (Fig. 59) clearly indicate the successful colonization of tea roots with AMF leading towards induced resistance against the pathogen.

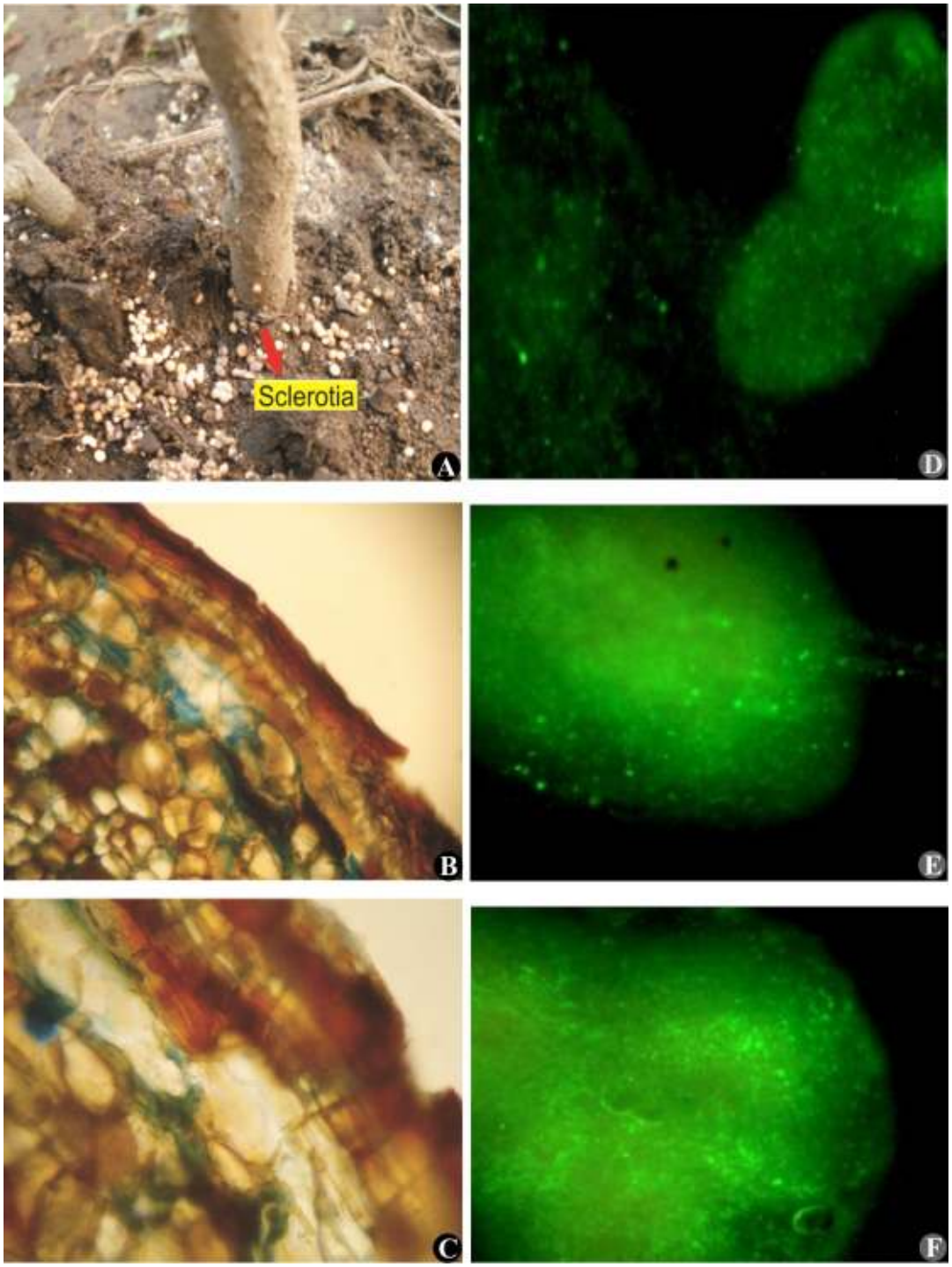

Fig. 56 Section of tea root tissue showing infection in tea root tissue (A-C) and indirect immunofluorescence test using PAb of *S. rolfsii*

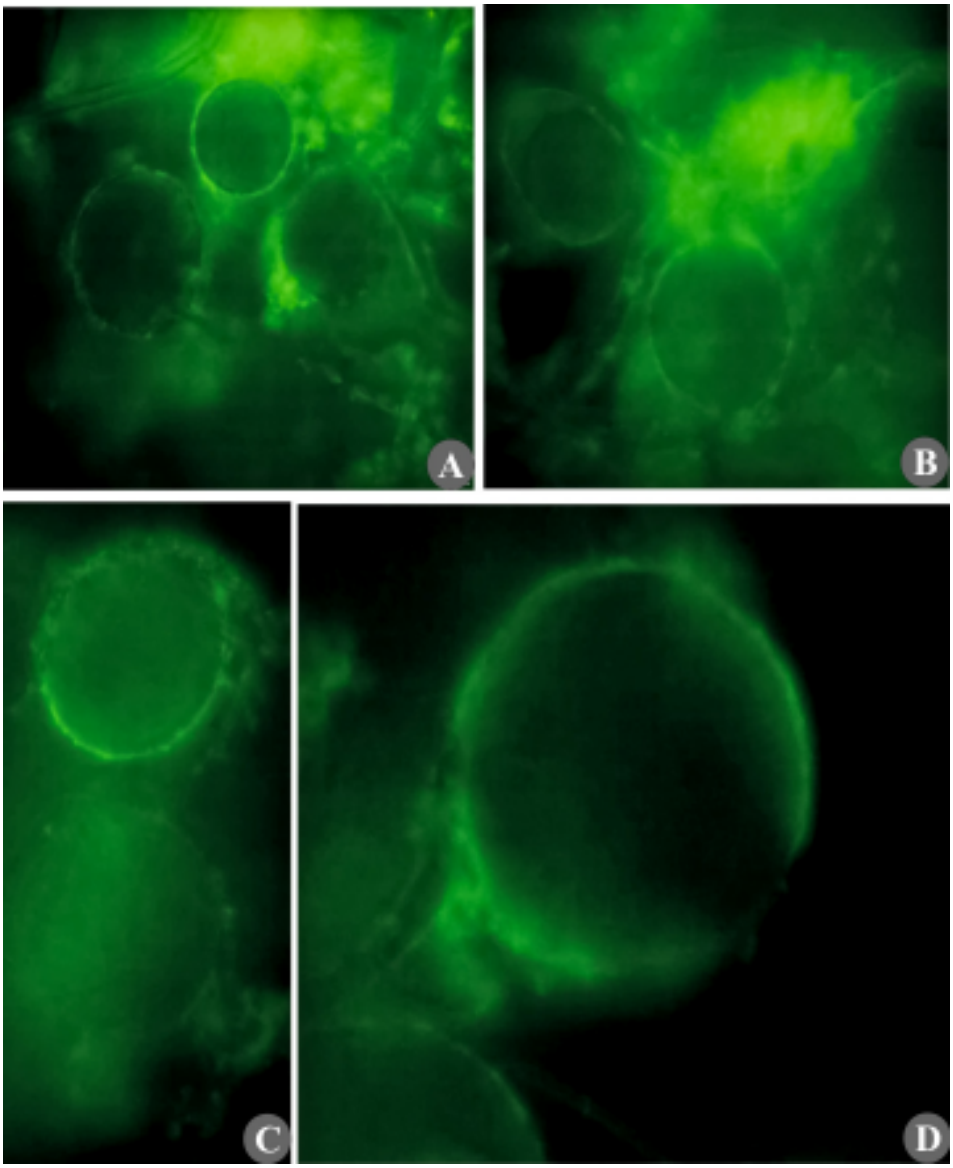

Fig. 57. Indirect immunofluorescence of spores of *Glomus mosseae* labelled with FITC and treated with PAb of *G. mosseae.* Spores in cluster (A-C), Enlarged view of spore showing subtending hyphae (D)

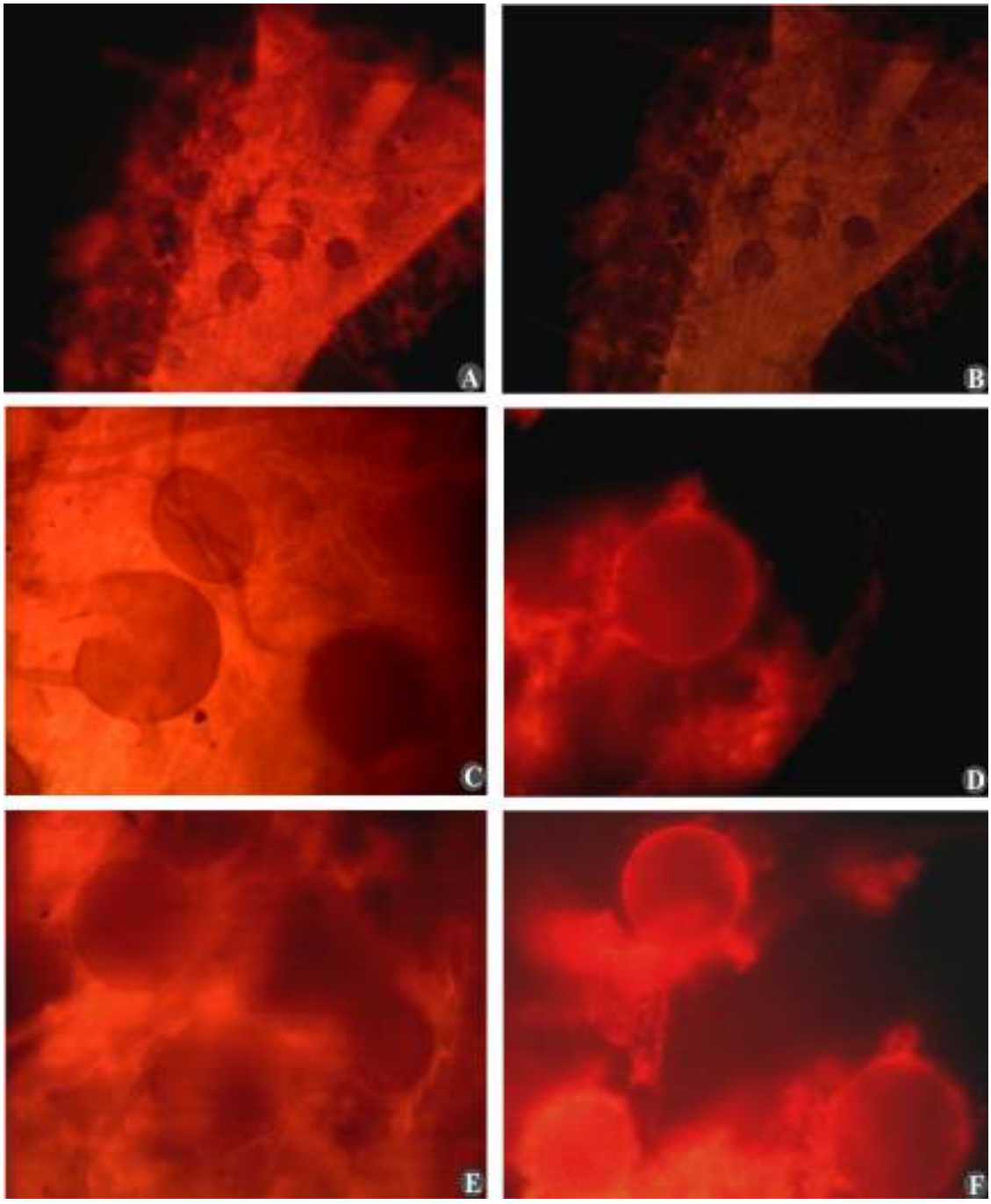

Fig. 58. Indirect immunofluorescence of tea root tissue labelled with RITC and treated with PAb of *G. mosseae*

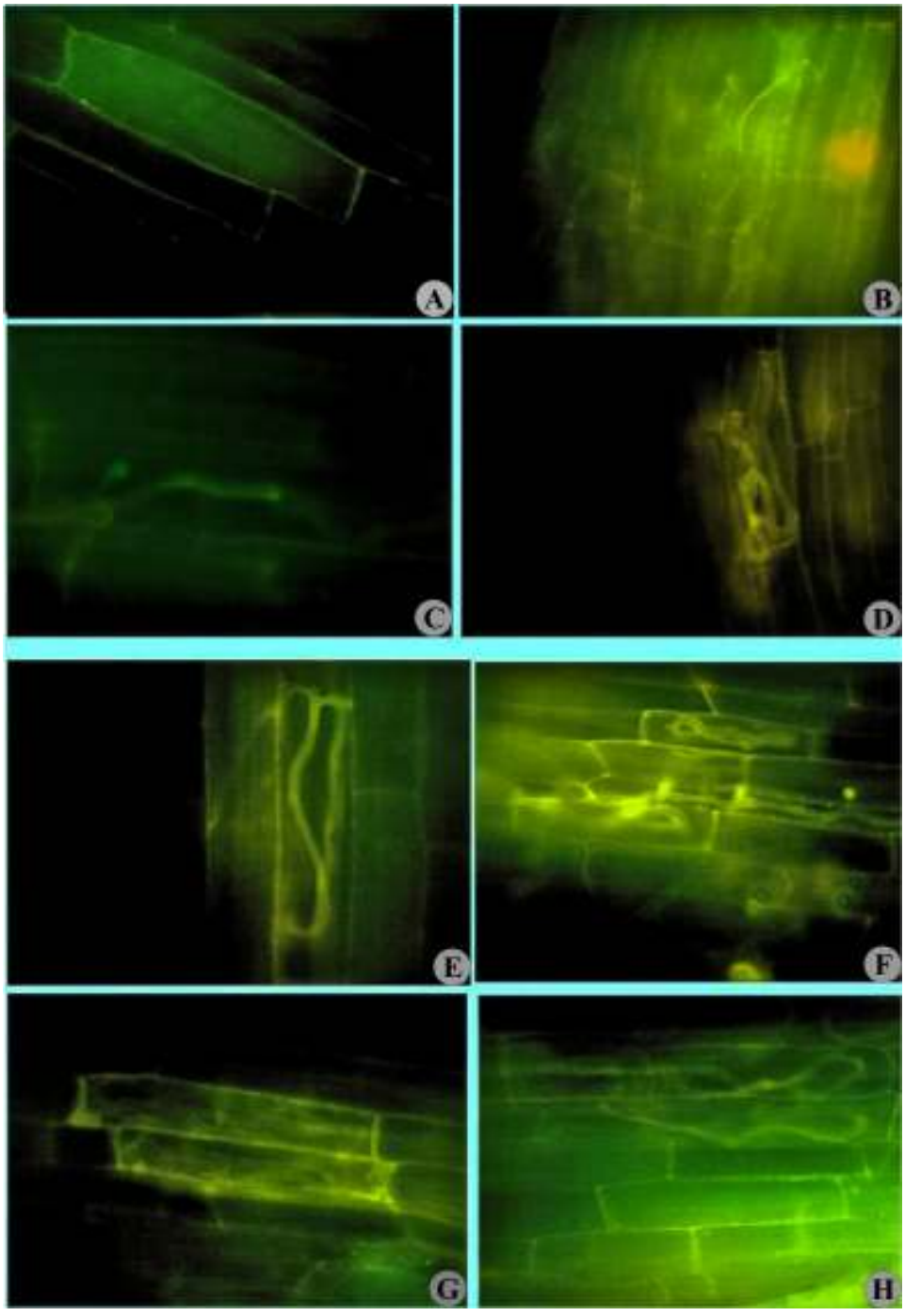

Fig. 59 (A-H). Indirect immunofluorescence of tea root tissue labelled with FITC and treated with PAb of *G. mosseae.* Healthy root tissue (A), AMF colonized tea root tissues showing branched hyphae (B, C and D) and arbuscules (E, F, G and H).

DISCUSSION

Arbuscular mycorrhizal fungi (AMF) inhabit the soils of virtually all terrestrial ecosystems, forming symbiotic associations with most plants. The host plants incur substantial carbon costs to sustain this symbiosis, but in return, they obtain multiple benefits from the fungal partners, above all, the provision of mineral nutrients. AMF may supply up to 90% of the host plant's nitrogen and phosphorus requirements Up to 20% of the photosynthesis products of terrestrial plants (approximately 5 billion tonnes of carbon per year) are estimated to be consumed by AM fungi. Therefore,
AM symbiosis contributes significantly to global phosphate and carbon cycling and influences primary productivity in terrestrial ecosystems. Arbuscular mycorrhizal fungi (AMF) spores provide a long-term reservoir of inoculums and play a key role in facilitating nutrient uptake by crops in low-input farming systems, a prerequisite to maintain sufficient productivity under stress conditions. Most plant families are colonized by AMF. Benefits of these fungi to their host plants include improving nutrient supply, protection from infection by soil-borne plant pathogens and protection from drought stress. Due to their widespread presence in soils, mycorrhizae play significant roles in the rhizosphere. During the process of plant colonization, AMF interact with bacteria and the fungal spores and hyphae provide sites where certain populations of bacteria can live. Bacteria can also be found in the cytoplasm of AMF spores. The beneficial effects of mycorrhizae in the rhizosphere are the result of synergistic interactions among all rhizosphere microbes, which are crucial for plant growth. Thus, the relationship between AMF and their associated bacteria may be of great importance for sustainable agriculture.

Tea, being perennial, is subjected to varying environmental conditions throughout its life as well as to numerous attacks by pest and pathogens, which in turn are influenced by various environmental conditions. Field grown tea plants are constantly subjected to advance environmental conditions, and constantly attacked by various root, shoot and fungal pathogens. Besides of their immobility plants have to make necessary metabolic and structural adjustments to cope with the stress conditions.

Keeping the above in mind, the present study was undertaken in order to select potential microorganisms such as Plant Growth Promoting Rhizobacteria (PGPR) and Arbuscular Mycorrhizal Fungi (AMF) from rhizosphere of different plantation crops such as Tea, Coffee, Rubber, Areca and Cinnamomum and make a detailed studies so that one or more of such microorganisms could be used as beneficial microorganisms.

In the present investigation, at first fifteen tea varieties, of which six UPASI varieties (UP-2, UP-3, UP-8, UP-9, UP-26 and BSS-2) and nine Tocklai varieties (TV-18, TV-9, T-17, TV-22, TV-23, TV-25, TV-26, TV-29 and TV-30) being grown in the experimental field (15 year old bush) were studied extensively to explore the diversity, mycorrhization and seasonal affect in AMF population. In addition 3 months old saplings of TV-18, T-17, AV-2, T-78, UP-3, UP-26, TV-25, TV-26, TV-9 and TV-20 were procured from Gaya Ganga Tea Estate, Silguri for various experimental needs.

AMF spores collected from tea, rubber, areca nut, coffee and cinnamomum roots were examined and spore population of rhizosphere of each plant was critically determined. Spore colour was determined under the dissecting microscope from spores suspended in water. All the spore types formed by members of this order were homologous. Some spores may be asexual, whereas others were very complex in structure, and may be sporangia or sexual structures. Morphological features of isolated AMF spores were critically examined with special reference to variation in size, colour, wall thickness, shape, wall layers specially germinal wall, coriaceous wall, amorphous wall and beaded wall. Scanning Electron Microscopy pictures were taken of various spores to study the texture character and ornamentation. Among Tea, Rubber, Areca and Coffee rhizosphere the genus *Glomus* was predominant followed by *Gigaspora, Acaulospora, Scutellospora* and very few *Entrophospora*. The spores were photographed and identified up to species level with the help of standard keys (Walker 1992). *Acaulospora bireticulata* were found to be present in all the samples whereas *A. scorbiculata* was found to be present in tea rhizosphere but absent in rubber, areca and coffee. *A. capsicula* was found to be persistent in all the studied plants except tea. Again *A. undulata* was only present in rubber rhizosphere. Of all the rhizosphere studied

Entrophospora colombiana and *Glomus pansihalos* were found only in the rhizosphere of Areca nut collected from Siliguri Foothills and Darjeeling Hills. Spores of *Glomus mosseae, Glomus aggregatum* and *G. constrictum* were found to be abundant in tea, coffee, rubber and areca nut rhizosphere but *Glomus intraradices* was found only in tea and coffee rhizosphere. *Gigaspora gigantea* and *G. margarita* were abundant in all the four plants studied but *Gigaspora rosea* was found only in tea and rubber. Similar results were also reported by Chakraborty and De (2013). The primary effect of AM symbiosis is to increase the supply of mineral nutrients to the plant, particularly those whose ionic forms have a poor mobility rate, or those which are present in low concentration in the soil solution. This mainly concerns phosphate, ammonium, zinc and copper (Barea, 1991). AMF can reduce or even suppress, damage caused by soil-borne plant pathogens (Hooker *et al*., 1994). AMF based bioformulation seems promising for promoting plant health and productivity for plantation crops which involve a transplant stage, as in horticultural systems where plants are produced in nursery beds.

Dark septate endophytes (DSE) are a group of hetero-geneous root-associated endophytic fungi which are characterized by melanized intercellular and intracellular runner hyphae and so-called microsclerotia (aggregation of dark, thick-walled, closely packed inflated cells) within epidermis and cortex of plant roots (Silvani et al., 2008). Moreover, their existence has been found prevalent all over the world (Jumpponen and Trappe, 1998; Wang and Zhao, 2005). Although, confusions of their taxonomic affinity and obscure effects on hosts remain a problem due to little understanding of their teleomorph (most DSE rarely sporulate or remain absolutely sterile under culture conditions) and their variable impacts on hosts ranging from positive to negative in different experimental conditions (Horton et al., 1998). Dark septate endophytes (DSE) comprise a miscellaneous group of root-inhabiting fungi. It is defined DSE as conidial or sterile ascomycetous fungi that colonize living plant roots without causing apparent negative effects such as tissue disorganization. This definition is likely to include a plethora of fungi whose functions and taxonomic affinities remain unknown. (Jumpponen and Trappe 1998) The DSH (Dark Septate hyphae) also formed arbuscules that are coiled in structure. Hyphae, originating from a spore or another colonized root, usually grow

along the epidermis, often following the groove between epidermal cells. Plant cell responses clearly associated with this stage have not been described yet. Intercellular and intracellular runner hyphae and microsclerotia were found abundant in BSS-2, UP-2, UP-3, UP-8, UP-9, TV-18, T-17, TV-22 and TV-26.

PGPRs have been reported to directly enhance plant growth by a variety of mechanisms; fixation of atmospheric nitrogen that is transferred to the plant, production of siderophores that chelate iron and make it available to the plant root, solubilisation of minerals such as phosphorus, and synthesis of phytohormones (Castillo *et al*. 2002). Direct enhancement of mineral uptake due to increases in specific ion fluxes at the root surface in the presence of PGPR has also been reported (Ait Barka *et al.* 2000; Bais *et al.* 2004). PGPR strength may us one or more of these mechanisms in the rhizosphere. A total no. of twenty two (22) bacteria were isolated from selected plantation crops. Isolated bacteria were studied under microscope after suitable staining and characterized based on morphological and biochemical studies following Bergey's manual of Systematic Bacteriology. Overall 15 bacterial isolates were gram positive, rod shaped whereas 7 isolates were gram negative. Among all the bacterial isolates two bacterial isolates designated as RHS/T-382 and TRS-6 obtained from the rhizosphere of tea showed positive response for all the PGPR tests like phosphate solubilization, protease, chitinase IAA and siderophore production. Scanning electron microscopic studies of both the bacterial isolates showed that they were characteristically rod shaped and the size of the bacterial cells ranged from 4-7 µm.

Most AMF form spores in the soils which are able to germinate and grow from a quiescent-like state in response to different edaphic and environmental conditions, but are unable to produce extensive mycelia and to complete their life cycle without establishing a functional symbiosis with a host plant. Their germination do occur but after a short period of time growth ceases. Spores of *G. mosseae* were allowed to grow on water agar media and water agar media supplemented with root exudates of 2-3 days old maize root. In the later case spores started to germinate, hyphal branching appeared but it ceased to further development after 10-12 days. In case of only water agar only 18 percent spores showed germination but when supplemented with root exudates it increased to 55

percent. As early as 1959, Barbara Mosse suggested the storage of collected spores on damp filter paper at 5°C for 6 weeks in order to obtain the regular germination of resting spores of *Glomus mosseae*. Eighty percent of spores treated in this way germinated within 3–4 days.

The causal organism (*Sclerotium rolfsii*) of sclerotial blight of tea are readily distinguished by rapidly growing silky-white hyphae that tend to aggregate into rhizomorphic cord like structure. In culture, the whole area of a Petri plate is rapidly covered with mycelium, including aerial hyphae which may cover the lid of the plate. Both in culture and in plant tissue, a fan-shaped mycelial expanse may be observed growing outward and branching acutely. At least two types of hyphae are produced. Sclerotia (0.5-2.0mm diameter) begin to develop after 6-7 days of mycelial growth. The pathogen was intially grown in different media *i.e*. Potato dextrose agar (PDA), Potato sucrose agar (PSA), Richard's Agar (RA), Carrot juice agar (CJA) Czapek-Dox agar (CDA), and Yeast extract- dextrose agar (YDA) in order to determine its growth rate. Maximum growth was recorded in PDA. Mycelial growth rate of *S. rolfsii* was further determined in PDA medium for a period of 40 days. Mycelial growth was recorded after 5,10,15,20,25,30,35 and 40 days of growth and incubated at 25± 1ºC. Maximum growth was recorded after 20 days of incubation after which it declined.

The effectiveness of the purified antigen of *S. rolfsii* in raising PAbs was checked by homologous cross reaction following agar gel double diffusion tests. The precipitin reaction done with PAb raised against mycelial protein yielded sharp band which was stained blue Dot immunobinding assay was performed using total soluble proteins of *S. rolfsii* used as homologous antigen source. Antigens were carefully spotted on nitrocellulose paper and probed with PAb of *S. rolfsii*. Results show clear and intense color reactions. Western blot analyses using polyclonal antibody of *S. rolfsii* revealed that the PAb collected after successful immunization of the rabbits for consecutive six weeks could show different levels of homologous reactions with the antigen of *S. rolfsii*. A sharp and intense band at 35 Kd was obtained 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

immunoflourescence of young and mature sclerotia of *S. rolfsii*. The young and old sclerotia along with the mycelia treated with PAbs and labeled with FITC showed apple green fluorescence where fluorescence was more intense on young sclerotia. Neither mycelia nor sclerotia showed any type of auto-fluorescence nor they showed any fluorescence when treated with normal serum followed by FITC.

The antagonistic effect of the both the isolates *B. pumilus* RHS/T-382 and *B. amyloliquefaciens*-TRS6 which showed positive results in all the tested PGPR characters were tested against tea root pathogens *viz*, *Sclerotium rolfsii, Fomes lamaoensis*, *Poria hypobrunnea* and *Spherostilbe repens*. Both the bacterial isolates were found to inhibit the test pathogens up to 84 %. Among both the isolates TRS6 was found to inhibit all the test pathogens more efficiently than RHS/T-382.

Screening for resistance of tea varieties against *S. rolfsii* was carried out in sick plot developed specifically for this pathogen. Varietal resistance test of tea against *Sclerotium rolfsii* was carried out in ten (10) tea cultivars including five Toklai varieties (TV-25, TV-26, TV-9, TV-20, TV-18), two Teen Ali varieties (T-17 and T-78), two Upasi varieties (UP-3 and UP-26) and one Assam variety (AV-2). Three year old plant roots were inoculated with *S. rolfsii* and disease assessment was done on the basis of visual observation of symptoms and disease index (ranging from 1-6) was calculated after 15,30 and 45 days following inoculation as well as on the basis of histoparthological studies of the infected root. Results show that among the tested tea varieties, TV-25, TV-26, TV-9 and TV-20 were found to be less susceptible in comparison with other tea varieties. Defoliation of leaves following infection with *S. rolfsii* was evident in TV-18, T-17, AV-2, T-78, UP-3, UP-26 and TV-25 after 30 days of inoculation in relation to healthy control plant. Disease symptoms occurred in those varieties within 7 days after transplantation following death of the plants, were selected as highly susceptible varieties, whereas the varieties showing resistant reactions were also categorized.

Artificial inoculation of tea roots with *Sclerotium rolfsii* was carried out in experimental field following sick soil technique. Seedlings of a susceptible tea variety were grown in the field and disease development was noticed. Histopathological studies revealed the establishment of pathogen in root tissues along with above ground symptom.

Biochemical changes following disease development was assayed in both the untreated healthy and inoculated tea varieties.

Total phenols from healthy and *Sclerotium rolfsii* inoculated tea roots of different varieties were extracted after 10 days of inoculation and estimated. Results revealed that total phenol decreased following inoculation with *Sclerotium rolfsii* in the susceptible varieties. However there is an increase in the phenol content of resistant varieties following inoculation. Among all the varieties tested TV -20 showed maximum increase in total phenol following inoculation with the pathogen.

Ortho-didydroxy phenols were also extracted from healthy and *Sclerotium rolfsii* inoculated tea roots of different varieties after 10 days of inoculation with the pathogen and estimated. Results revealed that ortho-dihydroxy content decreased in susceptible varieties and increase in resistant varieties following inoculation with *Sclerotium rolfsii*. Responses of TV-20 variety against the pathogen were towards increasing the levels of orthodihydroxy phenol. Results show that PAL activity increased after 14 days of inoculation markedly in all the varieties except in TV-25.

Four tea varieties (TV-25, TV-26, TV-9, and TV-20) were selected to test the effect of PGPR (*B. pumilus*) and AMF (*G. mosseae*) 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 that enhancement of plant growth by single as well as joint application of PGPR and AMF however the results reveals that the growth of tea saplings grown under same environmental and physical conditions were enhanced to a greater extent when both *B. pumilus* and *G. mosseae* were applied jointly. Among all the four varieties tested, maximum growth was noticed in TV 20 and TV 26. Statistical analysis (ANOVA) revealed that the decrease in soil P and increase in root and leaf P-content were significantly enhanced when both *G. mosseae* and *B. pumilus* were applied jointly. Similarly The growth enhancement in tea saplings in nursery conditions was also evaluated in terms of total phosphate mobilized by *B. pumilus* and *G. mosseae*. Total residual phosphate content in soil, roots and leaves were evaluated. The overall results reveal that the total soil P-content decreased while the root and leaf phosphate increased

following application of *B. pumilus* and *G. mosseae* singly or jointly which indicated efficient soil phosphate mobilization by both PGPR and AMF. Statistical analysis (ANOVA) revealed that the decrease in soil P and increase in root and leaf P- content were significantly enhanced when both *G. mosseae* and *B. pumilus* were applied jointly.
Productions of volatile compound by bacteria have also been shown to be an important mechanism of plant growth promotion.
As tea is cultivated mainly for its leaves, the induction of new shoots and more leaves have great impact in considering plant growth promotion. (Rao and Shukla 2002) observed that in water logged condition (+ 0.6 MPa level) the growth of seedlings, total N and P content, percentage of endomycorrhizal colonization, nitrogenase and NH_3 assimilating enzymes were found to be maximum in seedlings raised after dual inoculation with *Azospirillum brasilense* and *Glomus mossae* as compared to *Azospirillum* alone. (Chakraborty *et al*. 2009, Chakraborty *et al*. 1996) also evaluated one of the PGPRs, *Ochrobactrum anthropi* TRS-2 isolated from tea rhizosphere and its talc based formulation for growth promotion and management of brown root rot disease of tea caused by *Phellinus noxius*. *O. anthropi* could solubilize phosphate, produce siderophore and IAA *in vitro* and also exhibited antifungal activity against six test pathogens. Siderophores are low molecular weight molecules that are secreted by microorganisms to take up iron from the environment (Hofte 1993) and their mode of action in suppression of disease were thought to be solely based on competition of iron with the pathogens (Bakker *et al.* 1993; Duijff, 1999). Interestingly siderophores have also been shown to induce systemic resistence (Leeman *et al*. 1996; Bakker *et al*. 2003b). Siderophore producing bacteria were also isolated from tea rhizosphere previously. Saikia and Bezbarua (1995) isolated *Azotobacter* from iron rich tea garden acid soil which was demonstrated to produce siderophore. Bezbarua *et al* (1996) further isolated two *Pseudomonas* strains from tea rhizosphere which produce siderophore and inhibited growth of *F. lamaoensis*. *Pseudomonas putida* and *P. fluorescence* were shown to produce siderophores (Torres- Rubio *et al*. 2000). (Jagadeesh and Kulkarni 2003) reported that of 38 rhizobacterial strains isolated from tomato which showed antagonism to *Alstonia solanacearum*, 23 were siderophores producers. These include the ability to produce siderophores that chelate iron, making it unavailable to pathogens; the ability to

synthesize antifungal metabolites such as antibiotics, fungal cell wall-lysing enzymes, or hydrogen cyanide, which suppress the growth of fungal pathogens; the ability to successfully compete with pathogens for nutrients or specific niches on the root; and the ability to induce systemic resistance (Bashan and Holguin, 1998; Cornelis and Matthijs 2002). Biochemical and molecular approaches are providing new insight into the genetic basis of these traits, the biosynthetic pathways involved, their regulation, and importance for biological control in laboratory and field studies (Bashan and Holguin. 1998; Basnayake and Birch, 1995; Castillo *et al*., 2000; Cornelis and Matthijs, 2002). In a previous work, it was also shown that *Bacillus megaterium* could effectively control brown root rot of tea caused by *Fomes lamaoensis* (Chakraborty *et al*. 2006).

The major components analyzed in tea leaves in present study were defense enzymes, polyphenolics and catechins. In all tested varieties defense related enzymes viz. chitinase (CHT), β-1,3- glucanase (GLU), peroxidase (PO), phenylalanine ammonia layse (PAL), as well as phenolics increased significantly, especially in presence of the pathogen. In a similar study, two isolates of *B. pumilus* were reported to be best plant growth promoters and biocontrol agents downy mildew disease in pearl millet (Niranjana *et al.* 2003). They also reported increased activities of PAL, POX and β-1, 3- glucanase (GLU), but not of (CHI) activity. Induction of defense related enzymes by *P.fluorescens* in black pepper and *Phytopthora capsici* pathosystem was reported by (Paul and Sharma 2003). They obtained increased level of PO, PAL, PPO and Catalase in leaves apart from root of treated plants indicating the systemic protection offered to black pepper by PGPR strains. Peroxidase and polyphenol oxidase are important components of the defense mechanism of plants against pathogens.

Catechins are major flavor flavonoid components of tea and their quantitative changes with respect to different isomeric forms were analyzed by HPLC. It was observed that the treatment with the bacteria induced some new isomeric forms. Since tea leaves produced for their flavours enhancement of catechins isomer point to the fact that these are also enhanced during plant growth promotion. Catechins were analysed in HPLC from leaves of variety TV-20 that showed maximum response towards AMF and PGPR whose rhizosphere was soil drenched with *Bacillus pumilus* and roots were inoculated with spores of *G. mosseae*. Analysis revealed that a few isomers were

observed by the treatments, a few new ones developed and few were lost. Few were lost or there was suppression of few isomers by the treatment of *B. pumilus* and AMF alone in comparison to healthy plants. In TV-20 variety, in untreated control two isomers- gallo catechin (GC) and gallo catechin gallate (GCG) with retention times of 4.59 min and 13.20 min, whereas, in *B.pumilus* treatment, one isomer- gallocatechin (GC) with retention time of 4.59 min were detected. However, no major loss of isomers was noted due to treatments indicating that flavour components were not lost.

In another set of experiments with AMF and PGPR growth promotion of tea saplings by another dominant AMF of tea, *Glomus fasciculatum* and PGPR- *B. amyloliquefaciens* was tested in six tea varieties (TV-18, T-17, AV-2, T-78, UP-3 and UP-26) in pot conditions and growth enhancement was evaluated in terms of percent increase in height and leaf number over similar increase in control plants. A significant increase in growth and number of leaves in all the tea varieties treated with *G. fasciculatum* and B. *amyloliquefaciens* was observed. In single treatments *B. amyloliquefaciens,* showed better growth than *G. fasciculatum.* However growth enhancement was significantly higher when both the AMF and PGPR were applied jointly.

The growth enhancement in tea saplings in pot conditions was also evaluated in terms of total phosphate mobilized by *Glomus fasciculatum* and *B. amyloliquefaciens* in pot conditions. Total residual phosphate content in soil, roots and leaves were evaluated. The overall resuts reveal that the total soil P-content decreased whilke the root and leaf phosphate increased following application of *B. pumilus* and *G. mosseae* singly or jointly which indicated efficient soil phosphate mobilization by both PGPR and AMF. Statistical analysis (ANOVA) revealed that the decrease in soil P and increase in root and leaf P-content were significantly enhanced when both *Glomus fasciculatum* and *B. amyloliquefaciens* were applied jointly.

Rhizosphere of tea was inoculated by *G. fasciculatum* and *B. amyloliquefaciens* prior to challenge inoculation with *Sclerotium rolfsii*. Development of blight was determined after 15, 30 and 45 days of inoculation on the basis of disease index. Results revealed that both microorganisms could reduce sclerotial blight, but maximum suppression of disease was due to joint inoculation. In order to confirm the induction of enhanced activities of

defense enzymes due to treatment of *G. fasciculatum* and *B. amyloliquefaciens* with or both, immunological tests were done using PAbs raised against chitinase. Enzyme extracts were used as antigens and Dot Blot were carried out. Results revealed that ELISA values of reaction of PAbs of chitinase 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. Besides, immunodetection of *S. rolfsii* in soil by ELISA and dot blot showed a significant reduction of population due to joint application of *G. fasciculatum* and *B. amyloliquefaciens* (Chakraborty *et al*, 2012). *G. fasciculatum* and *B. amyloliquefaciens* alone could effectively reduce disease incidence under glass house condition. However combined inoculation with *G. fasciculatum* and *B. amyloliquefaciens* showed better results.

Application of *G. fasciculatum* and *B. amyloliquefaciens* prior to pathogen challenge in tea saplings in nursery conditions was found to elicit a series of biochemical responses in tea plants. Experiments were conducted to assess the effect of single as well as combined application of *G. fasciculatum* and *B. amyloliquefaciens* on biochemical components of tea leaves. Multifold increase in activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in roots as well as leaf of tea plants was observed on application of AMF and PGPR to soil followed by inoculation with *S. rolfsii*. Overall results show that the defense enzyme activities were higher in leaves than the roots. Total phenol content in the roots and leaves were also evaluated which was found to be higher in the leaves than the roots.

Effect of *Glomus mosseae* and *Bacillus pumilus* for management of sclerotial blight disease of tea was tested in glass house as well as in nursery conditions. Under nursery condition, *G. mosseae* and *B. pumilus* alone could effectively reduce disease incidence. However combined inoculation with *G. mosseae* ad *B. pumilus* showed 20% disease incidence recorded after 45 days of pathogen challenge. *In vivo* tests carried out by Chakraborty *et al*. (2004), with *S. marcescens* revealed that application of bacterium, either as soil drench to two year old potted plants, or to seedlings at the time of transplantation, increased plant growth of tested tea varieties. It also decreased brown root rot and stem canker diseases of tea, caused by *F. lamaoensis* and *P. hypobrumea*.

Experiments were conducted to assess the effect of single as well as combined application of *G. mosseae* and *B. pumilus* on biochemical components of tea leaves. Activities of some of the enzymes which are involved in phenol metabolism as well as in defense-i.e., peroxidase, phenylalanine ammonia lyase, chitinase, β-1,3 glucanase were also determined. Polyphenols are major constituents of tea leaves and hence phenol contents were determined. Multifold increase in activities of chitinase, β -1,3-glucanase, peroxidase and phenylalanine ammonia lyase in roots was observed on application of AMF and PGPR to soil followed by inoculation with *S. rolfsii* which was significantly higher in those plants which were jointly inoculated with AMF and PGPR. Apart from this the total phenol content in the roots and leaves were also evaluated and was found to be higher in plants treated joinltly with *G. mosseae* and *B. pumilus*. Similarly, catechins were also analysed from leaves of tea plants treated with *S. rolfsii*, *S. rolfsii* + *B. pumilus*, *S. rolfsii* + AMF and *S. rolfsii*+*B. pumilus*+AMF. There was suppression of few isomers in joint treatments in comparison to individual treatment by *S. rolfsii*. In *S. rolfsii* treatment, gallo catechin (GC) with retention time of 4.60 was predicted as one isomer of catechin. However, no major losses of isomers were noted due to treatments indicating that lavour components were not lost.

Internal transcribed spacer (ITS) regions have been used successfully to generate specific primers capable of differentiating closely related fungal species (Bryan *et al*., 1995). The ribosomal RNA genes (rDNA) possess characteristics that are suitable for the identification of fungal isolates at the species level. 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; Yao *et al*., 1992) arranged in tandem repeats with each repeat consisting of the 18S small subunit (SSU), the 5.8S, and the 28S large subunit (LSU) genes. Therefore we focused on the ITS regions of ribosomal genes for the construction of primers that can be used to identify *Sclerotium rolfsii*. In the broader context, taxon-selective amplification of ITS regions is likely to become a common approach in molecular identification strategies. ITS region of rDNA was amplified using genus specific ITS-1 and ITS4 primers. Amplified products of size in the range of 500bp were produced by the primers. The results are in accordance with Mukherjee (2002), Chakraborty et al. (2011) who

studied the identification and genetic variability of the fungal isolates. These results are in accordance with several workers viz. Muthumeenakshi (1994), Ospiana (1999), Lieck-Feedt (1999) and Venkateswarlu (2008) who observed the amplified rDNA fragment of approximately 500 to600 bp by ITS-PCR in fungi.

Direct sequencing of the PCR products of rDNA regions of both pathogen, PGPR and MHB isolates showed satisfactory homology with ex-type strains in NCBI genbank database on the basis of which they were identified as *Sclerotium rolfsii, Bacillus pumilus, B. amyloliquefaciens* and *B. flexus*. The priming site of the ITS1 and ITS4 primers were determined in order to confirm that the sequences obtained corresponded to the actual ITS 4 region. A multiple sequence alignment was carried out that included the ITS 1 region, including gaps and the complete sequences align. There were quite a number of gaps that were introduced in the multiple sequence alignment within the ITS-4 region that were closely related and similar sequence indicated. These isolates were used in the pair wise and multiple sequence alignment. From the sequence alignment, variations were observed between each isolates of *Sclerotium rolfsii, Bacillus pumilus, B. amyloliquefaciens* and *B. flexus*. In conclusion, above results strongly indicated a isolates of NCBI database with similar "DNA-based sequence", which could be accommodated with similar forms as referred in the species identifying concept.

In the present investigation PCR products of *Sclerotium rolfsii, Bacillus pumilus* and *B. amyloliquefaciens* were sequenced bidirectionally through the BigDye terminator technology (applied biosystem, at Chromus biotech, Bangalore India). The sequence information was then analysed through BLSATn program which indicated that the sequences (235, 834 and 985 bp) contains the genetic information of internal transcribe spacer region of rDNA gene of *Sclerotium rolfsii, Bacillus pumilus, B. amyloliquefaciens* (Accession no JQ429785 for *Sclerotium rolfsii,* JQ765580 for *B. pumilus,* JN983127 for *B. amyloliquefaciens*)

The bacterial community can change as a result of mycorrhizal establishment (Marcschner *et al*. 2001) and AMF may act as a vehicle for spreading rhizobacteria that promote plant growth to neighboring rhizospheres. This hypothesis would be supported if rhizobacteria that promote plant growth could be found adhering to spores and hyphal

structures. The attachment of bacteria to the spores and hyphae involves colonization of a solid substrate and eventually, the bacteria assemble into complex clusters termed biofilms, which contain polysaccharides. Bacteria often associate with eukaryotic cells to establish endocellular symbioses, but AMF spores are unique in that they host bacteria in their cytoplasm (Lumini *et al.,*2007). Intracellular structures similar to bacteria, called bacteria like organisms have been observed inside AMF spores with transmission electron microscope. Most AMF carry bacteria and this relatuionship can be dated back to the time when AMF established symbioses with emergent land plants (Bonfante, 2003). These bacteria can colonize the surface of AMF spores and hyphae and they can also be located inside the spore walls. In addition, the bacteria affect spore germination, hyphal growth and root colonization (Hori *et al*., 2008).

In the present investigation, SEM images of the surface of *Glomus mosseae* spores, one of the dominant AMF associated with tea roots were studied. Young, light yellow brown spore revealed with its sloughed and eroded outer hyaline layer covering the whole surface, but older, medium brown spore had a residual outer hyaline layer. However, old, dark orange brown spore that has lost almost all its outer hyaline layer. Bacterial cells of various shapes adhering to the surface of the laminated layer, with holes were visible, possibly corresponding to lysis zones in the spore wall. To identify PCR products of MHB isolate T/Gl/1 was sequenced bidirectionally through the Big Dye terminator technology (applied biosystem, at Chromus biotech, Bangalore India). The sequence information was then analysed through BLSATn program which indicated that the sequences (1373 bp) contains the genetic information of rDNA gene of *Bacillus flexus* .

Based on 16S rDNA sequences the bacteria associated with *Glomus mosseae* was identified as *Bacillus flexus* and deposited in NCBI(Accession no KF552068).This isolate promoted spore germination of *G. mosseae* markedly. Filippi *et.al.(*1998) demonstrated that many bacteria were attached to the hyaline wall kayer of *Glomus mosseae* spores and that up to 10^7 CFU/g chitinolytic microorganisms were present on the sporocarp surface. They also found bacterial forms in holes within the outer layer of the *Glomus mosseae* spores and suggested that the holes were formed by the bacterial lytic activity.

Despite some progress, knowledge of mechanisms involved in the relationship between AMF and bacteria is still limited. This is mostly due to the difficulties involved in cultivating some of the species *in vitro*. Based on 16S rRNA gene sequences, the endobacteria in *Gigaspora margarita* were identified as belonging to the genus *Burkholderia*. Normally, *Burkholderia* spp. is easily cultivated. However, the specific strains related to *Gigaspora margarita* spores may be difficult to grow *in vitro* outside the spores. Direct molecular approaches that avoid a cultivation step give a broader picture of bacterial communities. PCR-denaturing gradient gel electrophoresis (PCR-DGGE) analysis of 16S RRNA gene permits fingerprinting of the dominant bacteria of a given sample.

Roesti *et.al*. (2005) studied bacteria associated with spores of the *Glomus geosporum* and *G. constrictum* using PCR-DGGE analysis, which 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 exudate 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.The most dominant bacterial population was constituted by the genus *Flexibacter*. This genus is well known for its ability to degrade biomacromolecules in various habitats. The presence of active biopolymer degrading bacterial populations on the spore surface could support also spore germination by releasing nutrients or degrading toxic compounds that inhibit germination. Thus, the process of maturation and eventual germination of AMF spores might benefit from the activity of the surface microorganisms degrading the outer hyaline layer.

Flourescent antibody labelling with FITC is known to be one of the best techniques to detect a number of microorganisms in a given sample. In the present study polyclonal antibody raised against *S. rolfsii* and *G. mosseae* were used to detect their presence as well as their nature of colonization in the host root tissues with the help of two fluorescent dyes FITC. Tea roots following colonization with AMF alone or in combination with PGPR could induce resistance against the pathogen. This was evident

in the previous experiments when the treated tea leaves showed bright apple green fluorescence in mesophyll tissues when these were labelled with FITC and reacted with PAb of chitinase. The present investigation was designed to locate the pathogen in the rhizosphere as well as presence of AMF in rhizoplane as well as their cellular localization in root tissues using PAbs of the pathogen (*S.rolfsii*) and AMF (*G. mosseae*). In untreated inoculated tea plants, where disease was prevalent, sclerotial population was also evident when soil samples were examined by indirect immunofluorescence test using PAb of *S. rolfsii*.

However, in the AMF treated tea plants, when PAb of *G. mosseae* was used in indirect immunofluoescence test, bright apple green fluorescence developed which was distributed throughout the spore wall. The green fluorescent was also prominent in the substending hyphae. In case of RITC labelled fluorescent staining of AMF spores, a red coloured flourescence 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 tea were also treated with PAbs of *G. mosseae* and labelled with FITC to specifically locate the hyphal proliferation within the tissues. Observations of treated root tissues under UV- microscope showed bright apple green fluorescence of the hyphae, vesicles and arbuscules within the host tissue. Fluorescence was more prominent towards the cell wall in most of the tissues clearly indicate the successful colonization of tea roots with AMF leading towards induced resistance against the pathogen. Besides, based on the findings of this study in another dimension, it can also be concluded that *Bacillus flexus* isolated from surface sterilized spores of *Glomus mosseae* act as potential for enhancing AMF root colonization on tea seedlings. However, ecology of such mycorrhiza helper bacteria (MHB) and the mechanisms involved in their effect on mycorrhizal symbiosis as well as knowledge about the fate of introduced MHB in the soil and in the rhizosphere needs further attention.

Conclusion

- Tea, Rubber, Areca, Coffee and Cinnamon were selected for AMF and PGPR isolation. Depending on the abundance two AMF spores were selected, i.e. *Glomus mosseae* and *G. fasciculatum* were taken up for all the *in vitro* and *in vivo* studies.
- Scanning electron microscopic observation was made for the AMF spores. *G. mosseae*, *G. constrictum* and *G. intraradisces* obtained from tea rhizosphere showed difference in their wall characters and ornamentations. Similarly *Gigaspora gigantean*, *Glomus fasciculatum* and *Acaulospora* sp. isolated from Rubber root rhizosphere showed adhered hyphae with its sloughed and eroded outer hyaline layer covering the whole surface area. *G. constrictum* showed attached hyphae and few pores in the spore surface where MHB (Mycorrhiza Helper Bacteria) might persist. *Acaulospora bireticulata* with ornamentation consists of hyaline to round-tipped polygonal structures and the attached sporiferous sacule
- *In vitro* germination of AMF spores was studied to screen their ability to grow in an artificial medium without any host and with root exudates. Water agar when supplemented with three days old maize root extract showed a moderate increase in growth
- Among all the bacterial isolates two bacterial isolates designated as RHS/T-382 and TRS-6 obtained from the rhizosphere of tea showed positive tests for all the PGPR tests like phosphate solubilization, protease, chitinase IAA and siderophore production. Scanning electron microscopic studies of both the bacterial isolates showed that they were characteristically rod shaped and the size of the bacterial cells ranged from 4-7 μm.
- Both the isolates RHS/T-382 and TRS-6 grew best at pH 6.0 and did not grow well below pH 4.0 and above 8.0. Six different media (PDB, NB, KB, LB, NSB, and GYP) were selected to assess the growth of the PGPR and it was recorded that NB is the best medium for growth of isolates RHS/T-382 and TRS-6. The growth of isolates RHS/T-382 and TRS-6 were observed at different temperatures

ranging from 20 to 50 ºC. NB medium was inoculated with the bacteria and flask was incubated at 20,.25, 30, 35, 40,45,50ºC. Bacteria grew well within these ranges of temperature but grew best at 35 ºC. Maximum growth was recorded after 4 days and then growth gradually decreased.

- Antibiotic sensitivity test was performed to know the strength of isolates RHS/T-382 and TRS-6 against particular antibiotic. Antibiotics was mixed at rate of 15µg/ml in Nutrient Agar medium; Results presented in Table 10 shows that RHS/T-382 is highly sensitive to amoxicillin, streptomycin, gentamycin whereas TRS-6 is highly sensitive to Ampicillin, Chloramphenicol, Kanamycin and Tetracycline
- The bacterial isolates RHS/T-382 and TRS-6 obtained from tea rhizosphere were designated as potential PGPR and both the isolates were confirmed with the help of 16S rDNA sequences. The BLAST query of the 16S r DNA sequence of the isolates against GeneBank database confirmed the identity of the isolate RHS/T-382 as *Bacillus pumilus* and TRS-6 as *B. amyloliquefaciens*.The sequences have been deposited in NCBI, GeneBank database under the accession no. JQ765580 for *B. pumilus* and JN983127 for *B. amyloliquefaciens*
- A multiple sequence alignment of ITS gene sequences of the pathogen *S. rolfsii* (RHS/T-381) was also conducted. Phylogenitic analysis of *S. rolfsii* was carried out with the Ex-type strain sequences obtained from NCBI Genbank Database which showed maximum homology with the isolate RHS/T-381.
- *Sclerotium rolfsii* was grown in PDA medium for a period of 30 days, mycelial growth was recorded after 5,10,15,20,25,30,35 and 40 days of growth and incubated at 25± 1ºC. Maximum growth was recorded after 20 days of incubation after which it declined. *Sclerotium rolfsii* grew to a lesser or greater extent over a wide range of pH (3.0-8.0), maximum growth was observed at pH 5.5 and then growth gradually declined. *Sclerotium rolfsii* revealed maximum growth using lactose as the carbon source while no growth was observed in sorbose which was similar to control set. Fructose and sucrose also supported comparatively good growth. It was revealed that maximum growth in beef extract followed by yeast extract and then in peptone. Among the inorganic sources calcium nitrate

supported maximum growth. Other inorganic sources supported lower growth than organic sources, though no growth was observed in urea and insignificant growth was noted in basal medium without nitrogen.

- Western blot analyses using polyclonal antibody of *S. rolfsii* revealed that the PAb collected after successful immunization of the rabbits for consecutive six weeks could show different levels of homologous reactions with the antigen of *S. rolfsii*. A sharp and intense band at 35 Kda was obtained 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 indirectimmuno fluorescence of young and mature sclerotia of *S. rolfsii.* The young and old sclerotia along with the mycelia treated with PAbs and labeled with FITC showed apple green fluorescence where fluorescence was more intense on young sclerotia.
- The antagonistic effect of the both the isolates *B. pumilus* RHS/T-382 and *B. amyloliquefaciens*-TRS6 which showed positive results in all the tested PGP characters were tested against tea root pathogens *viz*, *Sclerotium rolfsii, Fomes lamaoensis*, *Poria hypobrunnea* and *Spherostilbe repens*.
- Artificial inoculation of tea roots with *Sclerotium rolfsii* was carried out in experimental field following sick soil technique. Seedlings of a susceptible tea variety were grown in the field and disease development was noticed. Histopathological studies revealed the establishment of pathogen in root tissues along with above ground symptom. Results revealed that total phenol decreased following inoculation with *Sclerotium rolfsii* in the susceptible varieties. However there is an increase in the phenol content of resistant varieties following inoculation. Among all the varieties tested TV -20 showed maximum increase in total phenol following inoculation with the pathogen. PAL activity increased after 14 days of inoculation markedly in all the varieties except in TV-25.
- Mass multiplication of AMF was carried out in Maize plant in glasshouse conditions. Root colonization by *G. mosseae* was confirmed with the help of indirect immuno-fluorescent staining of maize root tissues after treating them with PAbs of *G. mosseae.* Root tissues labeled with FITC when observed under UV-

microscope showed bright apple green fluorescence of the hyphae, vesicles and arbuscules within the host tissue which confirmed successful colonization by *G. mosseae.*

- The next level of experiments was conducted in glass house and nursery conditions to evaluate the effect of AMF and PGPR in growth of tea seedlings. In the first set of test, four tea varieties (TV-25, TV-26, TV-9, and TV-20) were selected to test the effect of PGPR (*B. pumilus*) and AMF (*G. nosseae*) 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 that enhancement of plant growth by single as well as joint application of PGPR and AMF however the results reveals that the growth of tea saplings grown under same environmental and physical conditions were enhanced to a greater extent when both *B. pumilus* and *G. mosseae* were applied jointly. Among all the four varieties tested, maximum growth was noticed in TV 20 and TV 26.
- In another set of experiments with AMF and PGPR growth promotion of tea saplings by another dominant AMF of tea, *Glomus fasciculatum* and PGPR-*B*. *amyloliquefaciens* was tested in six tea varieties (TV-18, T-17, AV-2, T-78, UP-3 and UP-26) in pot conditions and growth enhancement was evaluated in terms of percent increase in height and leaf number over similar increase in control plants. A significant increase in growth and number of leaves in all the tea varieties treated with *G. fasciculatum* and B. *amyloliquefaciens* was observed. In single treatments *B. amyloliquefaciens,* showed better growth than *G. fasciculatum.*
- The growth enhancement in tea saplings in nursery as well as pot conditions was also evaluated in terms of total phosphate mobilized by both AMF and PGPR. Total residual phosphate content in soil, roots and leaves were evaluated. The overall resuts reveal that the total soil P-content decreased while the root and leaf phosphate increased following application of AMF and PGPR singly or jointly which indicated efficient soil phosphate mobilization. Similarly alkaline phosphatase activities was found to be much more lesser than the acid

phosphatase activities of the rhizosphere soil. On the other hand *G. mosseae* and *B. pumilus* efficiently mobilized soil macro and micro nutrients in nursery conditions measured in terms of availability of chief macro nutrients like Nitrogen (N), Phosphorus (P) as well as Potassium (K) in the rhizosphere soil even after three months of inoculation. Oxidizable organic carbon percentage (OC%) and Moisture percentage were also evaluated.

- Catechins were analysed in HPLC from leaves of variety TV-20 that showed maximum response towards AMF and PGPR in nursery whose rhizosphere was soil drenched with *Bacillus pumilus* and roots were inoculated with spores of *G. mosseae*. Analysis revealed that a few isomers were observed by the treatments, a few new ones developed and few were lost. Few were lost or there was suppression of few isomers by the treatment of *B. pumilus* and AMF alone in comparison to healthy plants. In TV-20 variety, in untreated control two isomers- gallo catechin (GC) and gallo catechin gallate (GCG) with retention times of 4.59 min and 13.20 min, whereas, in *B.pumilus* treatment, one isomer- gallocatechin (GC) with retention time of 4.59 min were detected.
- Next level of *in vivo* tests was conducted to study the effct of AMF and PGPR in management of sclerotial blight disease of tea caused by *Sclerotium rolfsii*. In the first set of trial, *G. fasciculatum* and *B. amyloliquefaciens* were tested for their effect in inhibiting sclerotial blight of tea saplings in pot conditions in glass house when applied singly and in combination. The experiment was set in complete random block design and both AMF and PGPR were inoculated to the rjhizosphere of three year old tea plants prior to pathogen challenge. Under pot condition, *G. fasciculatum* and *B. amyloliquefaciens* alone could effectively reduce disease incidence. However combined inoculation showed better results. In the second set of experiments, *Glomus mosseae* and *Bacilus pumilus* were tested for their effect in inhibiting sclerotial blight of tea saplings in nursery conditions when applied singly and in combination. Under nursery condition, *G. mosseae* and *B. pumilus* alone could effectively reduce disease incidence. However combined inoculation with *G. mosseae* ad *B. pumilus* showed 20% disease incidence recorded after 45 days of pathogen challenge.

- Application of both the AMF and PGPR prior to pathogen challenges either in pot or nursery conditions were found to elicit a series of biochemical responses in tea plants. Multifold increase in activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in roots as well as leaf of tea plants was observed on application of AMF and PGPR to soil followed by inoculation with *S. rolfsii*. Overall results show that the defence enzyme activities was higher in leaves than the 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 tissues. For this leaf section of the most responsive tea variety, TV-20 was taken. Immunolocalization of chitinase in treated tea leaves were observed by immunofluorescence study.
- Spores of the arbuscular mycorrhizal fungus – *Glomus mosseae* were harvested from tea rhizosphere followed by single-spore-derived cultures with *Sorghum bicolor* as host plant. Scanning electron microscopy of *G. mosseae* spores revealed that the thin outer hyaline wall layer gradually degraded and replaced by mucilaginous products. The rough surface composed of the degraded and mucilated outer hyaline layer was present to different extents depending on the spore matuarity stage. Out of 20 light-coloured spores observed, only 5 had a smooth surface, 11 were covered with roughness of up to half of the visible surface by SEM and the surfaces of 4 were entirely rough. Bacterial cells of different sizes were present either in the sloughing hyaline layer or on the surface of the second, laminated wall layer. The spore surface also contained many holes, possibly corresponding to lysis zones. A total of three bacteria (TGL1, TGL2 and TGL3) were successfully isolated from *Glomus mosseae* spore originally obtained from tea root rhizosphere. All the three bacteria isolated were rod shaped and gram positive.
- MHB obtained from *G. mosseae* was identified on the basis of 16S rDNA sequences. The BLAST query of the 16S r DNA sequence of the isolate T/Gl/1designated as MHB against GenBank database confirmed the identity of the isolate as *Bacillus flexus*. The sequences have been deposited in NCBI,

GeneBank database under the accession no. KF552068. A multiple sequence alignment of ITS gene sequences of *B. flexus* was conducted. The result reveals that there were quite a number of gaps that were introduced in the multiple sequence alignment within the region that were closely related and similar sequence indicated the relationship among the isolates.

- Polyclonal antibody raised against *S. rolfsii* and *G. mosseae* were used to detect their presence as well as their nature of colonization in the host root tissues with the help of two fluorescent dyes FITC and RITC. Tea roots following colonization with AMF alone or in combination with PGPR could induce resistance against the pathogen. This was evident in the previous experiments when the treated tea leaves showed bright apple green fluorescence in mesophyll tissues when these were labelled with FITC and reacted with PAb of chitinase. The present investigation was designed to locate the pathogen in the rhizosphere as well as presence of AMF in rhizoplane as well as their cellular localization in root tissues using PAbs of the pathogen (*S.rolfsii*) and AMF (*G. mosseae*).
- In the AMF treated tea plants, when PAb of *G. mosseae* was used in indirect immunofluoescence test, bright apple green fluorescence developed which was distributed throughout the spore wall. The green fluorescent was also prominent in the substending hyphae. In case of RITC labelled fluorescent staining of AMF spores, a red coloured flourescence 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 tea were also treated with PAbs of *G. mosseae* and labelled with FITC to specifically locate the hyphal proliferation within the tissues. Observations of treated root tissues under UV- microscope showed bright apple green fluorescence of the hyphae, vesicles and arbuscules within the host tissue. Flourescence was more prominent towards the cell wall in most of the tissues clearly indicate the successful colonization of tea roots with AMF leading towards induced resistance against the pathogen.

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APPENDIX A: List of thesis related publications

In Journals

1. **Chakraborty BN and De U.** Association and diversity of AM fungi with plantation crops. *J. Pl.Dis.Sci.* **8(1)**: 24-28, 2013.

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

In proceedings volume

Chakraborty BN, Chakraborty U, De U, Chakraborty AP and Rai K. Dual application of *Glomus mosseae* and *Bacillus pumilus* in enhancing growth of tea and suppression of sclerotial blight disease In: *Microbial resources for crop improvement* (Eds. Chakraborty BN and Chakraborty U), Satish Serial Publishing House, New Delhi, pp.69-83, 2013.

APPENDIX B: List of Abbreviations

ACC- 1-amino-cyclopropane-1-carboxylic acid hydrochloride
APS- Ammonium per sulphate
B. megaterium- Bacillus megaterium
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
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)
0.1(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