Discussion

In agricultural management one of the most important goals is to ensure the crop of enough nutrients and to prevent it from diseases. Traditionally, these goals have been achieved by using pesticides and a high input of fertilizer. However, these management practices lead to a high loss of biodiversity, since soil microorganisms play an important role in nutrient cycling and can reduce diseases, a new approach is to manage the system by increasing the soil biodiversity. 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 rhizodeposition 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.

Nearly 5-23% of all photosynthetically fixed carbon is being transferred to the rhizosphere through root exudates (Marschner, 1995).an increase in root exudation of organic solutes could affect the rate of phyto-siderophore release. In turn, rhizosphere microorganisms may interact symbiotically with roots to enhance the potential for metal uptake (Yang Ching *et al.*, 2000).

Rhizosphere bacteria can have a profound effect on plant health. Rhizosphere colonization is important not only as the first step in pathogenesis of soil borne microorganisms , but also is crucial in the application of microorganisms for beneficial puposes (Lugtenberg *et al.*, 2001); most significant among these applications are biofertilization , phytostimulations, biocontrol and phytoremediation(Lugtenberg, 2000). The prospect of manipulating crop rhizosphere microbial populations by inoculation of beneficial bacteria to increase plant growth has shown considerable promise in laboratory and greenhouse studies, but responses have been variable in the field (Basnayake and Brich, 1995).

Although non chemical control of pests and diseases seems an attractive option in sustainable agricultural systems, its practical application is severely constrained by lack

of reproducible results in comparison to the use of agrochemicals. The biotic and abiotic factors in the environment, which influence the efficiency of pathogen suppression, are not well understood. Nevertheless it is known that some soil become suppressive to fungal, bacterial and nematode pests when susceptible host plants are grown continuously for several years and there is evidence that this is due to the development of soil biological communities which inhibit pests survival or infection of the hosts.

Keeping the above in mind, the present study was undertaken in order to select potential microorganisms from tea rhizosphere and make detailed studies so that one or more of such microorganisms could be used as PSF and PGPRs. Large number of microorganisms were isolated from the rhizosphere of tea plants growing in Temi Tea Estates (hilly terrain of Sikkim). The population of microorganisms was found to be maximum in 35-40 year old plants and minimum in 10 year old bushes. Population of VAM spores were also determined, and compared with VAM spores rhizosphere of tea growing in the plains. In the present study among the population of AM spores *Glomus* sp. was one of the largest genera found in hill soil and also in plains soils and was further identified by following Taxonomic keys develop by Gerdemann and Trappe (1974), Trappe and Schenck (1982) and Hall (1984). The *Glomus mosse* selected for further experimental purposes

In the present study, among isolated fungal and bacterial genera *Aspergillus* sp. and *Bacillus* sp. were the dominant genera respectively. The isolated bacteria and fungi were tested against root rot pathogens- *Ustulina zonata, Fomes lamaoensis,* and *Sclerotium rolfsii* for determining antagonistic activity. From among all the samples tested two fungal and two bacterial isolates were initially selected which showed maximum antagonistic activities. Besides antagonistic study, phosphate solubilizing activity of antagonistic isolates was also tested in Pikovaskaya's solid and liquid medium. The isolate Ttf 4 and Ttb 14 showed maximum phosphate solubilization then other tested isolates. On the basis of both antagonistic tests and phosphate solubilization the fungal isolate Tt4 was identified as *Aspergillus niger* based upon its colony morphology, spore characteristics and microscopic studies. The bacterial isolate Ttb 14 was identified as *B. pumilus* on the basis of its morphological and biochemical tests. They were further selected for various experiments.

The abundance, diversity, and distribution of native population and inoculants strains in agricultural fields have been characterized using a variety of methods. Screening of rhizosphere micro flora for antagonism against pathogenic fungi in order to select suitable biocontrol agents has also been previously reported by a large number of workers. Kobayashi *et al.* (2000) isolated three bacteria showing antagonism to *Rhizoctonia solani* from the rhizosphere soil of different crops which they identified as *Pseudomonas flurescens*, *Bacillus cereus* and *B. pumilus*. In another study, 11 *Bacillus pumilus* isolates were evaluated by Bargabus *et al.* (2004), of which 2 strains were found to be most effective against *Cercospora beticola*. The potential of various isolates of *Bacillus pumilus* has thus been recorded previously also.

Some of the phosphates solubilizing bacteria are found antagonistic to plant pathogens; the combinations of the two attributes will be of great advantage in crop production. In this present scenario, eco-friendly alternative strategies such as use of bacteria from phylloplane rhizosphere and endophytic bacteria have been explored by Mondol *et al.* (1999), Saha *et al.* (2001) and Bhowmik *et al.* (2002); Chakraborty *et al.* (2008) in a similar study isolated fifty four *Aspergillus* spp. form soils of agricultural fields of North Bengal out of which 70 fungal isolates showed phosphate solubilizing activity. Further, quantitative evaluation of phosphate solubilization in liquid medium supplemented with two phosphate source (tricalcium phosphate and rock phosphate) was done. Three isolates of *Aspergillus niger* (ES/L-04, RS/P-05 and FS/L 40) showed high levels of activity. The next best were five isolates of *A. melleus* (RHS/R-2.FS/ L-13, RS/P-14, FS/L-17 and FS/L-18). One isolates of *A. clavatus* (RHS/P-38) showed a minimum phosphate solubilization activity. Nine phosphorous solubilizing *Aspergillus* isolates were tested for their effect on growth and nodulation in soybean plants.

At the onset, the optimum conditions of the growth of the selected bacterium and fungus were determined. The bacteria grew best between 35° C in nutrient broth medium at pH 6 followed by incubation at 4 day shows maximum growth. Kobayashi *et al.* (2000) observed that *Bacillus cereus* isolates 96 and *B. pumilus* isolate 235 have an optimal temperature for growth at 30° C but survived even at 41 ° C and 50 ° C respectively. One advantage of *Bacillus* sp. is their ability to form spores which are long lived and are resistant to heat and desiccation (Osbern *et al.* 1995). Umamaheswari et al. (2003)

assessed the growth of different strains of fluorescent pseudomonads under different pH and temperature conditions. Optimum pH for the different strains ranged between 6 and 7. While *A. niger* grew best PDA and PDB in pH 4.5 almost no growth at pH2 and pH10, with regard to temperature 30 ° C is optimum for its growth..

As *A. niger* and *B. pumilus* can suppress root pathogen in dual culture technique in laboratory condition, the potential of the selected bacterial and fungal isolates in plant growth promotion and disease suppression, *in vivo* experiments were carried out on tea plants which are perennial and also on annual plants, like marigold, and soybean. Different varieties of tea plants were selected starting from young seedlings in nursery to two year old. Significant promotion of growth was obtained in seedlings, as well as in two year old plants after applications with PSF and PGPR. Besides tea, in the present study, both *A. niger* and *B. pumilus* increase growth in soybean and flowering in marigold. Results thus revealed that plant growth is promoted by the rhizobacteria and fungus in both annual and perennials. They could to some extent reduce charcoal stump root rot intensity caused by *U. zonata*. (Hazarika *et al*., 2000) reported that Antagonistic microflora, viz. *Trichoderma viride, Trichoderma harzianum, Gliocladium virens, Bacillus subtilis and Pseudomonas fluorescens* have been evaluated earlier against *Ustulina zonata*, causing charcoal stump rot disease of tea. All antagonistic microflora were most effective in inhibiting the mycelial growth of *U.zonata* in dual culture.

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*.

In a similar way isolated *G.mosseae* were tested for their effect on growth, development and to disease suppression in tea plants. Results shows positive effects after treatments. The most recognized AMF potential to mobilize plant nutrients, specially phosphorous is one among the many functional attributes that qualify them to be the plant growth promoting microorganisms par excellence. Soil inhabiting pathogenic microorganisms have coevolved with AM fungi and have been exposed to the mycorrhizal condition of roots over thousands of centuries as evidenced by the very fact

that root disease causing pathogens have successfully adopted to parasitize mycorrhizal roots (Graham, 2001). AM fungi, on the other hand, encourage the plant roots to rapidly absorb solubilized (Kucey, P. *et al.* 1989). Advantage of AM spores is it do not easily loose their viability, and germinate again, so once applied to the field, the spores will adhere to the soil lattices and proliferate thus increasing the efficiency of the soil VAM, is an excellent soil aggregator, it changes the macro compounds into micro compounds which can be easily assimilated by the plant, hence the plant health will increase vigorously. The results obtained in the present investigation have pointed to the possible use of a combination of two or more products commercially in tea plantations to increase productivity and yield without the use of additional chemical inputs.

In the present study, the selected bacterium and fungus which showed antagonistic behaviour were tested *in vitro* for other properties related to plant growth promotion and disease suppression mechanism. Results revealed that *B. pumilus* could solubilize phosphate followed by drop in pH, and produce siderophores and volatiles as well as sufficient amount of IAA. However no HCN was produced and *A. niger* could also to solubilize phosphate, produce Siderophores and volatiles. Ability of bacteria and fungi to solubilize phosphate is an important criterion when considering their use as biofertilizers. In a similar study, it was reported that *Aspergillus* and *Penicillum* isolated from agricultural soil showed a maximum level of phosphate solubilization activity *in vitro* when liquid medium was supplemented with both tricalcium phosphate and rock phosphate separately (Pradhan and Sukla 2005). Acid production and drop in the pH of the medium have been reported in earlier studies (Alla 1994; Whitelaw 2000). Though the drop in pH was from 7 to 3.2 in our study, no significant relationship could be established in terms of phosphate solubilization and drop in the pH of the liquid medium. A greater part of soil phosphorous (95-99) % is present in the form of insoluble phosphates and cannot be utilized by the plants (Vassileva *et al.* 2001). However, many soil fungi and bacteria are known to solubilize these inorganic phosphates (Illmer and Schinner 1992).

In another study it was reported that *A. niger* was one of the most efficient phosphatase producing fungi among many PSFs screened for efficiency; it was also observed that the reduction of the pH of the medium was maximum with *A. niger* isolate

which efficiently hydrolyzed different compounds of organic phosphorous (Tarafdar *et al.* 2003) Solubilization of dicalcium phosphate and tri-calcium phosphate by thermophilic bacteria, actinomycetes and fungi was investigated by Sujatha *et al.* (2004). In general dicalcium phosphate was solubilized more readily then tricalcium phosphate. *Bacillus stearothermophilus, Thermoactinomyces sacchari, Thermomonospora sp.* a *Malbranchea pulchella var. sulfurea* proved to be good phosphate solubilizers which coluld exploited for phosphate solubilization. (Mikanova and Novakova 2002) reported that microbial solubilization of hardly soluble mineral phosphates in soil is an important process in natural ecosystem and in agricultural soil. (Iman 2008) isolated *Aspergillus niger* and *Penicillium italicum* from the soil and rhizosphere of different plants. They were tested for their efficacy to solubilize tri-calcium-phosphate (TCP) *in vitro* as well as their effect *in vivo* to promote the growth of soybean (*Glycine max* L.) plants grown in soil amended with TCP. The results showed high solubilizing index in agar plates. Also, they effectively solubilized TCP in Pikovskaya's liquid medium (PVK) and released considerable amounts of P into medium. The efficacy of *Penicillium italicum* to solubilize and release the inorganic P was 275 µg P ml-1 whereas *Aspergillus niger* showed better efficiency and produced 490 µg P ml-1 after seven days of incubation. (Chen 2006) advocated that the ability of a few soil microorganisms to convert insoluble forms of phosphorus to an accessible form is an important trait in plant growth-promoting bacteria for increasing plant yields.

(Machuca *et al.* 2001) demonstrated that fungi such as *Trametes versicolor* and *Wolfiporia cocos* produce hydroxamate derivates and oxalic acid. *W. cocos* was positive in the CAS assay and it was found to produce siderophores in liquid medium with a simultaneous pH drop to 2.5 due to a high concentration of oxalic acid accumulated in the culture broth. Siderophore production by various fungi was shown on modified CAS agar- plate assays (Milagres *et al.* 1999). Bearing in mind that some of these fungi solubilize rock phosphate, presumably by releasing metal- chelating metabolites (Vassilev *et al.,* 2006a), we can expect their application as biocontrol microorganisms with simultaneous P- solubilizing activity. Cell wall degrading enzymes, such as β- 1, 3- glucanases, cellulases, proteases, and chitinases are known to be involved in the activity of some microorganisms against phytopathogenic fungi (Ordentlich *et al.,* 1988; Shapira *et al.* 1989; Harman *et al.* 1993; Chernin *et al.,* 1995; Dunn *et al.* 1997). Particularly,

microbial chitinases have attracted attention as potential enzymes to control phytopathogenic fungi and insect pests (Stleger *et al.,* 1986; Roco and Perez, 2001). Fungal microorganisms, such as *A. niger* and *Ph. Chrysosporium*, were shown to degrade intact fungal melanin present in phytopathogens (Butler *et al.,* 2005).

Disease suppression is attributed to the general microbial activity of the compost microflora. However, additional inoculation with other beneficial microorganisms including P- solubilizing microbial cultures proved successful (Zayed and Abdel- Motaal 2005).

Productions of volatile compound by bacteria have also been shown to be an important mechanism of plant growth promotion. 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* cloud effectively control brown root rot of tea caused by *Fomes lamaoensis* (Chakraborty *et al.* 2006).

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. In the present study, of the 18 fungal isolates only 5 and among the 19 bacterial isolates only 5 shows phosphate solubilizing activity. The fungal isolates (Ttf4) shows maximum halo zone and bacterial isolates (Ttb14) shows maximum halo zone.

It is apparent from the results of the present study as well as studies by a large number of previous workers that PGPRs and PSF have the ability to promote growth in plants, which in many cases is associated with pathogen suppression in soil (Chanway *et al.* 2000; Castillo *et al* 2002).PGPRs and PSF secrete one or more metabolites in the soil which then elicits the observed response in the host. Thus, these microorganisms or their products have the ability to elicit responses at molecular level which would include activation of a number of metabolic pathways in the host, the end product of which is finally expressed as increased growth of plants or reduced disease. ISR is effective against different types of pathogens but differs from SAR in that the inducing PGPR does not because visible symptoms on the host plant (Van Loon *et al.,* 1998). (Pieterse *et al.* 2002) confirmed that to protect themselves from the disease, plants have evolved sophisticated defense mechanisms in which the signal molecules salicylic acid, jasmonic acid and ethylene often plays crucial roles. The phenomenon of systemic acquired resistance (SAR) suggests that there is a signal that originates at the site of elicitor (biotic or abiotic) application and moves throughout the plant. The activation of SAR turns the compatible plant–pathogen interaction into an incompatible one, reported by (Uknes *et al.* 1992). This resistance was correlated with the accumulation of pathogenesis related

(PR) proteins, generally assumed to be markers of defense response added by (Ward *et al.* 1991).

Competition for colonization sites and nutrients, scavenging nutrients, niche exclusion, production of anti- fungal compounds, excretion of Siderophores, growth inhibition via the production of bacteriocins and antibiotics, and induction of plant resistance have been the mechanisms involved in the disease suppressions reported by (Ongena *et al.* 2002).

Strains isolated from naturally disease - suppressive soils, mainly fluorescent *Pseudomonas* spp. have been demonstrated to reduce plant diseases by suppressing soil-borne pathogens. Some of these biological control strains are also able to reduce disease caused by foliar pathogens by triggering a plant mediated resistance mechanism called induced systemic resistance (ISR). The potential of PGPR in relation to improved tea growth has also been recognized by (Pandey *et al.* 2000; Pandey and Palni (2002).

The major components analyzed in tea leaves in present study were defense enzymes, polyphenolics, proteins, chlorophyll 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, PO and β-1, 3- glucanase (GLU), but not of (CHI) activity. Induction of defense related enzymes by *P.flurescens* 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.

Accumulation of defense enzymes such as PAL, PPO, PO, in tea varieties following inoculation with *U. zonata* were determined. PPO usually accumulated following inoculation of plants. Among all the stress related enzymes, the role of peroxidese has been most thoroughly worked out. PO is a metallo- enzyme containing porphyrin bound iron. The enzyme acts on a wide range of substrates including phenols,

aromatic amines, amino acids and inorganic compounds. These are ubiquitous to plants and are characterized by a large number of isozymes. The induction of PO activity by pathogens and methyl jasmonate and existence of multiple molecular forms of peroxidase in tea has also been reported (Sharma and Chakraborty, 2004). Previous reports indicate that oxidative enzymes such as PPO and PO as well as those involved in phenolic biosnthesis such as PAL are involved in defense reaction in plants. (Bhattacharya and Ward 1987) reported that PAL activity in soybean was enhanced in the resistance response of soybean hypocotyls to *Phytophthora megasperma.* Considering that PAL is a key enzyme in the biosynthesis, not only of phytoalexins, but also of phenolics compounds have been associated with resistance responses in various host plants, it may be suggested that activity of PAL could be useful indicators of the activation of defense enzymes.

Flavonoids play an important role in growth, development and defense against microorganisms and pest (Dixon *et al* ,1999) and are involved in production of phytoalexins, act as UV protectants insect repellents and signal molecules in plant microbe interaction.(Winkel *et al.*, 2001). Besides, they also function as complex polymeric constituents of the leaf surface and support structures such as suberin, lignin and other cell- wall components (Dixon *et al.*, 1999).

No significant changes in protein content was observed by treatments. Chlorophyll content however increased with the application. 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.

Phenols are also known to play definite roles in a plant defense (Panwar and Vyas, 2002). Considering this in the present study phenol contents of the healthy PGPR treated, artificially inoculated (*U. zonata*) plants were determined. It has been reported previously that quinines in plant tissues react with proteins to form melanin and other tannins leading to the discoloration of damaged tea leaves. Many studies have demonstrated the importance of phenolic compounds in plant defense. In general, plant

phenolics have a diverse range of biological activity, depending on their structure, degree of polymerization; stereo isomeric differences etc. Interaction between phenolic compounds and environmental conditions determines their action. Polyphenols have a distinctive ability to engage in molecular recognition, or formation of intermolecular complexes with each other and with other molecules (Haslam, 1999). In the present study, total phenol content decreased with pathogen infection. However PAL activity increased significantly. The level of antifungal phenolics (pyrocatechol) in healthy and *U. zonata* infected tea varieties were estimated. Alteration of phenol metabolism following fungal infection has been observed in many disease and phenolics have been implicated in the defense reaction in several instances (Mahadvan, 1991). The involvement of phenol in the defense strategies of tea plants against foliar fungal pathogen (*Bipolaris carbonum, Pestalotiopsis theae, Glomerella cingulata*) has been described by (Chakraborty *et al.* 1995). Biochemical responses to tea plants exposed to biotic stress due to blister blight infection caused by *Exobasidium vexans* in the levels of phenols and enzymes activities were studied. (Sharma and Chakraborty, 2004).

The efficiency of eight arbuscular mycorrhizal species collected from rhizosphere soil of *Moringa concanecsis* from Indian Thar desert was tested. Mycorrhizal colonization resilted in increased accumulation of nutrients, chlorophyll, carotenoids, sugars and proteins. Among the eight AM fungi used, *Gigaspora margarita* (Becker & Hall) proved to be the most efficient. (Panwar and Vyas, 2002).

Since the isolated bacteria and fungi inhibited the growth of one or more fungi *in vitro*, it was expected that they would produce antifungal compounds. Keeping this in mind, active principles responsible for growth inhibition were extracted both from whole cells and cell free culture filtrates. It was observed that extracts from whole cells as well as cell free culture filtrates could inhibit spore germination and growth of several test fungi. Besides, the cell free culture filtrate was also extracted with various solvents and bioassayed. Though inhibition was obtained in most of the fractions, maximum inhibition was obtained in diethyl ether fraction of the bacteria. Results indicated that the antifungal compounds present in the bacterial and fungal cells were secreted into culture and these were solvent extractable. (Kyong and Dal 2003) also obtained an antifungal antibiotic from *B. megarterium* KL 39, which was isolated from a local soil of Korea. The crude

extract was reported to be active against a broad range of phytopathogenic fungi including *Rhizoctonia solani, Monilinia fruticola, Botrytis cinerea, Alternaria KiKuchiana, Fusarium oxysporium and F.solani.* They suggested that this antibiotic had a powerfull biocentrol activity against red pepper *phytophthora* blight disease. In an earlier study, (Chakroborty *et al.* 1998) extracted an antifungal compound from *Micrococcus luteus* which was originally isolated from tea phyllosphere. This compound also showed maximum activity in diethyl ether. In a similar study, using microorganisms from tea rhizosphere, (Barthaur and Bezbaruah 1997) isolated an antifungal compound from Proteus strain. This was shown to inhibit growth of several Fusarium sp. as well as tea root rot pathogens *F.lamaoensis and U.zonata.* it was also reported by (Kobayashi *et al.* 2000) that inhibition of *R. solani by P. flurescens, B. ceres and B. pumilus* was due to production and secretion of at least one antibiotic. Antifungal metabilites were also extracted from *P. fluorescens and B. subtilis* which inhibited growth of *Pythium aphanidermatum* and had maximum peak absorption of 200 nm (Kabitha *et al.* 2003). It is clear from the results of the present study and that of previous workers that different species of *Bacillus* produce various antifungal metabolites in culture.

As no microorganism survives individually in the soil they would no doubt be interacting among themselves in the rhizosphere,some of which would be antagonistic and some would be synergistic. In present study *in vivo* experiments were carried out in tea plants in order to find out the effect of dual application of *G. mossae, B. pumilus,* and *A. niger* results revealed that compared to individual treatment the effects of dual application shows good results. AM fungi and bacteria can interact synergistically to stimulate plant growth through a range of mechanisms that include improved nutrient acquisition and inhibition of fungal plant pathogens. These interactions may be of crucial importance within sustainable, low- input agricultural cropping systems that rely on biological processes rather then agrochemicals to maintain soil fertility and plant health.(Artursson, et al.2006). The synergistic effects of plant growth-promoting rhizobacteria and an arbuscular mycorrhizal (AM) fungus (*Glomus fasciculatum*) on plant growth, yield and nutrient uptake of sorghum plants were determined in field conditions by Mathur *et al.* (2009).

Direct contact between the spores and bacteria was necessary for the induction of spore germination in *Glomus clarum* (Xavier & Germida, 2003), indicating a ligand – receptor interaction betweem the two microbes.

Uses of bacterial consortia have sometimes shown to be better option than single ones especially when being applied as formulation. (Tilak *et al.*, 2006) observed that dual inoculation of pigeon pea with PGPR including *P. fluorescens* and *B. cereus* along with *Rhizobium* sp. increased growth nodulation and nitrogenous activity by various degree. They reported that the combination of *Rhizobium* sp. with *Azotobacter chroococcum* or *Azospirillum brasilens* registered a marginal but non significant increase over inoculation of *Rhizobium* alone. Most of the previous work in plant growth promoting rhizobacteria had focused on two genera- *Pseudomonas* and *Bacillus* and hence several reports are available regarding the PGPR activity of these two bacteria. in greenhouse experiment soybean plant growth and nodulation was obtained from the culture supernatant of *Serratia proteamaculans* strain 1-102. As tea is cultivated mainly for its leaves, the induction of new shoots and more leaves have great impact in considering plant growth promoton. (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) 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. Application of an aqueous suspension of *O. anthropi* to the rhizosphere of nursery grown tea seedlings of five varieties of tea (TV-18, T-17, HV-39, S-449 and UP-3) led to enhanced growth of the treated plants, as evidenced by increase in height, in the number of shoots and number of leaves per shoot. Treatment with *O. anthropi* also decreased brown root rot of tea. Multifold increase in activities of chitinase, β-1,3- glucanase, peroxidase and phenylalanine ammonia lyase in tea plants was observed on application of *O. anthropi* to soil followed by inoculation with *P. noxius*. A concomitant increase in accumulation of phenolics was also obtained. Further, talc based formulation of *O.*

anthropi was prepared and its survival determined every month up to a period of 12 months. *O. anthropi* could survive in the formulation up to a period of 9 months with a concentration of 7.0 $\log_{10}$ CFU g-1, after which there was a decline. Talc formulation was as effective as aqueous suspensions in both plant growth and disease suppression.

Two tea rhizosphere microorganisms, *Bacillus megaterium* and *Ochrobacterium anthropi* inhibited the growth of four tea pathogens (*Fomes lamaoensis, Sphaerostilbe repens, Poria hypobrumea* and *Sclerotium roulfsii*) to a certain degree in both solid and liquid medium. Application of *B. megaterium* and *O. anthropi*, either singly or in combination, to rhizosphere of *Camellia sinensis* promoted growth of seedling significantly, but the dual application was more affective. Besides, the bacteria could also control brown root rot of tea, caused by *F. lamaoensis*, B. *megaterium* was more effective than *O. anthropi.* Root colonization by the bacteria, followed by challenge inoculation with the pathogen, induced activities of defense enzymes β-1,3-glucanase, chitinase, phenylalanine ammonia lyase, as well as peroxidase in tea leaves (Chakraborty *et al.*, 2007)

Zaidi and Khan (2006) evaluated the effects of nitrogen fixing (*Bradyhizobium* sp.), phosphate solubilizing bacterium (*Bacillus subtilis*), phosphate solubilizing fungus (*Aspergillus awamori*) and AM fungus (*Glomus fasiculatum*) on the growth, chlorophyll content, seed yield, nodulation, grain protein, and N and P uptake of green gram plants grown in phosphorus-deficient soils. The triple inoculation of AM fungus, *Bradhyhizobium* sp. (vigna) and *B. subtilis* significantly increased dry matter yield, chlorophyll content in foliage and N and P uptake of green gram plants. Seed yield was enhanced by 24% following triple inoculation of *Bradyrhizobium* + *G. fasiculatum* + *B. subtilis*, relative to the control.

Interaction between VAM fungus *Glomus mosseae, Azospirillum brasilense and Azotobacter* in soil and their consequent effect on growth and nutrition of Neem seedling were studied under glass house conditions by (Sumana *et.al.,* 2002). Dual inoculation of *G. mosseae* and *A. chroococcum* resulted in maximum plant biomass, nitrogen and phosphorus uptake; biovolume index and quality of neem seedlings. It also increased the mycorrhizal root colonization and spore number in soil of the root-zoon. Dual inoculation of *G. mosseae* and *A. brasilense* improved plant growth more then the single inoculation

and uninoculated control. The VAM fungi and phosphate solubilizing bacteria (PSB) are the two groups of soil microorganisms associated with Phosphorous (P) nutrition of plants.

Studies including dual inoculation with AM fungi and other P-solubilizing microorganisms (Vassilev *et al* . 2005b) can be expected as the combinations of two such partners with complementary mechanisms might increase overall biocontrol and plant- growth – promoting efficacy, thus providing an environmentally safe alternative to chemicals

Bora *et al.* (2003) studied the effects of dual inoculation of vesicular arbuscular mycorrhiza (VAM) and phosphate solubilizing fungi on growth of young tea seedling and shows that dual inoculation in soil mixed with rock phosphate significantly enhanced the growth of tea seedling in terms of plant height , number of leaves, number of branches and dry weight of shoot and root uptake by seedling, mycorrhizal development in terms of spore density and percent colonization in plant roots was also enhanced by the combined inoculation treatment.

Certain bacteria which enhance the activity of AM fungi were described as mycorrhization helper bacteria (MHB) by Caroline and Bagyaraj (1995). These bacteria detoxify the substrate, making it more ideal for mycorrhizal infection and produce hydrolytic enzymes that dilate the cortical cell, thereby enhancing better colonization by mycorrhizal fungi. Further they are known to produce plant growth promoting substances (Duponnois and Garbye, 1991). Co inoculation of AM fungi with MHB was found to have synergistic interaction with consequential benefit on plant growth confirmed by (Jayanthi and Bagyaraj 1998).

As AM spore is difficult to multiply in vitro condition, their mass multiplication was done in Maize, Sorghum, *Cynodon dectylon.*

PSMs survive longer around mycorrhizal then non-mycorrhizal roots of maize and lavender and sometimes act synergistically with the mycorrhizal fungus to increase plant growth especially when rock phosphate was added to soil. Phosphate level in the roots was found to be more in those plants treated with *A. niger* and *B. pumilus.*

In the previous experiments it has been proved already that the dual application is effective then individual treatment, further test was carried out to find the combined effect of the Josh (VAM), Kalisena (*A. niger*), and PGPR (*B. pumilus*) treatments.

The growth of tea seedling inoculated individually with Josh, Kalisena and also two year old plant shows increase when compared to control. But the maximum growth was observed in combined treatment. When biochemical changes were analyzed results showed increase in chlorophyll content, followed by enzymes activities. Here also combined inoculation showed maximum increase.

Considering the result of above experiments it could be said that Josh (VAM), Kalisena and *B. pumilus* (PGPR), showed ability to increase growth of tea plants and induce resistance. Analysis of biochemical components have shown that these have a certain and obvious capability of inducing certain biochemical changes in the plant tissue system enhancing the production of compounds instrumental in aiding the host defense mechanism. The treatment with different combination/ coinoculation did bring about the increase in the defense related compounds like phenols and defense enzymes, yet the biochemical analysis showed certain fluctuations according to various plant materials. But VAM treated plants showed steady and increased concentration and activities of phenols and defense related enzymes. Mycorrhizal plants develop extensive root system as compared to non mycorrhizal plants, which ensures the plant with increased availability of nutrient there by helping the plant for better growth and development (Carling *et al.*, 1978). Mycorrhizal plants usually have more vascular bundles. Hence lignifications in the xylem is greater (Dehne, 1982) which may be instrumental in restricting pathogen proliferation. The possible mechanism may be competition for colonization sites, direct antibiosis and defense reaction colonization of roots by AM fungi induces biochemical changes within host tissues, these include stimulation of phenyl propanoid pathway and activation of defense related genes.

Kalisena SD is bio agent, reported to be very effective in controlling wilts, roots rots, damping- off and charcoal rot incited by *Fusarium, Rhizoctonia, Pythium* and *Macrophomina* on a wide range of crops including Muskmelon, Watermelon, Cauliflower, Guava, Sunflower and Potato have been reported. It survives in all types of soil and temperature with no specific moisture and pH range and it has two way actions

on crop plant: 1. As Biofungicide controls seven dreaded pathogen causing disease like Fusarium wilt, Sclerotinia rot, Rhizoctonia black scurf, damping off and many other soil borne pathogens, affecting agricultural crops. 2. As Bio-fertilizer increases phosphate solubilization improves germination and promotes plant growth. It releases growth promoting compound and increases crop yield.

VAM fungi from a special symbiotic relationship with plant roots can enhance growth and survivability of colonized plants. Many benefits are commonly found with VAM fungi that may result from use of the product Josh where active ingredient is VAM fungi with neutral carrier.

Talc based formulation of another PGPR (*Bacillus megaterium*) has been exploited for improvement of health status of tea plants against *Fomes lamaoensis* , brown root rot pathogen (Basnet *et. al*, 2009). This formulation was prepared using Carboxymethyl cellulose with charcoal powder as carrier and tested under green house conditions for their effect on growth promotion of tea seedlings. Observations were recorded after 2 and 4 months of application which revealed the significant growth promotion was accorded by application of bacterial suspensions. Selection of charcoal as carrier was done because of it being cost effective and commonly available. With increase in the focus of application of biofertilizers and biocontrol agents research on production of formulation have also increased. Inoculants development has been most successful to deliver biological control agents of plant disease that is organism capable of killing other organism pathogenic or disease causing to crops. Bacteria in the genera *Bacillus, Streptomyces, pseudomonas, Burkholderia and Agrobacterium* are the biological control agents predominantly studied and increasingly marketed. They suppress plant disease through at least one mechanism: induction of systemic resistance, and production of siderophores or antibiotics.

The application of five commercial chitosan based *Bacillus* formulations were found to be effective in increasing the growth and grain yield of rice. A formulation Elexa 1M was also reported to induce resistance to downy mildew disease and growth promotion in pearl millet (Sharathchandra *et. al.* 2004). Seed treatments with talc formulation of *B. subtilis* in glucose, talc and peat significantly enhance its biocontrol activity against *F. oxysporum* causing vascular wilt of lentil. It was also shown that

application of talc formulation of *P. fluorescens* along with *T. viride* either singly or combine decrease the sheath blight disease and increase plant growth and yield in rice (Mathivanan *et. al.* 2005). In the present study it was felt necessary to determine the sustainability of PGPR in the soil as this would be important in the field. Hence the survival of Bacteria applied either as aqueous solution in the soil or in the form of bioformulations was determined. The periods of survival of bacteria in the formulation were also determined. Determination of bacterial and fungal survival in soil and formulations were done by immunological techniques using antibodies raised against the bacteria and fungi, These techniques i.e. Elisa and Dot blot gave very specific and accurate results as the antibodies specifically reacted only with the specific bacteria and fungi. Results of both ELISA and Dot blot showed that they survived at high concentration even after six months on inoculation when the analysis was performed. Viability of the bacteria as determined by ELISA was evident in the formulations even after four months.

Talc–based formulations containing cells of *Pseudomonas flurorescens, Bacillus substilis and Saccharomyces cerevisae* were evaluated for their potential to attack the mango (*Mangifera indica* L.) anthracnose pathogen *Colletotricum Gloeosporiodes* Pebnz.under endemic conditions by Vivakananthan *et al.* (2004). The preharvest aerial spray was given at fortnightly and monthly intervals. The plant growth promoting rhizobacteria *Pseudomonas flurescens* (FP7) amended with chitin sprayed at fortnightly intervals gave the maximum induction of flowering, a yield attribute in the preharvest stage, consequently reduced latent symptoms were recorded at he postharvest stage. An enormous induction of the defence –mediating lytic enzymes chitinase and β- 1,3-glucanase was recorded in colorimetric assay and the expression of discrete bands in native PAGE analysis after FP7+ chitin treatment. The enhanced expression of defence-mediating enzymes may collectively contribute to suppress the anthracnose pathogen, leading to improved yield attributes.

The species of *Pseudomonas, Micrococcus, Bacillus, Aerobacter, Xanthomonas, Brevibacterium, Alcaligenes, Rhizobium, Rhodotorula, Penicillum, fusarium, sclerotium, aspergillus, pythium, phoma and cladosporium,* have been reported to be active in phosphate solubilization. The phosphate solubilizres also produce phytohormones and

growth promoting substances. The phosphate solubilizing bacteria are mostly of the genus *bacillus.* So far *Bacillus magaterium var. phposphaticum* has been used for preparation of the biofertilizer, known as Phosphobacterin. In addition to this, *B. firmus, B. circulans, B. subtilis and Pseudomonas striata* should also be considered suitable for inclusion in Indian conditions.

Population of *U. zonata* was also determined in the soil using PAbs raised against mycelia of U. *zonata* the causal agent of charcoal stump rot of tea. It was shown that populations of the pathogen reduce significantly in the bacteria and fungi treated soil, as detected on the analysis by ELISA and Dot blot. In an earlier study, (Mohandas *et al.* 2005) reported Immuno detection of *Phytophthora parasitica* var. *nicotianae* in papaya root pretreated with biocontrol agents. In plant treated with biocontrol and *Phytophthora* there was a considerable decrease in the *Phytophthora* population as seen by the number of florescent colonies as compared to plants treated with *Phytophthora* alone. A maximum reduction in *Phytophthora* population was observed in VAM + *Trichoderma* (19.6%) treated plant s followed by *Trichoderma* (86.2%) and *Pseudomonas* (79.3%) treated plants. The overall results of the present study have shown that rhizobacteria and fungi from tea rhizosphere *B. pumilus* and *A. niger* could induce plant growth promotion and disease reduction in tea and as well as in other crops. Regarding the mechanism of action of the bacteria it seems probable that these bacteria act through a combination of methods. It is difficult to predict the actual happening in the soil environment but probably the PGPR secrete metabolites in the soil which in turn elicit responses in the host.

It is also important in the studies on host parasite relationship to determine the cellular location of the pathogen. For this purpose in this study, Indirect immunofluorescence tests were conducted with cross sections of healthy and artificially inoculated (*U. zonata*) tea roots and mycelia. Transverse section from infected roots was made and PAbs raised against mycelial antigens of *U. zonata* were used for probing the fungal hyphae which penetrate the root tissues. Bright florescence was observed in the cross section of tea roots. Detection of pathogen in host tissues using antibody based immunoflurescent technique has been reported by several previous authors (Reddy and Ananthanarayan, 1984). Different test formats including indirect ELISA, western

blotting, dot blot and indirect immunoflurescence were assayed by (Wakeham and White 1996) for detection of resting spores of *Plasmodiophora brasica* in soil. In conclusion, it can be stated that charcoal stump rot can cause severe damage to tea plants, as primary root disease and such immunodetection technique makes it possible to detect micro quantities of the pathogen within root tissue and rhizosphere soil before much damage cause by the pathogen.

The overall results of the present study have shown that phosphate solubilizing microorganisms isolated from tea rhizosphere, could induce plant growth promotion and disease reduction in tea as well as in other crops. Different species of *Bacilli*, *Glomus* and *Aspergilli* are now widely used in other crops as plant growth promoters as well as biocontrol agents (BCA). Development of better formulations to ensure survival and activity in the field and compatibility with chemical and biological seed treatments is another area of focus; approaches include optimization of growth conditions prior to formulation and development of improved carries and application technology.

It is now widely recognized that biological control of plant pathogen is a distinct possibility for the future and can be successfully exploited in modern agriculture, especially within the framework of integrated disease management system. The rationale behind the disease control is to check pathogens growth in the host and improve the health status of the plant.