# DISCUSSION

Microorganisms in soil are critical to the maintenance of soil function in both natural and managed agricultural soils because of their involvement in such key processes as soil structure formation; decomposition of organic matter; toxin removal and the cycling of carbon, nitrogen, phosphorous and sulphur. In addition, microorganisms play key roles in suppressing soil borne diseases, in promoting plant growth and in changes in vegetation (Garbeva *et al.* 2004). Relationships are often observed between the extent of microbial diversity in soil, soil and plant quality and ecosystem sustainability. Soil represents a highly heterogeneous environment for the microbiota inhabiting it; the different components of the solid fractions in soil (sand, silt, clay and organic matter) provide myriads of different microhabitats (van Elsas and Trevors, 1997). The organisms resident in soil are exposed to abiotic and nutritional conditions that may vary even over the micrometer scale i.e. the scale experienced as their biosphere. These organisms collectively are the underlying catalysts of the biochemical processes in soil. Thus, the microbial processes in soil, including those resulting in disease suppressiveness, clearly take place at the scale of microhabitats and organismal biospheres. These processes are susceptible to major changes in surroundings, whereby a measurable effect will be the result of individual shifts at micrometer scale (Garbeva *et al.*, 2004).

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 purposes (Lugtenberg *et al.*, 2001). Most significant among these applications are biofertilization, phytostimulation, biocontrol and phytoremediation (Lugtenberg, 2000). Colonising microorganisms can be detected attached to the roots, as free organisms in the rhizosphere or as endophytes. The interactions between plants and microorganisms are immensely complex and very little is known about the sum of factors that lead to reliable biocontrol and biofertilisers applications.

In the present study, a large number of microorganisms were isolated from the rhizosphere of tea plants growing both in the hills and the terai regions. The isolated bacteria were tested against root rot pathogens- *Fomes lamaoensis*, *Poria*

*hypobrumea*, *Sclerotium rolfsii* I, *Sclerotium rolfsii* II and *Sclerotinia sclerotiorum* for determining antagonistic activity. From among all the samples tested four were initially selected which showed antagonistic activities. Of these four, two were finally selected for the present study and these were characterized and identified as *Bacillus megaterium* TRS 3 and *Ochrobactrum anthropi* TRS 2. Several previous authors have reported screening of rhizosphere microflora for antagonism against pathogenic fungi in order to select suitable biocontrol agents. Kobayashi *et al.* (2000) isolated three bacteria showing antagonism to *Rhizoctonia solani* from the rhizospheric soil of different crops which they identified as *Pseudomonas fluorescens*, *B. cereus* and *B. pumilus*. An evaluation of rice rhizosphere was conducted by Torres-Rubio *et al.*, (2000) from which 69 bacteria were isolated including *Pseudomonas* sp. and *Azotobacter* sp. Among 106 *Bacillus* strains isolated from various plant roots, Bae *et al.* (2003) selected three promising biocontrol agents screened against root rot pathogens *Cylindrocarpon destructans*.

At the onset, the optimum conditions of the growth of the selected bacteria were determined. Both the bacteria grew best between 20°C - 40°C in nutrient broth medium at pH 6. Kobayashi *et al.*(2000) observed that *B. cereus* isolate 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 & 7.

In the present study, the two bacteria which showed antagonistic behaviour were tested *in vitro* for other properties related to plant growth promotion and disease suppressing mechanism prior to their use *in vivo*. Results revealed that both the bacteria were able to produce IAA, volatiles, siderophores and solubilised phosphates *in vitro* but did not produce HCN and chitinase. Production of phytohormone IAA is wide spread among PGPR that inhabit the rhizosphere of crops. Torres-Rubio *et al.* (2000) also reported that all the microorganisms isolated from rice rhizosphere produced IAA in the medium. Khalid *et al.* (2004) evaluated thirty isolates from the rhizosphere soil of wheat plants for their potential to produce

auxins *in vitro*. They designated four isolates as plant growth promoting rhizobacteria (PGPR) based upon auxin production and growth promoting activity. Ability of bacteria to solubilise phosphate is an important criterion when considering their use as biofertiliser. Out of 37 *Acenetobacter* sp. isolated from rhizosphere of wheat, 36 were able to solubilise phosphates under different experimental conditions (Chopade, 2003). He reported that all the phosphate solubilising *Acenetobacter* strains had zone diameter of dissolution in the range 1-5 cm. Production of volatile compound by bacteria have also been shown to be an important mechanism of plant growth promotion. In confirmation with the result obtained in the present study, Ryu *et al.* (2003) reported that *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a released two compounds into culture which they identified as 3-hydroxy-2-butanone and 2,3-butanediol. Corsea *et al.*(2005) isolated rhizobacteria with properties related to plant growth promotion from the rhizosphere of the perennial legume *Chamaecytisus proliferus* spp. *proliferus* var. *palmensis* (tagasate) growing in field conditions. Among all isolatessof the species *Pseudomonas fluorescens* showed the maximum properties related to plant growth promotion, ACC deaminase activity, phtohormone production, nitrogen fixation, fungal growth inhibition and cyanogenesis and making it suitable for field testing. Siderophore production has also long been considered as one of the mechanisms of suppression of fungal growth in the rhizosphere. 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 resistance (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 produced siderophore and inhibited growth of *F. lamaoensis*. *P. aeruginosa, P. putida* and *P. fluorescens* 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 siderophore producers.

Since the two isolated bacteria *B. megaterium* and *O. anthropi* 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 extract 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 both the bacteria. Results indicated that the antifungal compounds present in the bacterial cells were secreted into culture and these were solvent extractable. Maximum absorption of the compound *B. megaterium* and *O. anthropi* were 236 and 230 nm respectively. Analysis by HPLC also revealed compound with very less retention time. Kyong and Dal (2003) also obtained an antifungal antibiotic from *B. megaterium* 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, Monillinia fructicola, Botrytis cinerea, Alternaria kikuchiana, Fusarium oxysporum* and *F. solani*. They suggested that this antibiotic had a powerful biocontrol activity against red pepper phytophthora blight disease. In an earlier study, Chakraborty *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, Barthakur and Bezbaruah (1997) isolated an antifungal 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. fluorescens, B. cereus* and *B. pumilus* was due to production and secretion of at least one antibiotic. In another study isolates of *B. subtilis* and *B. lentimorbus* which were antagonistic to *R. solani* were also reported to produce diffusible and volatile antibiotics (Montealegre *et al.* 2003). New antifungal compounds were isolated from *P. fluorescens* by Bajsa *et al.* (2003)

which inhibited *R. solani.* Antifungal metabolites were also extracted from *P. fluorescens* and *B. subtilis* which inhibited growth of *Pythium aphanidermatum* and had maximum peak absorption of 200 nm (Kabita *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. However, no report is yet available on the production of antifungal metabolites by *O. anthropi.*

Series of *in vivo* experiments were next carried out with the two selected bacteria to determine their plant growth promoting activity in the field. This was tested on tea plants which are perennial as well as on chickpea which is an annual plant. Different varieties of tea at various growth stages were selected starting from young seedlings in nursery to 10 years old bushes in the field. Significant promotion of growth was obtained in the seedling, in two year old potted plants as well as in the field. When the bacteria were applied as soil drench or foliar spray, both the bacteria promoted growth to a more or less similar degree. Though both the species had the ability for growth promotion individually, joint application with the two bacteria proved to be synergistic and enhanced growth promotion to even greater degree. 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. Uses of bacterial consortia have sometimes shown to be better option than single ones especially when being applied as formulations. 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. In case of chickpea, PGPRs were applied as seed bacterization and good promotion of growth in potted plant as well as in field were obtained. 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. Enebak *et al.* (1998) obtained both positive and negative result. 12 rhizobacterial strains were used as seed treatment in pine. According to them loblolly pine shoot length as well as above

and below ground biomass were reduced when seeds were treated with two bacterial strains, while three strains significantly increased the below ground biomass of seedling root systems. They suggested that the effect of rhizobacteria inoculation on seedling emergence and plant growth are independent and that the effects are seedling specific. Inoculation of sunflower seeds and soil with a strain of *Rhizobium* was observed to cause a significant increase in root drymass, both under normal and water stress conditions. This *Rhizobium* sp. secreted an exopolysaccharide which had the capacity for soil aggregation on roots which in turn affected nitrogen uptake and plant growth promotion (Alami *et al.*, 2000). In greenhouse experiment soybean plant growth and nodulation was obtained from the culture supernatant of *Serratia proteamaculans* strain 1-102. Authors of the study (Bai *et al.* 2002) suggested that PGPR cells produce low concentration of activator during culture process which was enhanced with the application of inducers, while the efficacy of the root activity of the activator was constant in both pouch and pot experiment. Leaf applications were not as effective as root applications. This is not in confirmatory with the present study where both soil and foliar application induced growth promotion. Ryu *et al.* (2003) also obtained growth promotion of *Arabidopsis* by *B. subtilis* and *B. amyloliquefaciens* which according to them was due to the production of volatiles by the bacteria. *B. amyloliquefaciens* was also able to promote growth in three varieties of barley (Park *et al.*, 2003). Kishore *et al.* (2005) isolated 393 groundnut associated bacteria representing the geocarposphere, phylloplane and rhizosphere. Maximum increase in plant biomass was obtained following treatment with a rhizosphere isolate identified as *B. firmis* and two phylloplane isolates *B. megaterium* and *P. aruginosa*. They concluded that identification of phylloplane bacteria as effective plant growth promoting rhizobacteria broadens the spectrum of PGPR available for field application. In this context, an interesting result obtained in the present study was that the suspension of PGPRs when applied as foliar spray was equally effective in increasing the leaf biomass of tea. As tea is cultivated mainly for its leaves, the induction of new shoots and more leaves would have great impact in considering plant growth promotion.

Besides plant growth promotion, the ability of the two PGPRs was also tested in reducing root rot diseases. Both *B. megaterium* and *O. anthropi* could reduce

brown root rot intensity caused by *F. lamaoensis* but of the two *B. megaterium* was more effective. Since *B. megaterium* was found to inhibit the growth of other pathogens also, its ability to suppress root rot of chickpea caused by *S. rolfsii* I, as well as crown rot of three orchids caused by *S. rolfsii* II, *S. sclerotiorum* were also tested. *B. megaterium* was found to suppress the diseases effectively. Hence it was concluded that though *B. megaterium* was isolated from tea rhizosphere, it had wide spectrum of activity and would be useful in future biocontrol strategies. *B. amyloliquefaciens, B. subtilis* and *B. pumilus* were observed to have the ability to reduce incidence of tomato mottle virus leading to a corresponding increase in fruit yield (Murphy *et al.* 2000). *P. fluorescens* was able to induce resistance in rice leaves against *Xanthomonas oryzae* (Vidyasekaran *et al.* 2001). Zhang *et al.* (2002) evaluated five PGPR strains, *S. marcescens, P. fluorescens* two strains of *B. pumilus* and *B. pasteuri* for reduction in blue mould `disease of tobacco. Of the five strains three were able to reduce severity. Field applications of biocontrol agents *P. fluorescens, B. subtilis* and *Trichoderma viridae* induced systemic resistance in banana cultivars against *Mycrosphaerella musicola* (Kannan *et al.*, 2003). Radhajeyalakshmi *et al.* (2003) observed that foliar application of culture filtrate of *P. fluorescens* and *B. subtilis* when sprayed twice at boot leaf stage and at 50 % flowering stage reduced seed infection of rice caused by *Helminthosporium oryzae, Saroflavum oryzae* and *Trichoconis padvickii.* Guo *et al.* (2004) also reported the ability of PGPRS *Serratia* sp., *Pseudomonas* sp. and *Bacillus* sp. to reduce wilt of tomato.

It is apparent from the present study as well as studies by a large number of previous workers that PGPRs have the ability to promote growth in plants, which in many cases is associated with pathogen suppression in the soil. These PGPRs secrete one or more metabolites in the soil which then elicit the observed response in the host. Whether it is growth promotion or disease suppression, the ultimate expression is 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 plant or reduced disease. Hence, in order to get a proper insight into the plant growth promotion and induced systemic resistance, analysis of the

biochemical changes especially those known to be involved in these mechanisms are essential. The major components analysed for tea leaves in the present study included 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 lyase (PAL) and polyphenol oxidase (PPO) increased significantly, especially in presence of the pathogen. *B. megaterium* enhanced the activity of chitinase to a greater extent than *O. anthropi.* Similar result was obtained with phenolics which though increased in all treatment were greatly induced by *B. megaterium* and that too in presence of the pathogen. In the study involving the induction of systemic resistance in rice leaves by *P. fluorescens* (Vidyashekaran *et al.*, 2001), increased activities of PO, PAL, 4-coumarate: 5 CO ligase and increased accumulation of lignin were observed. This was observed in the resistant reactions and not in the susceptible ones. *P. fluorescens* which induced systemic resistance in chickpea against *S. rolfsii* was found to induce accumulation of several phenolics and defense enzymes were observed to be enhanced in chickpea. Increased activity of chitinase, β-1,3-glucanase and peroxidase were obtained in sugar beet which was induced by treatment with *B. mycoides* (Bargabus *et al.*, 2002). Induction of defense related enzymes by *P. fluorescens* in balck pepper and *Phytophthora 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. The systemic nature of protection and growth promotion in the present study is also evident as the responses were analyzed in the leaves even when the application was in the rhizosphere. Two isolates of *B. pumilus* were reported to be best plant growth promoters and biocontrol agents against downy mildew disease in pearl millet (Niranjan *et al*, 2003). They also reported increased activities of PAL, PO and β-1,3-GLU but not of CHT activity. In another study, Radjacommare *et al.* (2005) reported the induction of defense enzymes, phenols and lignin in rice by *P. fluorescens* against *R. solani.* Treatment of finger millet with *P. fluorescens* induced systemic resistance against *Pyricularia grisea* and increased activities of defense enzymes CHT, b-1,3- Glu, Po and PAL. Ramanathan *et al.*, (2003) and Bargabus *et al.*, (2004) obtained systemic resistance

elicitation by *B. pumilus* in sugar beet which was marked by increase in CHT, β-1,3-GLU activities which was preceded by biphasic $H_2O_2$ production. Chakraborty *et al.* (2006) quoted increase in defense enzymes PO, CHT, β-1,3-GLU and PAL during plant growth promotion of tea and induction of resistance by *B. megaterium*.

Significant changes in either protein content or protein profile was observed by treatments with the two bacteria in the present study. 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 analysed by HPLC. It was observed that the treatment with the bacteria induced some new isomeric forms. This was more significant by foliar application and during joint inoculation. Since tea leaves are produced for their flavor enhancement of catechins isomers point to the fact that these are also enhanced during plant growth promotion. Besides tea, biochemical changes associated with reduction in disease development were analysed in chickpea and three orchids. In the, *B. megaterium* had caused reduction in disease development. Increase in activities of defense related enzymes CHT, GLU, PO, PAL and PPO along with increase in phenolics and decrease in chlorophylls were observed in all cases (Donate-Correa *et al.*, 2005). Thus results obtained with chickpea and orchids confirmed those of tea indicating that these mechanisms would be operative in different plant species.

Since both *B. megaterium* and *O. anthropi* applied either as soil drench or foliar spray could promote growth in all the tested plants, the next question was to determine whether these could be applied as suitable formulations in the rhizosphere. This information would be invaluable for commercial preparation of PGPRs. For this, formulations of the two PGPRs were prepared using carboxymethyl cellulose with talcum powder as carrier and tested under greenhouse conditions for their effect on growth promotion of tea seedlings. Observations were recorded after 2 and 4 months of application which revealed that significant growth promotion was accorded by application of bacterial suspensions. Selection of talcum as carrier was done because of it being cost effective, commonly available and inert. With increase in the focus of application of biofertilisers and biocontrol agents research on

production of formulations of PGPR have also increased. 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 ™ was also reported to induce resistance to downy mildew disease and growth promotion in pearl millet (Sharathchandra *et al.*, 2004). In a further study talc based formulation of *B. subtilis* and *P. fluorescens* either singly or mixed along with or without chitin and neem amendments for reducing root rot incidence of chillies along with plant growth promotion were evaluated by Bharati *et al.* (2004). According to them the PGPR mixed bioformulation of *P. fluorescens* + *B. subtilis* + neem +chitin was found to be the best one. New formulations of *B. subtilis* for management of tomato damping off caused by *Pythium aphanidermatum* were developed by Jayraj *et al.* (2005). Their formulation included a talc based powder, lignite based powder, lignite + fly ash based powder, wettable powder, bentonite paste and polyethylene glycol paste. All of these formulations were found to be effective and enhanced plant biomass in the glass house and field condition. Viability of propagules was maintained upto one year of storage. *B. subtilis* was also shown to survive in glucose and talcum powders at 8.6 and 7.6 $\log_{10}$ cfu $g^{-1}$ respectively for 1 year of storage compared to 3.5 $\log_{10}$ cfu $g^{-1}$ on a peat formulation (El-Hassan and Gowen, 2006). Seed treatments with talc formulations of *B. subtilis* in glucose, talc and peat significantly enhanced 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 combined decreased the sheath blight disease and increased plant growth and yield in rice (Mathivanan *et al.*, 2005). However, the joint application did not have any additive effect.

In the present study, it was felt necessary to determine the sustainability of PGPRs in the soil as this would be important in the field. Hence the survival of the 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 formulations were also determined. Determination of bacterial survival in soil and formulations was done by immunological techniques using antibodies raised against the two bacteria. These techniques i.e. ELISA and Dot blot gave very specific and accurate results, as the antibodies specifically reacted only with the specific bacteria.

Results of both ELISA and Dot blot showed that bacteria survived at high concentration even after six months of inoculation when the analysis was performed. Viability of the bacteria as determined by ELISA was evident in the bioformulations even after 4 months. Populations of *F. lamaoensis* were determined in the soil using dot using PAbs raised against *F. lamaoensis* the causal agent of brown root rot of tea. It was shown that the population of the pathogen reduced significantly in *B. megaterium* and *O. anthropi* treated soil, as detected on the analysis by ELISA and Dot blot. Thus these bacteria probably secreted antifungal metabolites into the soil which caused reduction in growth of the pathogen.

The overall results of the present study have shown that two rhizobacteria isolated from tea rhizosphere, *B. megaterium* and *O. anthropi* could induce plant growth promotion and disease reduction in tea as well as in other crops. Different species of *Bacilli* are now widely used in other crops as plant growth promoting and biocontrol agents. However, *O. anthropi* is not one of the commonly used PGPRs and hence this is one of the few studies reporting the use of *O. anthropi* as PGPR. Though both soil drench and foliar spray gave experimentally good result. soil drench is preferable mode of application. This is because tea being cultivated for its beverage produced from its leaves and soil drench induced systemic response transmitted to the leaves treatment of leaves can be avoided. 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 PGPRs secrete metabolites into the soil which in turn elicit responses in the host. The relative importance of importance of the metabolites in inducing plant growth promotion, as well as disease suppression is not yet clear. While *B. megaterium* was better as a biocontrol agent *O. anthropi* was very successful in plant growth promotion. All the elements commonly known to be involved in the induced systemic resistance have been enhanced. Thus these two PGPRs could be used in suitable formulations commercially which would benefit the tea industry where use of biological products to replace or supplement chemical use is the need of the hour.