INVESTIGATIONS ON PLANT GROWTH PROMOTING RHIZOBACTERIA OF TEA AND ELUCIDATION OF THEIR MECHANISM OF ACTION

THESIS SUBMITTED FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY IN SCIENCE (BOTANY)
OF THE UNIVERSITY OF NORTH BENGAL

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2006

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TO WHOM IT MAY CONCERN

This is to certify that Ms Merab Basnet has carried out her research work under our supervision. Her thesis entitled **'Investigations on Plant growth promoting rhizobacteria of tea and elucidation of their mechanism of action'**, is based on her original work and is being submitted to the University of North Bengal for the award of Doctor of Philosophy (Science) degree in Botany, in accordance with the rules and regulations of the University of North Bengal.

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Acknowledgement

I take this opportunity to thank all those who made this thesis possible. At the very outset, I would like to express my deep sense of gratitude to my supervisors Prof. Usha Chakraborty and Prof. Bishwanath Chakraborty, Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal. It wouldn't have been possible for me to complete my thesis without their noble guidance, encouragement and constant support.

I extend my heartfelt thanks to Dr. A. Saha, Head of the Department of Botany and other faculty members of. the department for providing necessary facilities to carry out my research work.

I record my gratitude to Prof. P. K. Sarkar, Co-ordinator, UGC-SAP for making availing the use of instruments.

The financial support received from the Department of Biotechnology, Ministry of Science and Technology, Govt. of India, New Delhi, India is gratefully acknowledged.

I extend my gratitude to Global Plant Clinic, Diagnostic Clinic and Advisory Laboratory, CABI, UK Centre, United Kingdom for identifying my bacterial cultures.

I owe special thanks to the managing partners of Janak Nursery, Siliguri for providing me with the orchid plants for my experimental purposes.

I would like to thank Dr. Monica Sharma and Ms. Belinda Lahon for all the help they offered me. I would like to record my thanks to all my other lab mates for their help and creating an atmosphere of friendliness. I wish to thank field men for maintaining my plants in good condition. I wish to especially acknowledge my friend Mr. Anirudra Gurung for being there for me in times of my need Thanks for the constant support and great time we had together.

I acknowledge the help and support I received from my dear sister Cyaria, who has a special place in my life.

Last, but not least, I wish to acknowledge my family for their constant encouragement and being there for me through my thick and thin.

Merab Basnet

Date : 8.11.2006 *(Merab Basnet)*

Place : Siliguri

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INTRO UCTION

Tea (*Camellia sinensis* (L.) O. Kuntze) is one of the important plantation crops of India. Since tea is grown in tropical agro climates, pests, weeds and disease causing organisms are capable of causing serious damage to the crops, for which extensive use of chemicals has been implemented in the past. Besides, as most of the nutrients in the soil are not in a readily available form, extra input of chemical fertilizers has been necessary for the optimum productivity of the tea plant. But the uses of fertilizers and pesticides have caused a serious problem of pollution and loss of land fertility (Bezbaruah *et al.*, 1996). In recent years concern over pesticide load in the environment and also pesticide residue in agricultural produce has led to a reduction in use of chemical fungicides commonly used to control plant pathogens. In its place major thrust is being given to biological control as a component of integrated disease management. In case of tea, the demand of organic tea in the world market is very high, and is increasing. Excessive use of chemicals and the presence of residue in the leaves is a major concern for consumers. Hence, biofertilisers, whether it is microorganism based or a plant based have the potential to be used in tea industry on a large scale. Tea plant is is a perennial and grows in several geographical regions of India, including the plains of Assam, North Bengal as well hills of Darjeeling and South India (Plate I) its rhizosphere is expected to be a rich source of microorganisms, some of which could be exploited for use as biofertiliser/ bioprotectant capable of improving the growth of the plant, either by suppression of pathogenic soil fungi or by growth promotion through other mechanisms.

The term 'Rhizosphere' was introduced by Hiltner in 1904, and is now defined as the volume of soil surrounding the plant root in which bacterial growth is stimulated. Rhizosphere is the habitat in which several biologically important processes and interactions takes place. It is the zone of intense activity of various groups of microorganisms. The root system is known to produce exudates with a number of sugars, organic acids and amino acids, favouring the population build up of rhizosphere microorganisms. These microorganisms grow in close association with the plant and are referred to as rhizobacteria (Bashan, 1998). They live at the expense of the plant, feeding on the nutrients released from the plant roots. Some of them affect the growth and development of plant.

Plate I (A-B): Tea gardens in hills (A) and plains (B).

These plant associated microorganisms mainly belong to the bacteria, fungi and actinomycetes. These may be beneficial or deleterious to the plant, or may not have any relation to the plant at all. The beneficial groups of microbes with the capacity to enhance plant growth by increasing seed emergence, plant weight and crop yields are designated as the plant growth promoting rhizobacteria (PGPR) (Kloepper, 1992). These bacteria appear completely harmless, do not cause any symptoms and yet induce substantial resistance against different pathogens (Van Loon, 1997). PGPRs have been applied to a wide range of agricultural species for the purpose of growth enhancement, including increased seed emergence, weight, crop yields and disease control (Glick, 1995).

The mechanisms by which PGPRs can influence plant growth may differ from species to species as well as from strain to strain. Symbiotic plant colonizers such as rhizobia mostly contribute to plant growth by nitrogen fixation. Free living rhizobacteria usually do not rely on single mechanism of promoting plant growth (Glick *et al.* 1999). There are several determinants for mechanisms of growth promotion that include bacterial synthesis of plant hormones like indole-3- acetic acid (IAA), cytokinin and gibberellin, breakdown of plant induced ethylene by bacterial production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase and increased mineral and N availability in the soil (Kloepper, 1992; Glick, 1995). Several indirect possible mechanisms have also been proposed that reduces the stress by limiting the damage caused by phytopathogens. These mechanisms may include suppression of diseases caused by the plant pathogens (Suslow and Schroth, 1982), competition with pathogenic microorganism by colonizing root.

Besides growth promotion, PGPRs have been tested and characterized for their activity as biocontrol agents against soil borne pathogens. It is clear that the biological control of soil borne plant pathogen by antagonistic microorganisms may offer a practical supplement or alternative to existing disease management strategies that depend heavily on chemical pesticides. Disease suppression by antagonistic bacteria depends on their ability to colonize roots and to produce substances inhibitory to pathogens. Such microorganisms can produce substances that limit the damage caused by pathogens, by producing antibiotics, siderophores and other lytic enzymes. These microorganisms can also function as competitors of pathogens for

nutrients and colonization sites. Potential biocontrol agents produce antibiotics, which play an important role in disease suppression and increase yield of the plants under greenhouse conditions. The most widely studied group of rhizobacteria with respect to production of antibiotics is that of the fluorescent pseudomonads (Haas and Keel, 2003; Dileep Kumar and Bezbaruah, 1997). Well-characterized antibiotics with biocontrol properties include phenazines, 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, lipopeptides, and hydrogen cyanide. *Bacillus megaterium* have also been reported to produce antibiotics against several fungal pathogens by Jung and Kim (2003).

Interest in biological control has increased recently by public concerns over the use of chemicals in the environment in general, and the need to find the alternatives to the use of chemicals for disease control (Whipps, 2001). The key to achieving successful, reproducible biological control is the gradual appreciation that knowledge of the ecological interactions taking place in soil and root environments is required to predict the conditions under which biocontrol can be achieved (Deacon, 1994; Whipps, 1997). It is also essential to know the interactions of the microorganisms and the host. In this context, the mechanisms of plant growth promotion and induced systemic resistance (ISR) by PGPRs have been extensively studied in the past decade. ISR occurs when the plant's defense mechanisms are stimulated and primed to resist infection by pathogens (Van Loon *et al.*, 1998). ISR developed by non-pathogenic fluorescent *Pseudomonas* bacteria, unlike the pathogen-induced systemic acquired resistance (SAR), is independent of salicylic acid (SA) but requires jasmonic acid (JA) and ethylene (ET) (Bakker *et al.* 2003a). PGPR mediated ISR results in reinforcement of plant cell wall by lignin, callose and phenolic compounds, alteration of physiological and biological reactions of plant cells, and production of antimicrobial substances, such as pathogenesis related proteins (PR) and phytoalexins (Ramamoorthy *et al.*, 2001).PGPRs act as a biological control and protects plants from the various pathogens in several crops by activating defense genes encoding chitinases, β-1,3 glucanase, b-1,4 glucanase, peroxidase, phenylalanine ammonia lyase and other enzymes which are involved in synthesis of phytoalexin (M'Piga *et al.*, 1997). The antagonists may produce mycolytic enzymes viz. β-1,3 glucanase, β-1,4 glucanase and lipases. Since the

mechanism of action of PGPRs are highly complex , it is quite essential that this type of work would require a study not only of any potential agent *per se*, but also its interactions with the crop, the natural resident biota and environment as well.

Considering the importance of using biological agents for growth promotion and disease suppression in tea, to reduce the use of chemicals, the present study was undertaken with the following objectives giving special emphasis on PGPRs and their mechanism of action:

i. Isolation of microorganisms from tea rhizosphere and their identification .
ii. Screening of isolated microorganisms against common soil fungi *Fomes lamaoensis, Sclerotium rolfsii* I, *Sclerotium rolfsii* II, *Sclerotinia sclerotiorum, Sphaerostilbe repens,* and *Poria hypobrumea in vitro.*
iii. Testing of plant growth promoting activity of the selected antagonists and selection of PGPRs; *in vivo* testing of the PGPRs for determination of disease suppressing activity; determination of population antagonists as well as pathogen in soil after definite time interval; *in vivo* determination of PGPR activity.
iv. Determination of biochemical changes in test plants induced by PGPRs.
v. Extraction of active principles from selected PGPRs and bioassay of isolated compounds and their characterization.

LITERATURE REVIEW

Numerous species of soil bacteria which flourish in the rhizosphere of plants, but which may grow in, on, or around plant tissues, stimulate plant growth by a plethora of mechanisms. These bacteria are collectively known as PGPR (plant growth promoting rhizobacteria). The search for PGPR and investigation of their modes of action are increasing at a rapid pace as efforts are made to exploit them commercially as biofertilizers. These microorganisms have important contributions toward the growth and development of plants. Plant growth promoting rhizobacteria increase plant growth directly by producing hormones, siderophores or by solubilising phosphates or indirectly either by the suppression of well-known diseases caused by major pathogens or by reducing the deleterious effects of minor pathogens. Considering the importance of the role of PGPRs in agriculture and understanding their mechanisms of action, several authors have reviewed this topic exhaustively (Lugtenberg *et al.*, 2001; Whipps, 2001; Haas and Keel, 2003; Morris and Monier, 2003; Morgan *et al.*, 2005). The main areas of focus of some of the recent reviews have been discussed below.

Rhizosphere colonization is one of the first steps in the parthenogenesis of soil borne microorganisms. It can also be crucial for the action of microbial inoculants used as biofertilisers, biopesticides, phytostimulators and bioremediators. *Pseudomonas*, one of the best root colonizers is therefore used as a model root colonizer. The review by Lugtenberg *et al.* (2001) focused on (a) the temporal spatial description of root colonising bacteria as visualized by confocal laser scanning microscopical analysis of autofluorescent microorganisms, and (b) bacterial genes and the traits used for the colonization of root and of animal tissues, indicating the general importance of a study. Finally, they identified several noteworthy areas for future research.

The loss of organic material from the roots provides the energy for the development of active microbial populations in the rhizosphere around the root. Generally, saproptrophs or biotrophs such as mycorrhizal fungi grow in the rhizosphere in response to this carbon loss, but plant pathogens may also develop and infect a susceptible host, resulting in disease. The review by Whipps (2001) examined the microbial interactions that can take place in the rhizosphere and that

are involved in biological disease control. The interactions of bacteria used as biocontrol agents of bacterial and fungal plant pathogens, and fungi used as biocontrol agents of protozoan, bacterial and fungal plant pathogens were considered. Whenever possible, modes of action involved in each type of interaction were assessed with particular emphasis on antibiosis, competition, parasitism, and induced resistance. The significance of plant growth promotion and rhizosphere competence in biocontrol was also considered. Multiple microbial interactions involving bacteria and fungi in the rhizosphere were shown to provide enhanced biocontrol in many cases in comparison with biocontrol agents used singly. The extreme complexity of interactions that occur in the rhizosphere was highlighted and some potential areas for future research in this area were discussed briefly.

Certain strains of fluorescent pseudomonads are important biological components of agricultural soils that are suppressive to diseases caused by pathogenic fungi on crop plants. The biocontrol abilities of such strains depend essentially on aggressive root colonization, induction of systemic resistance in the plant, and the production of diffusible or volatile antifungal antibiotics. Evidence that these compounds are produced *in situ* is based on their chemical extraction from the rhizosphere and on the expression of antibiotic biosynthetic genes in the producer strains colonizing plant roots. Well-characterized antibiotics with biocontrol properties include phenazines, 2,4-diacetylphloroglucinol, pyoluteorin, pyrrolnitrin, lipopeptides, and hydrogen cyanide. *In vitro*, optimal production of these compounds occurs at high cell densities and during conditions of restricted growth, involving (*i*) a number of transcriptional regulators, which are mostly pathway-specific, and (*ii*) the GacS/GacA two-component system, which globally exerts a positive effect on the production of extracellular metabolites at a posttranscriptional level. Small untranslated RNAs have important roles in the GacS/GacA signal transduction pathway. One challenge in future biocontrol research involves development of new strategies to overcome the broad toxicity and lack of antifungal specificity displayed by most biocontrol antibiotics studied so far (Haas and Keel, 2003).

Bacteria associated with plants have been observed frequently to form assemblages referred to as aggregates, microcolonies, symplasmata, or biofilms on

leaves and on root surfaces and within intercellular spaces of plant tissues. In a wide range of habitats, biofilms are purported to be microniches of conditions markedly different from those of the ambient environment and drive microbial cells to effect functions not possible alone or outside of biofilms. The review by Morris and Monier (2003) constructed a portrait of how biofilms associated with leaves, roots and within intercellular spaces influenced the ecology of the bacteria they harbor and the relationship of bacteria with plants. They also considered how biofilms may enhance airborne dissemination, ubiquity and diversification of plant-associated bacteria and may influence strategies for biological control of plant disease and for assuring food safety.

After an initial clarification of the term biofertilizers and the nature of associations between PGPR and plants (i.e., endophytic versus rhizospheric), the review by Vessey (2003) focused on the known, the putative, and the speculative modes-of-action of PGPR. These modes of action include fixing N_2, increasing the availability of nutrients in the rhizosphere, positively influencing root growth and morphology, and promoting other beneficial plant-microbe symbioses. The combination of these modes of actions in PGPR was also addressed, as well as the challenges facing the more widespread utilization of PGPR as biofertilizers.

Colonization of the rhizosphere by micro-organisms results in modifications in plant growth and development. The review by Persello *et al.* (2003) examined the mechanisms involved in growth promotion by plant growth-promoting rhizobacteria which are divided into indirect and direct effects. Direct effects include enhanced provision of nutrients and the production of phytohormones. Indirect effects involve aspects of biological control: the production of antibiotics and iron-chelating siderophores and the induction of plant resistance mechanisms. The study of the molecular basis of growth promotion demonstrated the important role of bacterial traits (motility, adhesion and growth rate) for colonization. New research areas emerge from the discovery that molecular signalling occurs through plant perception of eubacterial flagellins. Recent perspectives in the molecular genetics of cross-talking mechanisms governing plant-rhizobacteria interactions were also discussed.

Soil microbial populations are immersed in a framework of interactions known to affect plant fitness and soil quality. They are involved in fundamental activities that ensure the stability and productivity of both agricultural systems and natural ecosystems. Strategic and applied research has demonstrated that certain co-operative microbial activities can be exploited, as a low-input biotechnology, to help sustainable, environmentally-friendly, agro-technological practices. Much research is addressed at improving understanding of the diversity, dynamics, and significance of rhizosphere microbial populations and their co-operative activities. An analysis of the co-operative microbial activities known to affect plant development was the general aim of the review by Barea *et al.* (2005). In particular, they summarized and discussed significant aspects of this general topic, including (i) the analysis of the key activities carried out by the diverse trophic and functional groups of micro-organisms involved in co-operative rhizosphere interactions; (ii) a critical discussion of the direct microbe–microbe interactions which results in processes benefiting sustainable agro-ecosystem development; and (iii) beneficial microbial interactions involving arbuscular mycorrhiza, the omnipresent fungus–plant beneficial symbiosis. The trends of this thematic area will be outlined, from molecular biology and ecophysiological issues to the biotechnological developments for integrated management, to indicate where research is needed in the future.

The review by Morgan *et al.* (2005) looked briefly at plants and their rhizosphere microbes, the chemical communications that exist, and the biological processes they sustain. Primarily it is the loss of carbon compounds from roots that drives the development of enhanced microbial populations in the rhizosphere when compared with the bulk soil, or that sustains specific mycorrhizal or legume associations. The benefits to the plant from this carbon loss were discussed. Overall the general rhizosphere effect could help the plant by maintaining the recycling of nutrients, through the production of hormones, helping to provide resistance to microbial diseases and to aid tolerance to toxic compounds. When plants lack essential mineral elements such as P or N, symbiotic relationships can be beneficial and promote plant growth. However, this benefit may be lost in well-fertilized (agricultural) soils where nutrients are readily available to plants and symbionts reduce growth. Since these rhizosphere associations are common place and offer key

benefits to plants, these interactions would appear to be essential to their overall success.

The review presented below has been compiled on two important aspects of rhizosphere microflora related to the present work i.e. plant growth promotion and disease reduction.

Plant growth promotion by rhizobacteria

Abbass and Okon (1993) observed that treating seedling hypocotyls and roots of several plant species with cultures of *Azotobacter paspali* changed plant growth and development and significantly increased weight of shoot and roots. Morphological changes of root tips were already observed 5 days after inoculation. After 21 days the main effect was on the root surface area. Plant growth promotion was dependent on the inoculum size, indicating that for any given plant growth condition there is an optimal number of *A. paspali* for a positive effect on the plant.

Co-inoculation of plant growth promoting rhizobacteria (PGPR) with *Bradyrhizobium* has been shown to increase legume nodulation and nitrogen fixation at optimal soil temperatures. Nine rhizobacteria co-inoculated with *Bradyrhizobium japonicum* 532C were tested by Zhang *et al.* (1996) for their ability to reduce the negative effects of low root zone temperature (RZT) on soybean [*Glycine max*(L.) Merr.] nodulation and nitrogen fixation. Three RZTs were tested: 25 (optimal), 17.5 (somewhat inhibitory), and 15°C (very inhibitory). At each temperature some PGPR strains increased the number of nodules formed and the amount of fixed nitrogen when co-inoculated with *B. japonicum*, but the stimulatory strains varied with temperatures. The strains that were most stimulatory varied among temperatures and were as follows: 15°C, *Serratia proteamaculans* 1-102; 17.5°C, *S. proteamaculans* 1-102 and *Aeromonas hydrophila* P73; 25°C, *Serratia liquefaciens* 2-68.

Cook *et al.* (1998) reported that rhizobacteria, particularly *Pseudomonas* species were (i) able to colonize and maintain populations in the rhizosphere of wheat 5–10 cm and more below the seed, (ii) able to produce one or more antibiotics inhibitory to the target root pathogens, and (iii) tolerant to seed-treatment chemicals,

needed for immediate protection of germinating seeds. Their strains were from the rhizosphere of wheat growing in soil from fields where wheat had been grown continuously for many years, to help ensure that the strains are rhizosphere competent on the crop intended for protection. Initially, they concentrated on *P. fluorescens* 2-79 and *P. aureofaciens* 30-84 with ability to produce phenazine (PHZ) antibiotics. The second phase of their field work concentrated on *Bacillus* species L324-92 with antibiotic activity against three wheat root diseases, and on *P. fluorescens* Q69c-80 with no known ability to produce antibiotics, but widely effective in the field. The authors have reported that they are also now concentrating on *P. fluorescens* Q8R1-96 with ability to produce the antibiotic 2,4-diacetylphloroglucinol (PHL). The evidence is strong that PHL-producing strains like Q8R1-96 account for take-all decline. Used as a seed treatment, this strain produced the highest yields of wheat at every location where tested in 1997. They now have cultures of Q8R1-96 transformed to produce PHZ in addition to PHL.

Krebs. *et al.* (1998) isolated several *Bacillus* strains belonging to the *B. subtilis/amyloliquefaciens* group from plant-pathogen-infested soil possess plant-growth-promoting activity. Three out of the four strains investigated were identified as *B. amyloliquefaciens* and were able to degrade extracellular phytase (myo-inositol hexakisphosphate).

Lazarovits *et al.* (1998) developed a gnotobiotic bioassay, using potato plantlets derived from single-node explants grown in tubes containing solidified agar medium. Studies with this model system served to illustrate some important features that may be expected from PGPR per plant interactions. Growth-promotion of potato by PsJN (*Pseudomonas* sp. [strain PsJN]) was cultivar-specific. Inoculated plantlets of cv. 'Norchip' showed a five- to eightfold increase in root weight, cv. 'Kennebec' a two- to threefold increase, cv. 'Shepody' no response, and cv. 'Chaleur' a decrease of 50 %. PsJN was shown by other researchers to promote growth in a number of crop species, but to be cultivar-specific with them also. In their laboratory studies PsJN consistently promoted growth of potato, but in the field the response was site-specific. Bacterized plants had improved growth and yield of tubers in Alliston soils, but were reduced in Simcoe soil. This effect was reproduced under growth room conditions. Pasteurization of Simcoe soil eliminated the inhibitory impact of PsJN,

suggesting that the effect was determined due to presence of soil organisms. Therefore, they investigated the interactions of PsJN with other bacteria in the geocaulosphere. They harvested tubers at 2-day intervals from each soil, sterilized their surface, made a liquid homogenate, and plated the homogenates onto nutrient agar. A dozen bacteria isolates were recovered and identified by fatty acid profiles and biochemical tests. All genera isolated were found in tubers from both soils except *Pseudomonas acidovorans*, which was present only in tubers from Simcoe soil. The presence of PsJN significantly altered the types and populations of bacteria recovered from the tubers grown in non-pasteurized soil. However, four isolates, *Serratia proteamaculans, P. acidovorans, Alcaligenes piechaudii* and *Rahnella aquatilis*, showed antagonism to PsJN in assays on agar media. Several isolates also inhibited growth of potato nodal explants. Combinations of PsJN with each of the bacteria isolates showed that *Pantoea agglomerans*, which by itself had neutral or beneficial effects, when combined with PsJN was inhibitory to the growth of plantlets.

Cattelan *et al.* (1999) selected 116 isolates from bulk soil and the rhizosphere of soybean [*Glycine max* (L.) Merr.] and examined them for a wide array of traits that might increase early soybean growth in nonsterile soil (PGPR traits). A subsample of 23 isolates, all but one of which tested positive for one or more of these PGPR traits, was further screened for traits associated with biocontrol, (brady) rhizobial inhibition, and rhizosphere competence. Six of eight isolates positive for 1-aminocyclopropane-1-carboxylate (ACC, a precursor of ethylene) deaminase production, four of seven isolates positive for siderophore production, three of four isolates positive for ß-1,3-glucanase production, and two of five isolates positive for P solubilization increased at least one aspect of early soybean growth. One isolate, which did not share any of the PGPR traits tested *in vitro* except antagonism to *Sclerotium rolfsii* and *Sclerotinia sclerotiorum*, also promoted soybean growth. One of the 23 isolates changed bradyrhizobial nodule occupancy. Although the presence of a PGPR trait *in vitro* does not guarantee that a particular isolate is a PGPR, the results suggest that rhizobacteria able to produce ACC deaminase and, to a lesser extent, ß-1,3-glucanase or siderophores or those able to solubilize P *in vitro* may increase early soybean growth in nonsterile soil. A proteus strain inhibited mycelial

growth of *Fusarium oxysporum in vitro*. Seed bacterization showed significant plant growth promotion and *Fusarium*-wilt suppression activity of *Phaseolus mungo* in a gnotobiotic system. The culture filtrate of this strain exhibited three prominent bands in UV-VIS spectra between 300 and 400 nm. The growth promotion assay of the extracted compound against different indicator organisms indicated the production of a compound related to a 2-oxoacid-type siderophore. The HPLC of the purified ethyl acetate extract of the strains and standard 4-methyl-2-oxopentanoate (2-oxoisocaproate) revealed a single peak, similarly as the coinjection of the extract and the standard. The production of siderophore, probably 2-oxoisocaproate, was demonstrated by Barthakur (2000).

The studies conducted by Danuta *et al.* (2000) in the years 1997-1999 concerned the soil after cultivation of rye, winter wheat, triticale and potato. The purpose of the studies was to determine the composition of bacteria and fungi communities in the soil after the cultivation of these plant species. As a result of the microbiological analysis of the soil after rye cultivation 3.08×10^6 colonies of bacteria and 28.27×10^3 colonies of fungi were obtained. After triticale cultivation the soil contained 3.99×10^6 bacteria colonies and 38.74×10^3 fungi colonies, whereas after winter wheat cultivation there were 4.89×10^6 bacteria colonies and 26.19×10^3 fungi colonies. The soil after potato cultivation contained the greatest numbers of bacteria (5.01×10^6) and fungi (43.11×10^3) colonies. Among the obtained bacteria and fungi the greatest number of antagonistic microorganisms was found in the soil after winter wheat and rye cultivation, while the smallest number of antagonistic microorganisms was found in the soil after potato cultivation.

Experiments were conducted by Lee (2000a) to compare the germinability of pepper seeds primed by bacterial strain and solid matrix priming (SMP). Pepper seeds were soaked in the cell suspension of the bacterial strains for 1 hr and incubated at 28°C for certain period of time then dried in shade and stored. Seed priming with *Bacillus* strains showed even higher germination rate than SMP or chemical osmotic controllers. In pots experiments, pepper seeds primed by *Bacillus* sp. B2-13 showed more than 80% seedling emergency within 7 days, while SMP treatment was 11 days and untreated control was 13 days. When the bio-primed seeds were planted in pots, significant increase of shoot weight and length as well as

root weight and length were measured compare to other treatments. Bio-primed seedling revealed twofold more root biomass than untreated control.

In a further study by Lee (2000b) the effects of PGPR strains on the barley growth in Rice-Barley double cropping system was tested. The study was carried out in experimental farm of Gyeongnam Provincial RDA.The test variety was Jinyang, for brewing and PGPR strains were *Paenibacillus polymixa* E681 and *Pseudomonas fluorescens* B16. The tested barley seeds were soaked into bacterial suspension of E681 or B16 at 10^8 cells ml^{-1} for 5 hrs and dried in shade place for 12 hrs then sowed in the barely field at rate of 10kg seed $10a^{-1}$. The emergence rates of barley plants at 30 days after sowing were observed 211 m^{-2} in E681 treated plots, 233 m^{-2} in B16 treated plot and 191 m^{-2} in untreated control. The emergence rates of test plots after over-wintering were 288 m^{-2} in E681, 260 m^{-2} in B16 and 233 m^{-2} in untreated control. The plant height, average tillers and fresh weight of test plants were measured with time sequence. The dry weight of stems, leaves, and heads were also measured. The leaf dry weight of control plot was continuously reduced after heading, however, that one in bacteria treated plots were not reduced up to 7 days before harvest. Average head weight and total yield were significantly higher in bacteria treated plots.

According to Bai (2002) *Serratia proteamaculans* 1-102 (1-102) promotes soybean–bradyrhizobia nodulation and growth, but the mechanism is unknown. After adding isoflavonoid inducers to 1-102 culture, an active peak with a retention time of about 105 min in the HPLC fractionation was isolated using a bioassay based on the stimulation of soybean seed germination. The plant growth-promoting activity of this material was compared with 1-102 culture (cells) and supernatant under greenhouse conditions. The activator was applied to roots in 83, 830 and 8300 HPLC microvolts (μV) per seedling when plants were inoculated with bradyrhizobia or sprayed onto the leaves in same concentrations at 20 d after inoculation. The root-applied activator, especially at 1 ml of 830 μV per seedling, enhanced soybean nodulation and growth at the same level as 1-102 culture under both optimal and sub-optimal root zone temperatures. Thus, this activator stimulating soybean seed germination is also responsible for the plant growth-promoting activity of 1-102 culture. However, when sprayed onto the leaves, the activator did not increase growth and in higher

concentrations decreased average single leaf area. The results suggest that this inducible activator might be a lipo-chitooligosaccharide (LCO) analogue. LCOs act as rhizobia-to-legume signals stimulating root nodule formation. The activator could provide additional 'signal', increasing in the signal quality (the signal-to-noise ratio, SNR) of the plant–rhizobia signal exchange process.

The highest extracellular phytase activity was detected in strain FZB45, and diluted culture filtrates of this strain stimulated growth of maize seedlings under phosphate limitation in the presence of phytate by Idriss *et al.* (2002). The amino acid sequence deduced from the phytase *phyA* gene cloned from FZB45 displayed a high degree of similarity to known *Bacillus* phytases. Weak similarity between FZB45 phytase and *B. subtilis* alkaline phosphatase IV pointed to a possible common origin of these two enzymes. The recombinant protein expressed by *B. subtilis* MU331 displayed 3(1)-phytase activity yielding D/L-Ins(1,2,4,5,6)P5 as the first product of phytate hydrolysis. A phytase-negative mutant strain, FZB45/M2, whose *phyA* gene is disrupted, was generated by replacing the entire wild-type gene on the chromosome of FZB45 with a *km*::*phyA* fragment, and culture filtrates obtained from FZB45/M2 did not stimulate plant growth. In addition, the growth of maize seedlings was promoted in the presence of purified phytase and the absence of culture filtrate. These genetic and biochemical experiments provide strong evidence that phytase activity of *B. amyloliquefaciens* FZB45 is important for plant growth stimulation under phosphate limitation.Rhizobia form root nodules that fix nitrogen (N_2) in symbiotic legumes. Extending the ability of these bacteria to fix N_2 in non-legumes such as cereals would be a useful technology for increased crop yields among resource-poor farmers. Although some inoculation attempts have resulted in nodule formation in cereal plants, there was no evidence of N_2 fixation. However, because rhizobia naturally produce molecules (auxins, cytokinins, abscicic acids, lumichrome, rhiboflavin, lipo-chitooligosaccharides and vitamins) that promote plant growth, their colonization and infection of cereal roots would be expected to increase plant development, and grain yield.

The efficiency of 19 bacterial strains on the growth-promotion of micropropagated pineapple plantlets cv. *Perola* was tested by Marcelo *et al.* (2002) using different bacterization methods. Measurements of shoot length, leaf number,

leaf area, shoot dry weight and root dry weight at 30 days after transplanting were determined. Overall, the best bacterization methods were either root dipping or soil drenching plus root dipping. Bacterization by root dipping was chosen due to its practicability. The most efficient bacterial strains were C210, ENF16, RAB9 and ENF10. Increases as high as 163.6 %, 107.7 % and 87.0 % respectively for shoot dry weight, root dry weight and leaf area were obtained by applying the strain RAB9 by root dipping. All strains were compatible among them and combinations of ENF10 plus RAB9, ENF16 plus C210 and C210 plus RAB9 promoted root dry weight increases of 100.3%, 88.1% and 80.1%, respectively. Production of either IAA or HCN, and solubilization of phosphate by the strains were not detected under the experimental conditions used here. Only nitrogen amounts in bacterized plantlets had significantly differed from the controls. This work indicated that mixtures of the strains C210, ENF16, RAB9 and ENF10 applied by root dipping could be used to increase biomass production of micropropagated pineapple plantlets, reducing the acclimation period.

Experiments were conducted in pots to determine the growth effect of different rhizobacteria on maize under *Striga hermonthica* infestation. Babalola *et al.* (2003) selected three bacteria based on their plant growth promoting effects. Whole bacterial cells of the rhizobacteria were used to amplify 1-amino-cyclopropane-1-carboxylic acid (ACC) deaminase gene by polymerase chain reaction (PCR). Each bacterial inoculation increased agronomic characteristics of maize although not always to a statistically significant extent. The extent of growth enhancement differed between the isolates. *Enterobacter sakazakii* 8MR5 had the ability to stimulate plant growth; however in the PCR study, ACC deaminase was not amplified from this isolate, indicating that not all plant growth-promoting rhizobacteria contain the enzyme ACC deaminase. In contrast, an ACC deaminase specific product was amplified from *Pseudomonas* sp. 4MKS8 and *Klebsiella oxytoca* 10MKR7. This is the first report of ACC deaminase in *K. oxytoca*.

The inoculation effect of *Bradyrhizobium japonicum* and *Azotobacter chroococum* on soyabean (*Glycine max* (L) Merill var. Ransom) was studied by Bhattarai and Prasad (2003). Dual inoculation proved best in all the plant growth

parameters. Inoculation with *Azotobacter* alone was also little better than uninoculated control.

Three strains of plant growth promoting fluorescent Pseudomonads (HPR6, RRLJ008 and RRLJ134) were studied for their effect on growth and yield of French bean (*Phaseolus vulgaris* L.) under field conditions (Boruah *et al.*, 2003). They examined the effect of these strains on nature of root development and leaf palisade tube length. The strains induced positive response on growth and physiological parameters resulting in higher yield in P. vulgaris. Strain HPR6 produced the most promising results in thickening of leaf palisade layer, spreading of lateral roots and production of root hairs. The increase in specific leaf weight (SLW), net assimilation rate (NAR) and relative growth rate (RGR) by these strains were 68 %, 152 % and 167 %, respectively. The growth and yield parameters were also significantly improved compared to the uninoculated control. Antibiotic resistant mutant strains demonstrated that these bacteria effectively colonized the rhizosphere of French bean. The results suggest that the strains could be developed for field application on a large scale.

The effects of transplant type and soil treatment on growth and yield of strawberries (*Fragaria* x *ananassa* Duch.) produced in annual hill culture were evaluated for three years in Florida field trials. 'Sweet Charlie' and 'Camarosa' strawberry transplants were propagated by Burelle (2003) as bare root, plug, and plugs amended with a plant growth-promoting rhizobacterial (PGPR) treatment, LS213. The transplant treatments were evaluated in combination with methyl bromide, 1, 3-dichloropropene (Telone II), an unregistered iodine-based compound (Plantpro 45), and untreated soil. 'Camarosa' plugs amended with LS213 had higher overall yields than bare root transplants in all three years. Both 'Camarosa' and 'Sweet Charlie' plug and LS213 plug plants produced yields approximately two weeks earlier than bare root transplants in all years. Regardless of transplant type, and in both consecutive years of Plantpro 45 and Telone application, treatment with Plantpro 45 resulted in smaller and less healthy root systems than other soil treatments, and treatment with Telone resulted in yields comparable to methyl bromide.

Experiments were conducted during 2000 and 2001 to determine the effects of floral and foliar application of the bacterial strain *Bacillus* OSU 142 on the yield, growth and nutrient element composition of leaves of the apricot cultivar Hacihaliloglu grown in the Malatya province of Turkey. In 2000, trees were sprayed with a bacterial suspension at full bloom, and 30 and 60 days after full bloom. This experiment demonstrated significant differences in yield, shoot length and nutrient element composition of leaves only on trees treated at the full bloom stage. In view of this, the bacterial application was performed only at full bloom in 2001. The average increase in yield in 2000 and 2001 was 30 % and 90 %, respectively, compared with the untreated control. Shoot length development was significantly higher when trees were treated with OSU 142 at full bloom stage in both years. Similarly, N, P, K, Ca and Mg contents of leaves were higher on OSU 142-treated trees than on the untreated control. The results of this study by Esitken *et al.* (2003) suggest that OSU 142 has the potential to increase the yield of apricot trees.

In order to select potential Plant Growth Promoting Rhizobacteria (PGPRs), a selection of strains from the predominant genera in the rhizosphere of four lupine species, based on genetic divergence criteria, was carried out in a study by Gutierrez-Manero *et al.* (2003). This yielded 11 *Aureobacterium* (Aur), four *Cellulomonas* (Cell), two *Arthrobacter* (Arth), two *Pseudomonas* (Ps), and six *Bacillus* (Bc) strains. Cell-free culture filtrates of each bacterium were assayed for effects on germination, growth, and biological nitrogen fixation (BNF) of *Lupinus albus* L. cv. *Multolupa* seeds or seedlings. Four (Aur 6, Aur 9, Aur 11, and Cell 1) of the twenty-five strains assayed promoted germination. *Aureobacterium* 6 and Aur 9 also increased root surface, total nitrogen content, and BNF. As a result of the screening, and considering all the variables studied, authors suggested that Aur 6 can be considered a plant growth promoting rhizobacterium suitable for further field trials in other plants and in different production systems.

The study of the effect of the root exometabolites of tomato plants on the growth and antifungal activity of the plant growth-promoting *Pseudomonas* strains showed that the antifungal activity of plant growth-promoting rhizobacteria in the plant rhizosphere may depend on the sugar and organic acid composition of root exudates (Kravchenko *et al.*, 2003).

Mamatha *et al.* (2003) performed a greenhouse experiment in which *Bacillus coagulans* and *Pseudomonas fluorescens* were inoculated either singly or dually on growth and nutrition of sandalwood tree. Parameters such as plant height, number of leaves, biomass, P content, alkaline phosphatase & dehydrogenases and microbial population of the root zone soil revealed that all these parameters were higher in plants which were inoculated dually. Bacteria and actinomycetes populations were also higher in the root zone soil of plants, inoculated dually, but fungal and *Azotobacter* populations were not affected.

A bacterial screening was carried out by Manero *et al.* (2003) in the rhizosphere of two *Digitalis* species, *D. thapsi* and *D. parviflora*, both at the vegetative stage and at flowering. A total of 480 isolates were characterised at genus level, *Bacillus* being the dominant genera in all cases. Fifty percent of the *Bacillus* strains isolated from each species were analysed by PCR-RAPDs. At 85 % similarity, 12 groups separated for *D. thapsi* and 18 for *D. parviflora*. One strain of each group was selected for biological assay on *D. lanata*, evaluating growth promotion and cardenolide content in leaves after inoculation performed in the root system. The plant parameters evaluated were leaf surface area, shoot and root dry weight and leaf number. Lanatoside C content was evaluated by HPLC. Only 17 strains caused significant increases in at least one of the parameters evaluated. The most striking result was that some strains promoted growth and increased cardenolide content at the same time. This effect was detected on leaves while inoculation was carried out on roots. Interestingly, these two parameters are not enhanced simultaneously under regular conditions in pot or in tissue cultures.

The yield response of a wheat (Kirik) and a barley (Tokak 157/37) cultivar to inoculation with *Azospirillum brasilense* Sp246 and *Bacillus* sp. OSU-142 was studied by Ozturk *et al.* (2003) in relation to three levels of N fertilization (0, 40, and 80 kg ha^{-1}) under field conditions in Erzurum, Turkey, in 1999 and 2000. Seed inoculation with *A. brasilense* Sp246 significantly affected yield and yield components, both in wheat and barley. On average of years and N doses, inoculation with *A. brasilense* Sp246 increased spike number per m^2, grain number per spike, grain yield, and crude protein content by 7.2, 5.9, 14.7, and 4.1 % in wheat and by 6.6, 8.1, 17.5, and 5.1 % in barley, respectively, as compared to control. Inoculation

with *Bacillus* sp. OSU-142 significantly increased kernel number per spike in wheat, but no significant effect was determined in the other characteristics. Grain yields and yield components were also higher at all levels of nitrogen fertilizer in the inoculated plots as compared to the control. However, these increases diminished at high fertilizer levels. These results suggest that application of the growth promoting bacteria *A. brasilense* Sp246 may have the potential to be used as a biofertilizer for spring wheat and barley cultivation in organic and low-N input agriculture.

According to Penrose and Glick (2003) one of the major mechanisms utilized by plant growth-promoting rhizobacteria (PGPR) to facilitate plant growth and development is the lowering of ethylene levels by deamination of 1-aminocyclopropane-1-carboxylic acid (ACC) the immediate precursor of ethylene in plants. The enzyme catalysing this reaction, ACC deaminase, hydrolyses ACC to alpha-ketobutyrate and ammonia. Several bacterial strains that can utilize ACC as a sole source of nitrogen were isolated from rhizosphere soil samples. All of these strains were considered to be PGPR based on the ability to promote canola seedling root elongation under gnotobiotic conditions. The treatment of plant seeds or roots with these bacteria reduced the amount of ACC in plants, thereby lowering the concentration of ethylene.

Ramos *et al.* (2003) either inoculated alder seedlings with a suspension of *Bacillus licheniformis*, or left non-inoculated (controls) which were grown in two different soils under controlled conditions. For 8 weeks after inoculation, plant shoot and root systems were measured; nodules counted, and shoot and root length and surface area determined. In addition to plant growth, changes in the bacterial rhizosphere composition and inoculum levels were determined using the phospholipid fatty acid (PLFA) profile from the rhizosphere soil and from culturable bacteria from the rhizosphere (culturable PLFAs), respectively. They showed the differential effect of *B. licheniformis* on alder growth depending on the soil used. Increases in leaf surface area were significant only when grown in Soil A, while root growth increased in both soils. Effects were more pronounced in Soil A. Changes in the rhizosphere community after inoculation with *B. licheniformis* disappeared within a short period in both soils, 6 weeks in Soil A and only 2 in Soil B. *B. licheniformis* apparently survived at least 8 weeks in the rhizosphere, as revealed

by culturable PLFA profiles. Thus, increases in plant growth could be attributed to changes in the rhizosphere microbial communities, especially in the culturable fraction, due to the presence of the inoculated bacteria in soil. Given the different composition of soils, availability of nutrients must also be considered.

Two strains of *Azospirillum brasilense*, Sp245 and Sp7, were examined by Rothballer *et al.* (2003) for their endophytic potential on German, Brazilian and Israeli wheat cultivars. Plate count and Most Probable Number (MPN) methods were applied for quantification, as well as the fluorescent in situ hybridization (FISH) technique in combination with confocal laser scanning microscopy for the species specific detection and localization of the two *Azospirillum* strains in roots. Additionally, a plasmid bearing a constitutively expressed gfp gene was transformed into both strains, which enables visualization of the bacteria omitting the fixation process during the FISH protocol. The microscopic techniques showed that the potential of strain Sp245 to grow in the roots of all analyzed wheat varieties as an endophyte was greater than of Sp7, but overall cell densities were rather low under the applied experimental conditions. A plant growth promoting effect was clearly visible in all examined inoculated plants, irrespective of the *A. brasilense* strain used as inoculum.

Ryu *et al.* (2003) showed that some PGPR release a blend of volatile components that promote growth of *Arabidopsis thaliana*. In particular, the volatile components 2,3-butanediol and acetoin were released exclusively from two bacterial strains that trigger the greatest level of growth promotion. Furthermore, pharmacological applications of 2,3-butanediol enhanced plant growth whereas bacterial mutants blocked in 2,3-butanediol and acetoin synthesis were devoid in this growth-promotion capacity. The demonstration that PGPR strains release different volatile blends and that plant growth is stimulated by differences in these volatile blends establishes an additional function for volatile organic compounds as signaling molecules mediating plant-microbe interactions.

Plant-growth-promoting rhizobacteria (PGPR) are used on crops most often as seed treatments; however, an alternative application method for transplanted vegetables is mixing PGPR into the soilless medium in which the transplants are

grown. Studies were undertaken by Yan *et al.* (2003) to compare root colonization and persistence of rifampicin-resistant mutants of PGPR strains *Bacillus pumilus* SE34 and *Pseudomonas fluorescens* 89B61, SE34r and 89B61r, on tomato as a function of application method. When the bacteria were incorporated into PromixTM soilless medium at log 6, 7, and 8 colony-forming units g^{-1}, populations of strain SE34r per gram of medium maintained the initial inoculum densities, while populations of 89B61r decreased approximately one to two orders of magnitude by 4 weeks after planting. The populations of each PGPR strain colonizing roots after application into the soilless medium showed a similar pattern at 6 weeks as that at 4 weeks after planting, with higher populations on the whole roots and lateral roots than on the taproots. Strain SE34r but not 89B61r moved upwards and colonized the phyllosphere when incorporated into the soilless medium. Following application as seed treatment, populations of SE34r were significantly higher on upper roots and on the taproot than were populations following application through the soilless medium. Conversely, populations were higher on lower roots and lateral roots following application through the soilless medium than were populations following application as seed treatment. While strain SE34 enhanced plant growth with application both to the medium and as seed treatment, the level of growth promotion was significantly greater with application in the soilless medium. The results indicate that PGPR can be successfully incorporated into soilless media in vegetable transplant production systems.

Soil microbiota communities have demonstrated their crucial role in maintaining the soil ecological balance and therefore the sustainability of either natural ecosystems or agroecosystems. Rhizospheric microbe-plant interactions have a great influence on plant health and soil quality since these root-associated microorganisms are able to help the host plant to deal with drought, nutritional and soil-borne pathogen stress conditions. Plant growth-promoting rhizobacteria (PGPR) can be considered among rhizosphere-beneficial microorganisms. In a micropropagated plant system, bacterial inoculation at the beginning of the acclimatisation phase must also be observed from the perspective of the establishment of the soil microbiota rhizosphere. The objective of the work of

Jaizme *et al.* (2004) was to evaluate the effect of a rhizobacteria consortium of *Bacillus* spp. on the first developmental stages of two micropropagated bananas.

A study by Khalid *et al.* (2004) focused on the screening of effective PGPR strains on the basis of their potential for *in vitro* auxin production and plant growth promoting activity under gnotobiotic conditions. A large number of bacteria were isolated from the rhizosphere soil of wheat plants grown at different sites. Thirty isolates showing prolific growth on agar medium were selected and evaluated for their potential to produce auxins *in vitro*. Colorimetric analysis showed variable amount of auxins (ranging from 1.1 to 12.1 mg L^{-1}) produced by the rhizobacteria *in vitro* and amendment of the culture media with l-tryptophan (l-TRP), further stimulated auxin biosynthesis (ranging from 1.8 to 24.8 mg L^{-1}). HPLC analysis confirmed the presence of indole acetic acid (IAA) and indole acetamide (IAM) as the major auxins in the culture filtrates of these rhizobacteria. A series of laboratory experiments conducted on two cv. of wheat under gnotobiotic (axenic) conditions demonstrated increases in root elongation (up to 17.3 %), root dry weight (up to 13.5 %), shoot elongation (up to 37.7 %) and shoot dry weight (up to 36.3 %) of inoculated wheat seedlings. Linear positive correlation ($r = 0.99$) between in vitro auxin production and increase in growth parameters of inoculated seeds was found. Based upon auxin biosynthesis and growth-promoting activity, four isolates were selected and designated as plant growth-promoting rhizobacteria (PGPR). Auxin biosynthesis in sterilized vs nonsterilized soil inoculated with selected PGPR was also monitored that revealed superiority of the selected PGPR over indigenous microflora. Peat-based seed inoculation with selected PGPR isolates exhibited stimulatory effects on grain yields of tested wheat cv. in pot (up to 14.7 % increase over control) and field experiments (up to 27.5 % increase over control); however, the response varied with cv. and PGPR strains. It was concluded that the strain, which produced the highest amount of auxins in nonsterilized soil, also caused maximum increase in growth and yield of both the wheat cv. Their study suggested that potential for auxin biosynthesis by rhizobacteria could be used as a tool for the screening of effective PGPR strains.

Matiru and Dakora (2004) used light, scanning, and transmission electron microscopy to show that roots of sorghum and millet landraces from Africa were

easily infected by rhizobial isolates from five unrelated legume genera. With sorghum, in particular, plant growth and phosphorus (P) uptake were significantly increased by rhizobial inoculation, suggesting that field selection of suitable rhizobia/cereal combinations could increase yields and produce fodder for livestock production.

In order to examine naturally occurring variation in the ability of *Triticum aestivum* L. (hexaploid wheat) to support certain strains of *P. fluorescens*, Okubara *et al.* (2004) have surveyed 27 Pacific Northwest (PNW) cultivars for the ability to undergo root colonization with the aggressive colonizer *P. fluorescens* strain Q8r1-96, and *P. fluorescens* strain Q2-87, a less effective colonizer. In seed inoculation experiments, Q8r1-86 colonized roots of all of the cultivars equally or more effectively than did Q2-87 in a non-pasteurized, non-agricultural soil. Seven cultivars supported significantly ($P<0.05$) higher rhizosphere populations of Q8r1-96 than Q2-87 within 14 days post-inoculation (dpi), two cultivars supported relatively high population densities of each bacterial strain, and three cultivars supported low population densities of the strains. Population densities normalized to root weight reached maximum steady-state levels within 4 dpi, and differential colonization was seen as early as 7 dpi. In pairwise comparisons, the bacterial treatments differentially affected the root morphology of some of the cultivars at 14 dpi. However, principal components (factor) and correlation analysis showed that preferential colonization by Q8r1-96 was independent of root fresh weight, total length, surface area, volume, and average diameter, and that differential colonization was not correlated with changes in any specific root morphometric variable. Variation in root colonization of specific cultivars suggests useful genetic stocks for mapping and identifying host genes involved in wheat–rhizosphere interactions.

Rhizobacteria with properties related to plant growth-promotion were isolated from the rhizosphere of the perennial legume *Chamaecytisus proliferus* ssp. *proliferus* var. *palmensis* (tagasaste) growing in field conditions. Donate-Correa *et al.* (2005) collected samples in two localities of the Tenerife Island: La Laguna and El Tanque, NE and NW at 600 and 1000 meters above sea level, respectively, and in two seasons, winter and summer. The strains were isolated by using culture dependent procedures, and identified by phenotypic (culturable and biochemical)

and genotypic (ERIC-PCR fingerprinting) features. The rhizosphere isolates formed a diverse community of mainly Gram-negative bacteria, with members of genera *Pseudomonas*, *Burkholderia* and *Sphingomonas* being predominant. A high level of selectivity was found in the rhizosphere environment as compared to the non-rhizosphere soil where Gram-positives were more abundant. Species richness (number of species) and species abundance were related to the sampling season and the locality, thus, samples obtained in winter at both sites had larger counts than samples obtained in summer, and the higher species richness was found in La Laguna. The species *Pseudomonas fluorescens* showed the highest number of properties related to plant growth promotion (PGP): 1-aminocyclopropane-1-carboxylate (ACC) deaminase activity, phytohormone production, nitrogen fixation, fungal growth inhibition and cyanogenesis, thus it seems to be the most suitable microorganism to be tested in PGP-field experiments.

Paenibacillus polymyxa is a plant growth-promoting rhizobacterium with a broad host range, but so far the use of this organism as a biocontrol agent has not been very efficient. Timmusk *et al.* (2005) showed that this bacterium protected *Arabidopsis thaliana* against pathogens and abiotic stress. They studied colonization of plant roots by a natural isolate of *P. polymyxa* which had been tagged with a plasmid-borne *gfp* gene. Fluorescence microscopy and electron scanning microscopy indicated that the bacteria colonized predominantly the root tip, where they formed biofilms. Accumulation of bacteria was observed in the intercellular spaces outside the vascular cylinder. Systemic spreading did not occur, as indicated by the absence of bacteria in aerial tissues. Studies were performed in both a gnotobiotic system and a soil system. The fact that similar observations were made in both systems suggests that colonization by this bacterium can be studied in a more defined system. They discussed the problems associated with green fluorescent protein tagging of natural isolates and deleterious effects of the plant growth-promoting bacteria.

In search of efficient PGPR strains with multiple activities, a total of 72 bacterial isolates belonging to *Azotobacter*, fluorescent *Pseudomonas*, *Mesorhizobium* and *Bacillus* were isolated from different rhizospheric soil and plant root nodules in the vicinity of Aligarh by Ahmad *et al.* (2006). These test isolates were biochemically characterized. These isolates were screened *in vitro* for their

plant growth promoting traits like production of indoleacetic acid (IAA), ammonia (NH_3), hydrogen cyanide (HCN), siderophore, phosphate solubilization and antifungal activity. More than 80 % of the isolates of *Azotobacter*, fluorescent *Pseudomonas* and *Mesorhizobium ciceri* produced IAA, whereas only 20% of *Bacillus* isolates was IAA producer. Solubilization of phosphate was commonly detected in the isolates of *Bacillus* (80 %) followed by *Azotobacter* (74.47 %). *Pseudomonas* (55.56 %) and *Mesorhizobium* (16.67 %). All test isolates could produce ammonia but none of the isolates hydrolyzed chitin. Siderophore production and antifungal activity of these isolates except *Mesorhizobium* were exhibited by 10–12.77% isolates. HCN production was more common trait of *Pseudomonas* (88.89 %) and *Bacillus* (50 %). On the basis of multiple plant growth promoting activities, eleven bacterial isolates (seven *Azotobacter*, three *Pseudomonas* and one *Bacillus*) were evaluated for their quantitative IAA production, and broad-spectrum (active against ≥three test fungi) antifungal activity. Almost at all concentration of tryptophan (50–500 µg/ml), IAA production was highest in the *Pseudomonas* followed by *Azotobacter* and *Bacillus* isolates. *Azotobacter* isolates (AZT_3, AZT_{13}, AZT_{23}), *Pseudomonas* (Ps_5) and *Bacillus* (B_1) showed broad-spectrum antifungal activity on Muller-Hinton medium against *Aspergillus*, one or more species of *Fusarium* and *Rhizoctonia bataticola*. Further evaluation of the isolates exhibiting multiple plant growth promoting (PGP) traits on soil–plant system is needed to uncover their efficacy as effective PGPR.

A study was conducted by Cakmakci *et al.* (2006) with sugar beet in greenhouse and field at two soil type with different organic matter (containing 2.4 and 15.9 % OM, referred as the low- and high-OM soil) conditions in order to investigate seed inoculation of sugar beet, with five N_2-fixing and two phosphate solubilizing bacteria in comparison to control and mineral fertilizers (N and P) application. Three bacterial strains dissolved P; all bacterial strains fixed N_2 and significantly increased growth of sugar beet. In the greenhouse, inoculations with PGPR increased sugar beet root weight by 2.8–46.7 % depending on the species. Leaf, root and sugar yield were increased by the bacterial inoculation by 15.5–20.8, 12.3–16.1, and 9.8–14.7 %, respectively, in the experiment of low- and high-OM soil. Plant growth responses were variable and dependent on the inoculants strain,

soil organic matter content, growing stage, harvest date and growth parameter evaluated. The effect of PGPR was greater at early growth stages than at the later. Effective *Bacillus* species, such as OSU-142, RC07 and M-13, *Paenibacillus polymyxa* RC05, *Pseudomonas putida* RC06 and *Rhodobacter capsulatus* RC04 may be used in organic and sustainable agriculture.

In a study by Chen *et al.* (2006), isolation, screening and characterization of 36 strains of phosphate solubilizing bacteria (PSB) from Central Taiwan were carried out. Mineral phosphate solubilizing (MPS) activities of all isolates were tested on tricalcium phosphate medium by analyzing the soluble-P content after 72 h of incubation at 30°C. Identification and phylogenetic analysis of 36 isolates were carried out by 16S rDNA sequencing. Ten isolates belonged to genus *Bacillus*, nine to genus *Rhodococcus*, seven to genus *Arthrobacter*, six to genus *Serratia* and one each to genera *Chryseobacterium*, *Delftia*, *Gordonia* and *Phyllobacterium*. In addition, four strains namely, *Arthrobacter ureafaciens*, *Phyllobacterium myrsinacearum*, *Rhodococcus erythropolis* and *Delftia* sp. were reported for the first time as phosphate solubilizing bacteria (PSB) after confirming their capacity to solubilize considerable amount of tricalcium phosphate in the medium by secreting organic acids. P-solubilizing activity of these strains was associated with the release of organic acids and a drop in the pH of the medium. HPLC analysis detected eight different kinds of organic acids, namely: citric acid, gluconic acid, lactic acid, succinic acid, propionic acid and three unknown organic acids from the cultures of these isolates. An inverse relationship between pH and P solubilized was apparent from this study. Identification and characterization of soil PSB for the effective plant growth-promotion broadens the spectrum of phosphate solubilizers available for field application.

Seventeen rhizobacteria isolated from different ecological regions, i.e. Brazil, Indonesia, Mongolia and Pakistan were studied to develop inoculants for wheat, maize and rice. Almost all the bacterial isolates were Gram-negative, fast-growing motile rods and utilized a wide range of carbon sources. These isolates produced indole-3-acetic acid at concentrations ranging from 0.8-42.1 μg ml^{-1}, irrespective of the region. Fifteen isolates fixed N at rates ranging from 20.3-556.8 nmole C_2H_2 reduced h^{-1} $vial^{-1}$. Isolate 8N-4 from Mongolia produced the highest amount of

indole-3-acetic acid (42.1 μg ml^{-1}, produced siderophores (0.3 mg ml^{-1}) and was the only isolate that solubilized phosphate (188.7 μg P ml^{-1}). Inoculation of the wheat variety *Orkhon* with 8N-4 isolate resulted in the maximum increase in plant biomass, root length, and total N and P contents in plants. Random amplified polymorphic deoxyribonucleic acid (RAPD) analysis, conducted with 60 decamer primers, revealed a high level of polymorphism among the bacterial isolates from different geographic regions and a low level of polymorphism among isolates from the same region. The complete 16S rRNA gene sequence analysis demonstrated that 8N-4 is a *Bacillus pumilus* strain (Accession number AY548949). It was concluded that *Bacillus pumilus* 8N-4 can be used as a bio-inoculant for biofertilizer production to increase the crop yield of wheat variety *Orkhon* in Mongolia (Hafeez *et al.*, 2006).

Five bacterial strains with phosphate-solubilizing ability and other plant growth promoting traits increased the plant biomass (20–40 %) as tested by paper towel method. Glasshouse and field experiments were conducted using two efficient strains *Serratia marcescens* EB 67 and *Pseudomonas* sp. CDB 35. Increase in plant biomass (dry weight) was 99 % with EB 67 and 94 % with CDB 35 under glasshouse conditions. Increase in plant biomass at 48 and 96 days after sowing was 66 % and 50 % with EB 67 and 51 % and 18 % with CDB 35 under field conditions. Seed treatment with EB 67 and CDB 35 increased the grain yield of field-grown maize by 85 % and 64 % compared to the uninoculated control. Population of EB 67 and CDB 35 were traced back from the rhizosphere of maize on buffered rock phosphate (RP) medium and both the strains survived up to 96 days after sowing (Hameeda *et al.*, 2006).

Field trials were conducted by Kokalis-Burelle *et al.* (2006) in Florida on bell pepper (*Capsicum annuum*) to monitor the population dynamics of two plant growth-promoting rhizobacteria (PGPR) strains (*Bacillus subtilis* strain GBO3 and *Bacillus amyloliquefaciens* strain IN937a) applied in the potting media at seeding and at various times after transplanting to the field during the growing season. In-field drenches of an aqueous bacterial formulation were used for the mid-season applications. The effects of the applied PGPR and application methods on bacterial survival, rhizosphere colonization, plant growth and yield, and selected indigenous rhizosphere microorganisms were assessed. The Gram-positive PGPR applied to the

potting media established stable populations in the rhizosphere that persisted throughout the growing season. Additional aqueous applications of PGPR during the growing season did not increase the population size of applied strains compared to treatments only receiving bacteria in the potting media; however, they did increase plant growth compared to the untreated control to varying degrees in both trials. Most treatments also reduced disease incidence in a detached leaf assay, indicating that systemic resistance was induced by the PGPR treatments. However, treatments did not result in increased yield, which was highly variable. Application of the PGPR strains did not adversely affect populations of beneficial indigenous rhizosphere bacteria including fluorescent pseudomonads and siderophore-producing bacterial strains. Treatment with PGPR increased populations of fungi in the rhizosphere but did not result in increased root disease incidence. This fungal response to the PGPR product was likely due to an increase in nonpathogenic chitinolytic fungal strains resulting from the application of chitosan, which is a component of the PGPR formulation applied to the potting media.

A study was conducted by Shaharoona *et al.* (2006) to test the hypothesis that the bacterial strains possessing 1-aminocyclopropane-1-carboxylic acid (ACC)-deaminase activity may also promote growth of inoculated plants and could increase nodulation in legumes upon co-inoculation with rhizobia. Several rhizobacteria were isolated from maize rhizosphere through enrichment on ACC as a sole N source. Purified isolates were screened for growth promotion in maize under axenic conditions and for in vitro ACC-deaminase activity. A significant positive correlation was observed between in vitro ACC-deaminase activity of bacterial cells and root elongation. None of the isolates produced auxins. *Bradyrhizobium japonicum* produced lesser amount of auxins but did not carry ACC-deaminase activity. Results of pot experiment revealed that co-inoculation with *Bradyrhizobium* and plant growth promoting rhizobacteria (PGPR) isolates enhanced the nodulation in mung bean compared with inoculation with *Bradyrhizobium* alone. It is highly expected that inoculation with rhizobacteria containing ACC-deaminase hydrolysed endogenous ACC into ammonia and alpha-ketobutyrate instead of ethylene. Consequently, root and shoot growth as well as nodulation were promoted.

The effect of different plant-growth promoting rhizobacteria (*Azotobacter chroococcum, Azospirillum brasilense*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Bacillus cereus*) on pigeonpea (*Cajanus cajan* (L) Milsp.) cv. P-921 inoculated with *Rhizobium* sp. (AR-2–2 k) was assessed. A glasshouse experiment was carried out by Tilak *et al.* (2006) with a sandy-loam soil in which the seeds were treated with *Rhizobium* alone or in combination with several PGPR isolates. It was monitored on the basis of nodulation, N_2 fixation, shoot biomass, total N content in shoot and legume grain yield. The competitive ability of the introduced *Rhizobium* strain was assessed by calculating nodule occupancy. The PGPR isolates used did not antagonize the introduced *Rhizobium* strain and the dual inoculation with either *Pseudomonas putida*, *P. fluorescens* or *Bacillus cereus* resulted in a significant increase in plant growth, nodulation and enzyme activity over *Rhizobium*-inoculated and uninoculated control plants. The nodule occupancy of the introduced *Rhizobium* strain increased from 50 % (with *Rhizobium* alone) to 85 % in the presence of *Pseudomonas putida*. This study enabled us to select an ideal combination of efficient *Rhizobium* strain and PGPR for pigeonpea grown in the semiarid tropics.

Yanni *et al.* (2006) studied the natural and intimate associations between rhizobia and rice (*Oryza sativa* L.) and assessed their impact on plant growth in order to exploit those combinations that can enhance grain yield with less dependence on inputs of nitrogen (N) fertilizer. Diverse, indigenous populations of *Rhizobium leguminosarum* bv. *trifolii* (the clover root-nodule endosymbiont) intimately colonize rice roots in the Egyptian Nile delta where this cereal has been rotated successfully with berseem clover (*Trifolium alexandrinum* L.) since antiquity. Laboratory and greenhouse studies have shown with certain rhizobial strain–rice variety combinations that the association promotes root and shoot growth thereby significantly improving seedling vigour that carries over to significant increases in grain yield at maturity. Three field inoculation trials in the Nile delta indicated that a few strain–variety combinations significantly increased rice grain yield, agronomic fertilizer N-use efficiency and harvest index. The benefits of this association leading to greater production of vegetative and reproductive biomass more likely involve rhizobial modulation of the plant's root architecture for more efficient acquisition of certain soil nutrients [e.g. N, phosphorus (P), potassium (K),

magnesium (Mg), calcium (Ca), zinc (Zn), sodium (Na) and molybdenum (Mo)] rather than biological N_2 fixation. Inoculation increased total protein quantity per hectare in field-grown grain, thereby increasing its nutritional value without altering the ratios of nutritionally important proteins. Studies using a selected rhizobial strain (E11) indicated that it produced auxin (indoleacetic acid) and gibberellin [tentatively identified as gibberellin (GA 7)] phytohormones representing two major classes of plant growth regulators. Axenically collected rice root exudate significantly enhanced E11's production of this auxin. This strain extensively colonized the rice root surface under gnotobiotic culture conditions, producing distributions of spatial patchiness that would favour their localized erosion of the epidermal surface, colonization of small crevices at epidermal junctions as a possible portal to enter into the root, and quorum sensing of diffusible signal molecules indicating that their nearest bacterial neighbours are in close proximity in situ. Studies of selected rhizobial endophytes of rice indicated that they produced cell-bound cellulase and polygalacturonase enzymes that can hydrolyze glycosidic bonds in plant cell walls, and non-trifolitoxin bacteriocin(s) that can inhibit other strains of clover rhizobia. Strain E11 was able to endophytically colonize rice roots of varieties commonly used by Filipino peasant farmers, and also to stimulate genotype-specific growth-promotion of corn (*Zea mays*, maize) under field conditions. An amalgam of these results indicate some rhizobia have evolved an additional ecological niche enabling them to form a three-component life cycle including a free-living heterotrophic phase in soil, a N_2-fixing endosymbiont phase within legume root nodules, and a beneficial growth-promoting endocolonizer phase within cereal roots in the same crop rotation. Our results further indicate the potential opportunity to exploit this newly described, plant¿rhizobia association by developing biofertilizer inoculants that may assist low-income farmers in increasing cereal production (especially rice) with less fertilizer N inputs, fully consistent with both sustainable agriculture and environmental safety.

Biological Control

Pseudomonas fluorescens strains which effectively inhibited mycelial growth of *Fusarium udum*, the pigeonpea (*Cajanus cajan*) pathogen, were isolated from the rhizoplane of different crops (Vidhyasekaran, 1997). Various powder formulations of two efficient *P. fluorescens* strains were developed. All freshly prepared powder formulations were effective in controlling the disease, but their efficacies varied depending upon the length of storage. Talc formulations were effective even after 6 months of storage, while peat formulations were effective up to 60 days of storage. The shelf life of vermiculite, lignite, and kaolinite formulations was short. Unformulated bacterial suspensions could not be stored even for 10 days, at which time their efficacy was completely lost. The bacterial strains survived in pigeonpea rhizosphere throughout the crop-growth period. The talc-based powder formulations effectively controlled pigeonpea wilt and increased yield in two field trials. Development of powder formulations of *P. fluorescens* will aid large-scale application of biological control in farmers' fields.

Selected PGPR strains belonging to diverse Gram-positive and Gram-negative genera can, upon seed treatment or soil drench treatment to plant root systems were reported by Kloepper *et al.* (1998), reduce the incidence of distally infecting pathogens. Single PGPR strains have been shown to reduce pathogen infection and symptoms of multiple diseases on cucumber and tomato. Cucumber diseases affected in both greenhouse and field studies in multiple years include foliar diseases (angular leaf spot, caused by *Pseudomonas syringae* pv. *lachrymans*; and anthracnose, caused by *Colletotrichum orbiculare*); systemic wilt diseases (cucurbit wilt, caused by *Erwinia tracheiphila*; and Fusarium wilt, caused by *Fusarium oxysporum* f.sp. *cucumerinum*), and the systemic viral disease caused by cucumber mosaic virus (CMV). In the case of cucurbit wilt, disease control is linked to PGPR-mediated reductions in plant preference by the insect vectors, the striped and spotted cucumber beetles. In field and greenhouse studies, PGPR treatments led to significant reduction in beetle feeding, which was associated with PGPR-mediated reductions in cucurbitacin C, a feeding attractant. With tomato, protection has been noted in the greenhouse or field against CMV; bacterial spot, caused by *Xanthomonas axonopodis* pv. *vesicatoria*; tomato mottle geminivirus; and bacterial

speck, caused by *P. syringae* pv. *tomato*. Mode of action studies support the conclusion that the observed systemic biocontrol results from ISR, since measurable biochemical and cytological changes occur in the plant in relation to host recognition of the inducing PGPR strains. The specific plant changes vary somewhat among PGPR strains and are the focus of intense current investigation. They have observed enhanced peroxidase activity and lignification in cucumber and induction of PR1a promoter in transgenic tobacco containing a GUS reporter gene. In this tobacco system, all PGPR strains which enhanced protection against wildfire disease – caused by *P. syringae* pv. *tabaci* – in the greenhouse induced GUS activity, whereas control strains lacking disease protecting activity did not induce GUS activity significantly relative to controls.

Plant growth-promoting rhizobacteria (PGPR) strains INR7 (*Bacillus pumilus*), GB03 (*Bacillus subtilis*), and ME1 (*Curtobacterium flaccumfaciens*) were tested singly and in combinations for biological control against multiple cucumber pathogens. Investigations under greenhouse conditions were conducted with three cucumber pathogens—*Colletotrichum orbiculare* (causing anthracnose), *Pseudomonas syringae* pv. *lachrymans* (causing angular leaf spot), and *Erwinia tracheiphila* (causing cucurbit wilt disease)—inoculated singly and in all possible combinations. There was a general trend across all experiments toward greater suppression and enhanced consistency against multiple cucumber pathogens using strain mixtures. The same three PGPR strains were evaluated as seed treatments in two field trials over two seasons, and two strains, IN26 (*Burkholderia gladioli*) and INR7 also were tested as foliar sprays in one of the trials. In the field trials, the efficacy of induced systemic resistance activity was determined against introduced cucumber pathogens naturally spread within plots through placement of infected plants into the field to provide the pathogen inoculum. PGPR-mediated disease suppression was observed against angular leaf spot in 1996 and against a mixed infection of angular leaf spot and anthracnose in 1997. The three-way mixture of PGPR strains (INR7 plus ME1 plus GB03) as a seed treatment showed intensive plant growth promotion and disease reduction to a level statistically equivalent to the synthetic elicitor Actigard applied as a spray (Raupach and Kloepper, 1998).

According to Van Loon *et al.* (1998) nonpathogenic rhizobacteria can induce a systemic resistance in plants that is phenotypically similar to pathogen-induced systemic acquired resistance (SAR). Rhizobacteria-mediated induced systemic resistance (ISR) has been demonstrated against fungi, bacteria, and viruses in *Arabidopsis*, bean, carnation, cucumber, radish, tobacco, and tomato under conditions in which the inducing bacteria and the challenging pathogen remained spatially separated. Bacterial strains differ in their ability to induce resistance in different plant species, and plants show variation in the expression of ISR upon induction by specific bacterial strains. Bacterial determinants of ISR include lipopolysaccharides, siderophores, and salicylic acid (SA). Whereas some of the rhizobacteria induce resistance through the SA-dependent SAR pathway, others do not and require jasmonic acid and ethylene perception by the plant for ISR to develop. No consistent host plant alterations are associated with the induced state, but upon challenge inoculation, resistance responses are accelerated and enhanced. ISR is effective under field conditions and offers a natural mechanism for biological control of plant disease.

According to Braun-Kiewnick *et al.* (2000) strains of *Pantoea agglomerans* (synanamorph *Erwinia herbicola*) suppressed the development of basal kernel blight of barley, caused by *Pseudomonas syringae* pv. *syringae*, when applied to heads prior to the *Pseudomonas syringae* pv. *syringae* infection window at the soft dough stage of kernel development. Field experiments in 1994 and 1995 revealed 45 to 74 % kernel blight disease reduction, whereas glasshouse studies resulted in 50 to 100 % disease control depending on the isolate used and barley cultivar screened. The efficacy of biocontrol strains was affected by time and rate of application. Percentage of kernels infected decreased significantly when *P. agglomerans* was applied before pathogen inoculation, but not when coinoculated. A single *P. agglomerans* application 3 days prior to the pathogen inoculation was sufficient to provide control since populations of about 10(^7) CFU per kernel were established consistently, while *Pseudomonas syringae* pv. *syringae* populations dropped 100-fold to 2.0 × 10(^4) CFU per kernel. An application to the flag leaf at EC 49 (before heading) also reduced kernel infection percentages significantly. Basal blight decreased with increasing concentrations (10(^3) to 10(^7) CFU ml^{-1}) of

P. agglomerans, with 10(^7) CFU/ml providing the best control. For long-term preservation and marketability, the survival of bacterial antagonists in several wettable powder formulations was tested. Over all formulations tested, the survival declined between 10- to >100-fold over a period of 1.5 years (r = –0.7; P = 0.000). Although not significant, storage of most formulations at 4°C was better for viability (90 to 93 % survival) than was storage at 22°C (73 to 79 %). However, long-term preservation had no adverse effect on biocontrol efficacy.

The efficacy of various *P. fluorescens* isolates was tested for the management of fruit rot of chilli caused by *Colletotrichum capsici*. Among the various isolates tested *P. fluorescens* isolates *viz.* Pf1 and ATR increased the plant growth and produced the maximum amout of indole acetic acid. *P. fluorescens* Pf1 effectively inhibited the mycelial growth of the pathogen under *in vitro* conditions and decreased the fruit rot incidence under greenhouse condition. Seed treatment plus soil application of talc based formulation of *P. fluorescens* isolate Pf1 effectively reduced the disease incidence. Expression of various defense related enzymes and chemicals was found involved in the induction of systemic resistance against pathogen infection. Induction of various defense related genes has been discussed for the suppression of pathogen infection by Ramamoorthy and Samiyappan (2001).

Efficacy of seven strains of *Pseudomonas fluorescens* (*Pfs*17), plant growth-promoting rhizobacteria (PGPR), were tested by Sarma *et al.* (2002) under field conditions for their ability to protect *Cicer arietinum* against *Sclerotium rolfsii* infection. Best protection was observed in strain *Pfs*3 where 23 % seedling mortality was recorded in comparison to 44 % in non-treated control. To correlate the induction of phenolic compounds by the PGPRs with disease resistance, qualitative and quantitative alterations of phenolic compounds in different parts of *C. arietinum* were estimated following PGPR application as seed treatment. High performance liquid chromatographic (HPLC) analysis of the leaves, collars and roots of the PGPR-treated and non-treated (control) plants showed the presence of gallic, ferulic, chlorogenic and cinnamic acids with varied amounts in the PGPR-treated as well as non-treated (control) plants. Maximum accumulation of cinnamic acid was observed in plants treated with *Pfs*3 strain (1660 ng g^{1} fresh wt.) which was almost 19.5 times higher than untreated control plants and also significantly high when compared to

other PGPR treatments. *Pfs*3 also caused maximum accumulation of total phenolics and gallic acid in all chickpea plant parts as compared to other treatments and untreated control. A direct relationship between the level of total phenolics and seedling survivability was observed. PGPR-mediated induction of phenolic compounds as a biochemical barrier in *C. arietinum* against *S. rolfsii* infection is envisaged.

Shternshis *et al.* (2002) tested three products based on compounds of biological origin for their ability to control the raspberry midge blight in the Siberian region of Russia. *Bacillus thuringiensis* sub sp. *israelensis* (Bacticide) and *Streptomyces avermitilis metabolies* (Phytovern) were used against *Thomasiniana theobaldi* (a general member of the midge blight) and chitinase was used against fungi (mainly *Didymella applanata*) associated with *T. theobaldi*. The Bacticide (0.2 %) and Phytoverm (0.2%) sprays caused a two fold decrease in midge blight severity and the same effect was obtained with chemical insecticides. The chitinase (1%) spray caused a four fold decrease in the severity of midge blight. In addition. Chitinase and Phytovem caused a significant suppression of the independent spur blight. These studies form the basis for further evaluation of ecologically safe control of the raspberry midge blight.

Bansal *et al.* (2003) tested the efficacy of *Azotobacter chroococcum* against tomato wilt pathogen (*Fusarium oxysporum* f. sp. *lycopersici)* during rabi 2000-01 and 2001-02 in pot house under artificial inoculum conditions. Tomato seedlings var. local, treated with *A. chroococcum* before transplanting along with soil application of nitrogen @ 60, 80 and 100 kg ha^{-1} showed complete inhibition of plant mortality (7.36%) was also observed when seedlings were reated with *A. chroococcum* only as compared to the seedlings without any treatment (17.35%). It may be attributed to the production of antifungal substances by *A. chroococcum.*

Bhatia *et al.* (2003) observed maximum colony growth inhibition due to *Pseudomonas* PS 2 (74 %) as compared to PS 1 (71 %) on trypticase soy agar (TSM) plates after 5 days of incubation. Light and scanning electron microscope examination showed hyphal coiling, vacuolation and granulation of cytoplasm resulting in lysis of hyphae of *Macrophomina phaseolina* by pseudomonads. Cell

free culture filtrates of strains PS1 and PS 2 restricted the growth of mycelium of *M. phaseolina*, PS 1 and PS 2 caused maximum colony growth inhibition by 57 and 61% respectively at 20% conc. of culture filtrate after 4 days of incubation. Volatile substances produced by PS 1 and PS 2 also inhibited the colony growth of *M. phaseolina* by 25 and 32% respectively. Inhibitory effect of volatile substances, however, decreased with advancing in incubation period. Colony growth of *M. phaseolina* was significantly decreased by PS 1 and PS 2 as compared to control both in iron sufficient and iron deficient conditions. PS 2 showed higher antagonistic activity than PS 1, as evidenced by pronounced colony growth inhibition.

Fourteen plant growth promoting rhizobacteria (PGPR) isolated from rhizotic zones of field-grown green gram (*Vigna radiata* (L.) Wilczek) plants were examined by Gupta *et al.* (2003) for their growth-promoting attributes and ability to affect the growth *in vitro* of 10 strains of *Bradyrhizobium* sp. (*Vigna*). None of the rhizosphere bacteria was found to repress or stimulate the growth of any of the *Bradyrhizobium* strains tested. However, they produced antibiotics and siderophores and plant growth promoting substances. *Ex planta* and plant nitrogen fixation and phosphate solubilization was not detected by any of the isolates. Under *in-vitro* conditions, nine isolates inhibited growth of soil-borne fungal pathogens; one of them identified as *Bacillus* sp. antagonized all the fungi tested on two different media. All PGPR isolates were tested both in sterile and unsterile soil for their ability to promote nodulation, nitrogen fixation, growth, and yield of green gram in the presence of two *Bradyrhizobium* sp. (*Vigna*) strains S 24 and Cog 15. In sterile soil, all PGPR isolates had a positive effect on shoot biomass development, acetylene reduction assay (ARA), and N content when co-inoculated with *Bradyrhizobium* strain Cog 15, but could influence only shoot biomass development in the presence of strain S 24. In unsterile soil, PGPR isolates had a nodule-stimulatory effect on strain Cog 15 and a plant growth promoting effect on strain S 24, after 50 and 90 days of plant growth. Five isolates EG-RS-3, EG-RS-4, and NG-ER-7 (*Bacillus* spp), and KG-ER-1 and EG-ER-2 (*Enterobacter* spp) significantly increased yield of green gram in unsterile soil.

Murphy *et al.* (2003) evaluated combinations of two strains of plant growth-promoting rhizobacteria (PGPR) formulated with the carrier chitosan for the ability

to induce growth promotion of tomato plants and resistance to infection by Cucumber mosaic virus (CMV). Each PGPR combination included GB03 (*Bacillus subtilis*) and one of the following PGPR strains: SE34 (*B. pumilus*), IN937a (*B. amyloliquefaciens*), IN937b (*B. subtilis*), INR7 (*B. pumilus*), or T4 (*B. pumilus*). The PGPR combinations formulated with chitosan are referred to as biopreparations. Tomato plants treated with each of the biopreparations appeared phenotypically and developmentally similar to nonbacterized control plants that were 10 days older (referred to as the older control). When plants were challenged with CMV, all plants in the biopreparation treatments and the older control treatment had significantly greater height, fresh weight, and flower and fruit numbers than that of plants in the CMV-inoculated same age control treatment. CMV disease severity ratings were significantly lower for biopreparation-treated and older control tomato plants than for that of same age control plants at 14 and 28 days postinoculation (dpi). CMV accumulation in young noninoculated leaves was significantly less for all biopreparation-treated plants and those in the older control than for the same age control plants at 14 dpi and for four of the five biopreparation treatments at 28 dpi. In those tomato plants shown to be infected, the amount of CMV in noninoculated leaves was significantly lower for three of the biopreparation treatments and the older control treatment at 14 dpi and biopreparation G/INR7 treatment at 28 dpi when compared with the control treatment. These data show that treatment of tomato plants with biopreparations results in significant enhancement of growth and protection against infection by CMV.

A series of laboratory, greenhouse and field experiments were conducted by Niranjan, *et al.* (2003) on the strains of plant growth promoting rhizobacteria (PGPR).The PGPR were tested as suspensions of fresh cultures and talc-based powder formulations. Evaluations were conducted on pearl millet (*Pennisetum glaucum*) for growth promotion and management of downy mildew caused by *Sclerospora graminicola.* All treatments with fresh suspensions and powdered formulations showed enhancement in germination and vigor index over the respective untreated controls. With fresh suspensions, maximum vigor index resulted from treatments by *Bacillus pumilus* strain INR7 followed by *B. subtilis* strain IN937b (64 and 38% higher than the untreated control, respectively). With powdered

formulation, treatment with strain INR7 also resulted in the highest germination and vigor indexes, which were 10 and 63%, respectively, over the untreated control. Under experimental plot conditions, prominent enhancement in growth also was observed in the disease tests. Yield was enhanced 40 and 37% over the untreated control by seed treatment with powdered formulations of strains INR7 and SE34, respectively. The same strains also increased yield by 36 and 33%, respectively, when applied as fresh suspensions. Studies on downy mildew management resulted in varied degrees of protection by the PGPR both under greenhouse and field conditions. With fresh suspensions, treatment with INR7 resulted in the highest protection (57%), followed by *B. pumilus* strain SE34 and B. subtilis strain GBO3, which resulted in 50 and 43% protection, respectively, compared with the untreated control. With powdered formulation, PGPR strain INR7 suppressed downy mildew effectively, resulting in 67% protection, while SE34 resulted in 58% protection, followed by GBO3 with 56% protection. Treatment with Apron (Metalaxyl) resulted in the highest protection against downy mildew under both greenhouse and field conditions. Thus, the present study suggests that the tested PGPR, both as powdered formulations and fresh suspensions, can be used within pearl millet downy mildew management strategies and for plant growth promotion.

Five plant growth promoting rhizobacterial formulations, each consisting of two *Bacilli* strains with chitosan as a carrier were tested for their capacity to promote growth and induce resistance against downy mildew in pearl millet under both greenhouse and field conditions. Three modes of applications were tested: seed treatment, soil amendment, and seed treatment+soil amendment. In general, irrespective of application method, most of the formulations, in comparison with the control, increased plant growth and vigor as measured by seed germination, seedling vigour, plant height, fresh and dry weight, leaf area, tillering capacity, number of earheads, length and girth of earhead, 1000 seed weight and yield. The time of flowering was also advanced by 4-5 days over the control. Likewise all the formulations significantly reduced downy mildew incidence relative to the nontreated control. However, the rate of growth enhancement and disease suppression varied considerably with the formulations. Formulations LS256 and LS257 besides being the best growth promoters were also the most efficient

resistance inducers. None of the formulations matched the level of the fungicide metalaxyl in offering protection against downy mildew. Among the application methods tested, soil amendment was found to be the most suitable and desirable way of delivering the formulations. Combination of seed treatment and soil amendment produced the same effect that was produced by soil amendment alone. This study by Raj *et al.* (2003) demonstrates a potential role for plant growth promoting rhizobacterial formulations in downy mildew management.

Greenhouse experiments showed that four mixtures of plant growth-promoting rhizobacteria (PGPR) strains (all *Bacillus* spp.) elicited induced systemic resistance in several plants against different plant pathogens. Based on these findings, Jetiyanon *et al.* (2003) sought to determine if systemic resistance induced by these PGPR would lead to broad-spectrum protection against several pathogens under field conditions in Thailand. Experiments were conducted during the rainy season (July to October 2001) and winter season (November 2001 to February 2002) on the campus of Naresuan University, Phitsanulok, Thailand. The specific diseases and hosts tested were southern blight of tomato (*Lycopersicon esculentum*) caused by *Sclerotium rolfsii*, anthracnose of long cayenne pepper (*Capsicum annuum* var. *acuminatum*) caused by *Colletotrichum gloeosporioides*, and mosaic disease of cucumber (*Cucumis sativus*) caused by Cucumber mosaic virus (CMV). Results showed that some PGPR mixtures suppressed disease more consistently than the individual PGPR strain IN937a. One PGPR mixture, *Bacillus amyloliquefaciens* strain IN937a+*B. pumilus* strain IN937b, significantly protected (P=0.05) plants against all tested diseases in both seasons. Further, cumulative marketable yields were positively correlated with some treatments.

Biochemical changes in banded leaf and sheath blight affected maize plants caused by *Rhizoctonia solani* f. sp. *sasakii* grown out of seeds treated with *Pseudomonas fluorescens* were studied by Shivakumar and Sharma (2003). There was an increase in phenolic content in maize leaf sheaths inoculated with *R. solani* or in those of maize plants raised from *P. fluorescens* treated seeds. Increase in phenolic content was observed, in leaf sheaths of plants raised from *P. fluorescens* treated seeds when inoculated with *R. solani*. Peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) activities increased when leaf

sheaths were inoculated with the pathogen and plants raised from *P. fluorescens* treated seeds showed higher activity. However leaves from *P. fluorescens* treated seeds did not show any further increase in PO and PPO activities after inoculation with *R. solani*. The bacterized seed with *P. fluorescens* led to accumulation of higher phenolic compounds and higher activity of PO, PPO and PAL, that may play a role in defense mechanism in plants against pathogen.

Root colonization by certain non-pathogenic bacteria can induce systemic resistance to pathogen infections in plants. In a split-root assay with tomato plants, Siddiqui and Shaukat (2003) investigated which determinants of the rhizobacterium *Pseudomonas aeruginosa* IE-6S+ were important for induction of resistance to the root-knot nematode *Meloidogyne javanica*. *P. aeruginosa* IE-6S+ produced 3.9+-1.1 μg ml^{-1} salicylic acid (SA) in a liquid casamino acid medium under laboratory conditions. The bacterial inoculant induced resistance equivalent to the application of 10 mM synthetic SA. However, SA at this concentration did not produce significant mortality of *M. javanica* juveniles *in vitro*. Soil iron (2.4 mM FeCl3cntdot6H2O) did not markedly alter the resistance that *P. aeruginosa* IE-6S+ induced in tomato roots, which suggested that *P. aeruginosa* IE-6S+ activity was not iron-regulated. However, the resistance reaction was greatly enhanced when IE-6S+ and SA were co-inoculated with 0.5% Tween-20. While IE-6S+ colonized the tomato rhizosphere at 6.38 log cfu g^{-1} fresh weight of root during the first 3 days after inoculation, the bacterial populations declined steadily, reaching a mean population density of 4.73 log cfu g^{-1} fresh weight of root at 21 days. The bacterium was not isolated from the unbacterized half of the split root system.

The ability to colonize roots is a sine qua non condition for a rhizobacteria to be considered a true plant growth-promoting rhizobacteria (PGPR). A simple screening method to detect such a potential ability of PGPR is described by Silva *et al.* (2003). Tomato seeds were surface sterilized for 30 s in 50% ethanol and this was followed by 3 min dipping in 2% NACIO. They were then washed three times in sterile water, left immersed in a propagule suspension of the rhizobacteria for 24 h, and transferred onto sterile 0.6% water-agar in tubes. The young, developing root system showed a tendency to grow downwards in the agar-gel column. Testing 500

rhizobacteria isolated from tomato rhizosphere for their ability to induce systemic resistance against *Pseudomonas syringae* pv. *tomato*, 28 of them did reduce infection to less than 40% and all 28 colonized roots according to the described bioassay.

Two plant growth-promoting rhizobacteria (PGPR), viz., *Pseudomonas fluorescens* strain Pf4 and *P. aeruginosa* strain Pag, protected chickpea (*Cicer arietinum*) plants from *Sclerotium rolfsii* infection when applied singly or in combination as seed treatment (Singh *et al.*, 2003). Pag gave the best protection to the seedlings, applied either singly (mortality 16%) or in combination with Pf4 (mortality 17%) compared with 44% and 24% mortality in control and Pf4 treatment, respectively. The two PGPR strains induced the synthesis of specific phenolic acids, salicylic acid (SA), as well as total phenolics at different growth stages of chickpea seedlings with varied amount. The maximum amount of total phenolics was recorded in all the aerial parts of 4-week-old plants. Gallic, ferulic, chlorogenic, and cinnamic acids were the major phenolic acids detected in high-performance liquid chromatography (HPLC) analysis. Induction of such phenolic acids in the seedlings was observed up to 6 weeks in comparison with control. Salicylic acid (SA) was induced frequently during the first 3 weeks of growth only. Between the two strains, Pag was more effective in inducing phenolic acid synthesis applied either singly or in combination with strain Pf4 during the entire 6 weeks of growth of chickpea. In the presence of a culture filtrate of *S. rolfsii*, the two *Pseudomonas* strains induced more phenolic acids in treated than in non-treated and control plants. The occurrence of salicylic acid was frequent in the first 24 h, but infrequent at 48 and 96 h. Foliar spray of *Pseudomonas* strains also enhanced the phenolic acid content as well as total phenolics within 24 h of application. Gallic, chlorogenic, and cinnamic acids were consistently discerned in the treated leaves, whereas SA was absent even up to 96 h of application. Resistance in chickpea plants by *Pseudomonas* strains through induction of phenolic compounds as well as induced systemic resistance via SA-dependent pathway was evident.

Aflatoxin contamination of groundnut, caused by *Aspergillus flavus* (Af) group of fungi, is a major problem in the rain fed agriculture in the seni arid tropics. Biological control could be one of the components of integrated management to reduce pre harvest kernel investment in the field. Thakur *et al.* (2003) evaluated six

Trichoderma and three *Pseudomonas* strains that were identified as highly antagonistic to Af 11-4 (a highly toxigenic strain) *in vitro*, in field to determine their biocontrol potential. The antagonists were applid as seed dressing and soil application in flowering in Af-sick pots. All the antagonists significantly reduced as seed infection in all three field experiments. Two *T. viridae*(Tv 17 and Tv 23), one *T. harzianum* (Th 23) and one *Pseudomonas* (pf 2) isolates provided greater protection to seed infection by Af 11-4 than others. The reduced seed contamination occurred due to significant reduction in Af population in the rhizosphere of groundnut.

Greenhouse experiments were conducted by Anith *et al.* ((2004) to study the effect of plant growth promoting rhizobacteria (PGPR; *Bacillus pumilus* SE 34, *Pseudomonas putida* 89B61, BioYield, and Equity), acibenzolar-S-methyl (Actigard), and a soil amendment with S-H mixture (contains agricultural and industrial wastes such as bagasse, rice husk, oyster shell powder, urea, potassium nitrate, calcium super phosphate, and mineral ash) on bacterial wilt incidence caused by *Ralstonia solana-cearum* (race 1, biovar 1) in susceptible tomato (*Lycopersicon esculentum* cv. Solar Set). In experiments with PGPR, *Pseudomonas putida* 89B61 significantly reduced bacterial wilt incidence when applied to the transplants at the time of seeding and 1 week prior to inoculation with *Ralstonia solanacearum*. BioYield, a formulated PGPR that contained two *Bacillus* strains, decreased disease significantly in three experiments. Equity, a formulation containing more than 40 different microbial strains, did not reduced wilt incidence compared with the untreated control. With inoculum at low pathogen densities of 1×10^5 and 1×10^6 CFU m^{-1}, disease incidence of Actigard-treated plants was significantly less than with nontreated plants. This is the first report of Actigard-mediated reduction of bacterial wilt incidence in a susceptible tomato cultivar. When PGPR and Actigard applications were combined, Actigard plus *P. putida* 89B61 or BioYield reduced bacterial wilt incidence compared with the untreated control. Incorporation of S-H mixture into infested soil 2 weeks before transplanting reduced the bacterial wilt incidence in one experiment. Combination of Actigard with the S-H mixture significantly reduced bacterial wilt incidence in tomato in two experiments.

Endophytic actinobacteria isolated from healthy cereal plants were assessed for their ability to control fungal root pathogens of cereal crops both *in vitro* and in planta. Thirty eight strains belonging to the genera *Streptomyces*, *Microbispora*, *Micromonospora*, and *Nocardioidies* were assayed by Coombs *et al.* (2004) for their ability to produce antifungal compounds *in vitro* against *Gaeumannomyces graminis* var. *tritici* (*Ggt*), the causal agent of take-all disease in wheat, *Rhizoctonia solani* and *Pythium* spp. Spores of these strains were applied as coatings to wheat seed, with five replicates (25 plants), and assayed for the control of take-all disease in planta in steamed soil. The biocontrol activity of the 17 most active actinobacterial strains was tested further in a field soil naturally infested with take-all and *Rhizoctonia*. Sixty-four percent of this group of microorganisms exhibited antifungal activity *in vitro*, which is not unexpected as actinobacteria are recognized as prolific producers of bioactive secondary metabolites. Seventeen of the actinobacteria displayed statistically significant activity in planta against *Ggt* in the steamed soil bioassay. The active endophytes included a number of *Streptomyces*, as well as *Microbispora* and *Nocardioides* spp. and were also able to control the development of disease symptoms in treated plants exposed to *Ggt* and *Rhizoctonia* in the field soil. The results of this study indicate that endophytic actinobacteria may provide an advantage as biological control agents for use in the field, where others have failed, due to their ability to colonize the internal tissues of the host plant.

A pool of 11 randomly selected, uncharacterized *Bacillus pumilus* isolates from sugar beet were evaluated by Bargabus, *et al.*(2004) using a high-throughput screen that utilized laboratory-based tests for 2 pathogenesis-related proteins, chitinase and β-1,3-glucanase, and biphasic hydrogen peroxide production. The screen was followed by a glasshouse test for induction of systemic acquired resistance for control of Cercospora leaf spot in sugar beet. These isolates were compared to the known biological control agent, *Bacillus mycoides* isolate Bac J, and a chemical inducer of resistance, acibenzolar-*S*-methyl. All laboratory-based screens identified *B. pumilus* isolates 203-6 and 203-7, which reduced Cercospora leaf spot symptoms by approximately 70%, even when spatially separated from the casual agent, *Cercospora beticola*. This level of control was similar to *B. mycoides* isolate Bac J and acibenzolar-*S*-methyl. In all cases, systemic resistance elicitation

was marked by an increase in 2 pathogenesis-related proteins, chitinase and β -1,3-glucanase, and was preceded by biphasic hydrogen peroxide production, also found in incompatible plant–pathogen interactions in which systemic resistance is induced. A combination of glycol chitin and aniline blue plate assays correctly identified all in planta inducers of systemic resistance as measured by control of Cercospora leaf spot in classical challenge assays for systemic acquired resistance without the inclusion of false positive identifications, reducing the workload in subsequent disease challenge assays by nearly 70%.

Jeun *et al.* (2004) cytologically compared the expression of induced resistance between cucumber plants induced with either plant growth-promoting rhizobacteria (PGPR) or chemicals. Inoculation with PGPR strains *Serratia marcescens* (90–166) and *Pseudomonas fluorescens* (89B61) induced systemic protection in the aerial part of cucumber plants against the anthracnose pathogen *Colletotrichum orbiculare*. Disease development was significantly reduced in these plants compared to control plants that were not inoculated with the PGPR strains. Inoculation with the PGPR strains caused no visible toxicity, necrosis, or other morphological changes. Induction with DL DL-3-aminobutyric acid (BABA) or amino salicylic acid (ASA) also significantly reduced disease development. Soil drenched with 10mM BABA and 1.0mM ASA-induced resistance in cucumber leaves without any toxicity to the plants. Higher concentrations of ASA (up to 10mM) were phytotoxic, resulting in plant stunting and blighted appearance of leaves. Cytological studies using fluorescent microscopy revealed a higher frequency of autofluorescent epidermal cells, which are related to accumulation of phenolic compounds, at the sites of fungal penetration in plants induced with PGPR and challenged by the pathogen. Neither spore-germination rate nor formation of appressoria was affected by PGPR treatments. In contrast, both BABA and ASA significantly reduced spore-germination rate and appressoria formation, while there were no differences from controls in the frequency of autofluorescent epidermal cells at the sites of fungal penetration. Their findings suggest that PGPR and chemical inducers cause different plant responses during induced resistance.

Lysobacter enzymogenes C3, the only biocontrol agent previously known to induce resistance in tall fescue against *Bipolaris sorokiniana*, was compared in growth chamber experiments with other strains of *L. enzymogenes*, strains of plant growth promoting rhizobacteria (PGPR) that induce systemic resistance in dicot plants, and the synthetic elicitor 1,2,3-benzothiadiazole-7-thiocarboxylic acid-S-methyl-ester (BTH). The treatments were evaluated for induction of localized or systemic resistance against *B. sorokiniana*, in an experiment conducted by Kific-Ekici and Yuen (2004) when applied to leaves and roots. In addition, the effects of induced resistance on pathogen conidial germination on the phylloplane were assessed. None of the bacterial or chemical treatments induced systemic resistance when applied to a leaf. Strains of *L. enzymogenes* differed in their ability to cause localized disease inhibition following foliage treatment and to induce systemic resistance in leaves when applied to roots. In contrast to C3, two other strains of *L. enzymogenes* were ineffective in inducing systemic resistance. PGPR strains varied in effectiveness in causing localized disease inhibition when applied to leaves. Most of the bacterial strains increased peroxidase activity in the treated leaves, providing evidence that localized disease inhibition may have been plant mediated. The involvement of localized induced resistance was confirmed in *P. fluorescens* WCS417r, which did inhibit *B. sorokiniana* conidial germination or hyphal growth in vitro. Soil drenches with nearly all PGPR strains resulted in systemic resistance in leaves, but the treatments varied as to the timing and strength of induced systemic resistance. BTH induced localized resistance when applied to leaves but did not activate resistance in leaves when applied to roots. All cases of induced resistance were associated with an inhibition of conidial germination on leaf surfaces and, thus, this reaction appears to be a hallmark of induced resistance in the *B. sorokiniana*-tall fescue pathosystem.

Salicylic acid (SA)-mediated induction of systemic resistance by *Pseudomonas aeruginosa* strain 7NSK2 and *P. fluorescens* strain CHA0 against soil-borne fungi and viruses have been reported by Siddiqui and Shaukat (2004). The role of SA biosynthesis in the enhancement of defence mechanism against plant-parasitic nematodes by these bacterial strains in tomato is not known. To better understand the importance of SA in rhizobacteria-mediated suppression of root-knot nematodes,

biocontrol potential of SA-negative or SA-overproducing mutants against *Meloidogyne javanica* was evaluated with their respective wild type counter parts. Culture supernatant of 7NSK2, CHA0 and their respective mutants caused significant mortality of *M. javanica* juveniles *in vitro*. SA deletion in 7NSK2 and SA overproduction in CHA0 did not influence bacterial efficacy to cause nematode deaths. Similarly, culture supernatants resulting from King's B liquid medium amended with $FeCl_3$ did not influence nematicidal activity of the bacterial strains. Strain CHA0 induced juvenile deaths more than 7NSK2 did. In pot experiments, the bacterial strains applied in unsterilized sandy loam soil markedly reduced final nematode population densities in roots and subsequent root-knot infection in tomato seedlings. SA-negative or overproducing derivatives prevented tomato roots in kinetics similar to those with their respective wild types. When soil iron concentration was lowered by the addition of ethylenediamine di(o-hydroxyphenylacetic acid), nematode biocontrol by the bacterial strains (both wild type and mutants) remained unaltered. To understand the mechanism involved in rhizobacteria mediated suppression of root-knot nematode in tomato, bacterial performance was assessed in a split root trial in which one-half of the root system was treated with bacterium while the other inoculated with nematode. Compared with the controls, application of the bacterial cell suspension to one-half of the root system lowered the populations of root-knot nematode in non-bacterized nematode-treated sections indicating enhanced defence in the non-bacterized half. With respect to nematode infection, mutants induced systemic resistance to a similar extent as that caused by the wild types in both wild type tomato and *NahG* tomato plants. It is concluded that fluorescent pseudomonads induce systemic resistance against root-knot nematode via a signal transduction pathway, which is independent of SA accumulation in roots.

Talc-based bioformulations containing cells of *Pseudomonas fluorescens*, *Bacillus subtilis* and *Saccharomyces cerevisiae* were evaluated for their potential to attack the mango (*Mangifera indica* L.) anthracnose pathogen *Colletotrichum gloeosporioides* Penz. under endemic conditions by Vivekananthan *et al.* (2004). The preharvest aerial spray was given at fortnightly and monthly intervals. The plant growth-promoting rhizobacteria *Pseudomonas fluorescens* (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 the 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.

Bhatia *et al.* (2005) isolated ten isolates of fluorescent pseudomonads from rhizosphere of sunflower, potato, maize and groundnut. All the isolates produced fluorescent pigment in succinate broth and displayed siderophore production. Production of hydrocyanic acid (HCN) and indole acetic acid (IAA) by all the isolates was reduced besides phosphate solubilisation. Out of the ten strains, *Pseudomonas* PS I and PS II were found most potential. Bacterisation of sunflower seeds with fluorescent *Pseudomonas* PS I and PS II resulted in increased seed germination, root length, shoot length, fresh and dry weight of roots ans shoots, and yield of sunflower. Seed bacterisation with strains of fluorescent *Pseudomonas* PS I and PS II reduced incidence of collar rot by 69.8% and 56.9% respectively, in *Sclerotium rolfsii*-infested soil, making the organism a potential biocontrol agent against collar rot of the sunflower.

Patterns of colonization of *Vitis vinifera* L. cv. Chardonnay plantlets by a plant growth-promoting bacterium, *Burkholderia* sp. strain PsJN, were studied under gnotobiotic conditions. Wild-type strain PsJN and genetically engineered derivatives of this strain tagged with *gfp* (PsJN::*gfp*2x) or *gusA* (PsJN::*gusA*11) genes were used to enumerate and visualize tissue colonization. The rhizospheres of 4- to 5-week-old plantlets with five developed leaves were inoculated with bacterial suspensions. Epiphytic and endophytic colonization patterns were then monitored by dilution plating assays and microscopic observation of organ sections. Bacteria were chronologically detected first on root surfaces, then in root internal tissues, and finally in the fifth internode and the tissues of the fifth leaf. Analysis of the PsJN colonization patterns showed that this strain colonizes grapevine root surfaces, as

well as cell walls and the whole surface of some rhizodermal cells. Cells were also abundant at lateral root emergence sites and root tips. Furthermore, cell wall-degrading endoglucanase and endopolygalacturonase secreted by PsJN explained how the bacterium gains entry into root internal tissues. Host defense reactions were observed in the exodermis and in several cortical cell layers. Bacteria were not observed on stem and leaf surfaces but were found in xylem vessels of the fifth internode and the fifth leaf of plantlets. Moreover, bacteria were more abundant in the fifth leaf than in the fifth internode and were found in substomatal chambers. Thus, it seems that *Burkholderia* sp. strain PsJN induces a local host defense reaction and systemically spreads to aerial parts through the transpiration stream (Compant *et al.*, 2005).

Integration of foliar bacterial biological control agents and plant growth promoting rhizobacteria (PGPR) was investigated by Ji *et al.* (2005) to determine whether biological control of bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato*, and bacterial spot of tomato, caused by *Xanthomonas campestris* pv. *vesicatoria* and *Xanthomonas vesicatoria*, could be improved. Three foliar biological control agents and two selected PGPR strains were employed in pairwise combinations. The foliar biological control agents had previously demonstrated moderate control of bacterial speck or bacterial spot when applied as foliar sprays. The PGPR strains were selected in this study based on their capacity to induce resistance against bacterial speck when applied as seed and soil treatments in the greenhouse. Field trials were conducted in Alabama, Florida, and California for evaluation of the efficacy in control of bacterial speck and in Alabama and Florida for control of bacterial spot. The foliar biological control agent *P. syringae* strain Cit7 was the most effective of the three foliar biological control agents, providing significant suppression of bacterial speck in all field trials and bacterial spot in two out of three field trials. When applied as a seed treatment and soil drench, PGPR strain *Pseudomonas fluorescens* 89B-61 significantly reduced foliar severity of bacterial speck in the field trial in California and in three of six disease ratings in the field trials in Alabama. PGPR strains 89B-61 and *Bacillus pumilus* SE34 both provided significant suppression of bacterial spot in the two field trials conducted in Alabama. Combined use of foliar biological control agent Cit7 and PGPR strain

89B-61 provided significant control of bacterial speck and spot of tomato in each trial. In one field trial, control was enhanced significantly with combined biological control agents compared to single agent inoculations. These results suggested that some PGPR strains may induce plant resistance under field conditions, providing effective suppression of bacterial speck and spot of tomato, and that there may be some benefit to the integration of rhizosphere-applied PGPR and foliar-applied biological control agents.

The deliberate targeting of the stigma with the biocontrol agent in this pathosystem prompted Ngugi *et al.* (2005) to evaluate potential negative impacts on pollination and pollination-related fruit characteristics. Application of Serenade to the stigmatic surface of detached blueberry flowers in the laboratory had no effect ($P > 0.05$) on the number of pollen tubes entering the style or their growth rates within the stylar canal. There was also no reciprocal effect, i.e., population dynamics of *B. subtilis* were unaltered by the presence of pollen. Application of the biocontrol product to open flowers, regardless of whether it was done 1 day before or immediately prior to pollination, did not impact fruit set or the number of seeds per berry, but marginally ($P = 0.048$) affected fruit weight in one of two experimental runs in the greenhouse; fruit weights in the two Serenade timing treatments were significantly different from each other but neither was different from that of the control that received pollen only. In a field experiment in which honey bees were utilized to vector the biocontrol product to open flowers, application of Serenade did not affect fruit weight but significantly reduced fruit set from 49.1 to 38.1% ($P = 0.0382$) and seed number to about half of that of the untreated control ($P = 0.0109$). However, fruit weights and seed numbers in the experiment were low even in treatments receiving no Serenade, indicative of poor pollination overall. Taken together, these results indicate that application of Serenade has no inherently adverse effects on pollination and associated fruit characteristics, but caution should be exercised in applying this product in conditions otherwise unfavorable for adequate pollination.

The *Pseudomonas fluorescens* isolate 1 (Pf1) was found to protect the ragi [*Eleusine coracana* (L.) Gaertner] blast fungus, *Pyricularia grisea*. Induction of

defense proteins *viz.* chitinase, β-1,3 glucanase, peroxidase (PO) and polyphenol oxidase (PPO) by the Pf1 isolate was studied against *P. grisea* by Radjacommare (2005). Chitinase in a resistant, susceptible and commonly used cultivar with and without challenge inoculation of *P. grisea*, revealed changes in the isoform pattern by UV illumination after staining the gel with fluorescent brightner 28. Native PAGE (polyacrylamide gel electrophoresis) of PO showed the single isoform in all the treatments including the control and a significant increase in the intensity of the band in the inoculated control and Pf1 treatment in all the varieties. Isoform analysis of PPO showed the induction of PPO in *P. fluorescens* treated plants challenged with *P. grisea*. Application of Serenade, a commercial biofungicide formulation containing the bacterium *Bacillus subtilis*, to the stigmatic surface of open blueberry flowers suppresses floral infection by the mummy berry fungus *Monilinia vaccinii-corymbosi*.

One of 500 rhizobacteria isolated from soil, rhizosphere and rhizoplane of healthy tomato plants was previously selected by Romeiro *et al.* (2005) in laboratory, greenhouse and field tests as a good inducer of systemic resistance. This plant growth-promoting rhizobacterium (PGPR) was identified as *Bacillus cereus* by fatty-acid analysis. *Bacillus cereus* bacterial cells were removed from liquid culture by centrifugation and the supernatant repeatedly dialyzed (cut-off = 12 000 daltons) against distilled water. Dialysates applied to roots protected tomato plants against leaf fungal and bacterial pathogens, evidence that macromolecules synthesized by the PGPR and released into the environment act as elicitors of systemic resistance.

The aim of a study by Demoz and Korsten (2006) was therefore to determine the ability of *Bacillus subtilis* B246, commercially registered as Avogreen and used as a biocontrol agent against avocado pre- and postharvest diseases, to attach, colonize, and survive on avocado flowers and to study the interaction of the SER pathogens and the antagonist on avocado flowers. Avocado flowers inoculated with a liquid commercial formulation of the antagonist were observed at different time intervals under the scanning electron microscope (SEM). Population dynamics of the antagonist on the flowers were determined by means of total viable counts using reference cultures and background counts from the control. Flowers were also

inoculated with antagonist-pathogen (*Dothiorella aromatica* and *Phomopsis perseae*) combinations to determine in vivo interactions. The SEM observations and population dynamics study confirmed that the antagonist could effectively attach, colonize, and survive on avocado flowers. It could also attach to conidia and hyphae of the pathogens and cause cell degradation. These modes of action can give new insights into the control of pathogens by *B. subtilis*.

Different formulations of *Bacillus licheniformis* were evaluated on their own and in combination with prochloraz and stroburilin for their ability to reduce mango post-harvest fruit diseases [anthracnose and stem-end rot (SR)] when applied as a dip treatment in a mango pack house. Untreated fruit and fruit treated with either prochloraz or stroburilin alone served as controls. In these trials treatments integrating chemical pesticides with *B. licheniformis* controlled anthracnose and SR as effectively as the chemical control. The antagonist was more effective especially in the control of post-harvest diseases when fruit were kept in cold storage to simulate export conditions. In two of the three trials, results obtained when fruit was treated with the antagonist in combination with the commercial chemical were comparable to that obtained with the commercial chemical control. In this study by Govender and Korsten (2006), it was found that the antagonist when used in mango pack house treatments could provide an effective alternative to fungicides. Furthermore, the powder formulation of the antagonist can be successfully incorporated into the existing pack line.

In greenhouse experiments, plant growth promoting rhizobacteria (PGPR) *Serratia marceseens* NBRI1213 was evaluated for plant growth promotion and biologic control of foot and root rot of betelvine caused by *Phytophthora nicotianae* (Lavania *et al.*, 2006) . Bacterization of betelvine (*Piper betle* L.) cuttings with *S. marcescens* NBRI1213 induced phenylalanine ammonia-lyase, peroxidase, and polyphenoloxidase activities in leaf and root. Qualitative and quantitative estimation of phenolic compounds was done through high-performance liquid chromatography (HPLC) in leaf and root of betelvine after treatment with *S. marcescens* NBRI1213 and infection by *P. nicotianae*. Major phenolics detected were gallic, protocatechuic, chlorogenic, caffeic, ferulic, and ellagic acids by comparison of their retention time with standards through HPLC. In all of the treated plants, synthesis of phenolic

compounds was enhanced compared with control. Maximum accumulation of phenolics was increased in *S. marcescens* NBRI1213-treated plants infected with *P. nicotianae*. In a greenhouse test, bacterization using *S. marcescens* NBRI1213 decreased the number of diseased plants compared with nonbacterized controls. There were significant growth increases in shoot length, shoot dry weight, root length, and root dry weight, averaging 81 %, 68 %, 152 %, and 290 %, respectively, greater than untreated controls. This is the first report of PGPR-mediated induction of phenolics for biologic control and their probable role in protecting betelvine against *P. nicotianae*, an important soil-borne phytopathogenic fungus.

Bacillus licheniformis N1, which has previously exhibited potential as a biological control agent, was investigated to develop a biofungicide to control the gray mold of tomato caused by *Botrytis cinerea*. Various formulations of *B. licheniformis* N1 were developed by Lee *et al.* (2006) using fermentation cultures of the bacteria in Biji medium, and their ability to control gray mold on tomato plants was evaluated. The results of pot experiments led to the selection of the wettable powder formulation N1E, based on corn starch and olive oil, for evaluation of the disease control activity of this bacterium after both artificial infection of the pathogen and natural disease occurrence under production conditions. In plastic-house artificial infection experiments, a 100-fold diluted N1E treatment was found to be the optimum biofungicide spray formulation. This treatment resulted in the significant reduction of symptom development when N1E was applied before *Bo. cinerea* infection, but not after the infection. Both artificial infection experiments in a plastic house and natural infection experiments under production conditions revealed that the N1E significantly reduced disease severity on tomato plants and flowers. The disease control value of N1E on tomato plants was 90.5 % under production conditions, as compared to the 77 % conferred by a chemical fungicide, the mixture of carbendazim and diethofencarb (1:1). The prevention of flower infection by N1E resulted in increased numbers of tomato fruits on each plant. N1E treatment also had growth promotion activity, which showed the increased number of tomato fruits compared to fungicide treatment and non-treated control and the increased fruit size compared the non-treated control under production conditions. This study suggests that the corn starch-based formulation of *B. licheniformis*

developed using liquid fermentation will be an effective tool in the biological control of tomato gray mold.

Pieterse *et al.* (2006) developed an *Arabidopsis*-based model system using *Fusarium oxysporum f sp raphani* and *Pseudomonas syringae pv tomato* as challenging pathogens, in order to study the molecular basis underlying the systemic resistance. Colonization of the rhizosphere by the biological control strain WCS417r of *P. fluorescens* resulted in a plant-mediated resistance response that significantly reduced symptoms elicited by both challenging pathogens. Moreover, growth of *P. syringae* in infected leaves was strongly inhibited in *P. fluorescens* WCS417r-treated plants. Transgenic *Arabidopsis* NahG plants, unable to accumulate SA, and wild-type plants were equally responsive to *P. fluorescens* WCS417r-mediated induction of resistance. Furthermore, *P. fluorescens* WCS417r-mediated systemic resistance did not coincide with the accumulation of PR mRNAs before challenge inoculation.. The result indicated that *P. fluorescens* WCS417r induces a pathway different from the one that controls classic systemic acquired resistance and that this pathway leads to a form of systemic resistance independent of SA accumulation and PR gene expression.

Plant growth-promoting rhizobacteria (PGPR) bioformulations (*Pseudomonas* and *Bacillus)* were tested for their efficacy against blister blight (*Exobasidium vexans*) disease in tea (*Camellia sinensis*) under field conditions for two seasons. Among the bioformulations tested, foliar application of *Pseudomonas fluorescens* Pf1 at 7-d intervals consistently reduced the disease incidence of blister blight for two seasons, almost comparable with that of chemical fungicide. In addition to disease control, it also increased tea yield significantly compared to the untreated control. Induction of defense enzymes such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, chitinase, β-1,3-glucanase and phenolics were studied. The enzyme accumulation was greater in *P. fluorescens* Pf1-treated plants compared to control. The study revealed the probable influence of plant growth promotion and induced systemic resistance (ISR) in enhancing the disease resistance in tea plants against blister disease by PGPR bioformulations (Saravanakumar *et al.*, 2006).

Pseudomonas corrugata, a soil bacterium originally isolated from a temperate site of Indian Himalayan Region (IHR) was examined by Trivedi *et al.*, (2006) for its antagonistic activities against two phytopathogenic fungi, *Alternaria alternata* and *Fusarium oxysporum*. Although the bacterium did not show inhibition zones due to production of diffusible antifungal metabolites, a reduction in growth between 58 % and 49 % in both test fungi, *A. alternata* and *F. oxysporum*, was observed in sealed petri plates after 120 h of incubation due to production of volatile antifungal metabolites. Reduction in biomass of *A. alternata* (93.8) and *F. oxysporum* (76.9) in Kings B broth was recorded after 48 h of incubation in dual culture. The antagonism was observed to be affected by growth medium, pH and temperature. The reduction in fungal biomass due to antagonism of bacteria was recorded maximum in the middle of the stationary phase after 21 h of inoculation. The production of siderophore, ammonia, lipase and chitinase in growth medium by *P. corrugata* were considered contributing to the antagonistic activities of the bacterium.

MATERIAL AND METHODS

3.1. Collection of soil samples

Soil samples were collected from the rhizosphere of tea plants of different ages growing in different tea states, as follows: Hansqua, Chandmani, Falakata, Nagrakata tea estates (90, 80, 50 and 40 year old bushes respectively), as well as Tea Experimental Garden, University of North Bengal (10 year old bushes), from the plains and Margaret's hope Tea Estate, Darjeeling (75 year old bushes) from the hilly regions at an altitude of 2000 metres above sea level. The soil samples collected from these plantation areas were used for determining microbial population and isolation of microorganisms.

3.2. Isolation of rhizosphere bacteria

Isolation of rhizobacteria was made from the rhizosphere of healthy tea plants. Five grams of soil particles loosely adhering to the roots were collected. The soil suspension was prepared by dissolving the soil sample in 30 ml of sterile distilled water using magnetic stirrer for 1 h. The suspension was allowed to settle down till the two distinct layers were clearly visible. Then the upper light brown colored layer was pippeted out and serial dilutions were made. 1ml each of 10^{-3} and 10^{-4} dilutions was actually used for isolation by dilution plate technique (Kobayashi *et al.*, 2000) using Nutrient Agar (NA- peptone 5 g, NaCl 5 g, beef extract 1.5 g, yeast extract 1.5 g, agar 12 g and distilled water 1 L; pH 7.2 ± 0.2), King's B media (KB- Peptone 20 g, K_2HPO_4, anhydrous 1.5 g, $MgSO_4$. $7H_2O$ 1.5 g, Glycerol 15 ml in 1 L dist. Water, pH 7.4 ± 0.2) and Potato Dextrose Agar (PDA- Potato 400 g, Dextrose 20 g, agar 20 g, distilled water 1 L; pH 7.3 ± 0.2) as the growth media. The petriplates were then placed in an incubator for observation of the microbial growth after 24, 48 and 96 h.

3.3. *In vitro* antagonistic tests of rhizosphere bacteria

3.3.1. Solid medium

The efficacy of individual bacterial strain, isolated from tea rhizosphere, for inhibiting growth of the pathogens was tested *in vitro* in dual culture using PDA or NA. Each bacterial isolate was streaked at one side of the agar plate about 1cm away

from the edge and 7 mm diameter plug of the pathogen taken from growing edge of the fungal culture was inoculated at the other half of the petriplate or by placing the fungal inoculum in the centre and streaking the bacteria around the fungal inoculum. For each test three replicate plates were used. The plates were incubated for 5-7 days (depending upon the growth rate of the pathogen) at 26°C and inhibition zone towards the fungal colony in individual plate was quantified. Results were expressed as mean of percentage of inhibition of the growth of the pathogen ± standard error in presence of the bacterial isolates.

3.3.2. Liquid medium

Both the fungal inoculum (7 mm plug) and the bacteria were inoculated in broth (PDB and NB) and incubated for 6-8 days. The fresh weight as well as dry weight of the fungal mycelium was taken. The mycelium grown without the bacterial strain in the similar medium was taken as the control sample. Three replicates were taken in each case.

3.4. Identification of selected antagonists

3.4.1. Microscopic observation

For microscopic identification of bacteria, these were gram stained and observed under microscope. Gram technique involves the application of two dyes. crystal violet and safranin.

The slide was placed on a rack and flooded the entire slide with crystal violet and allowed the crystal violet to stand for about a minute. When the time had elapsed, the slide was washed with water for 5 s. Next, the slide was flooded with the iodine solution and allowed it to stand for a minute as well. After a minute the slide was rinsed with water for 5 s, after which ethanol was added. The ethanol was added drop wise until no blue-violet color was emitted from the specimen. Finally the slide was rinsed with the water for 5 s, dried and observed under microscope.

3.4.2. Biochemical tests

i. Endospore stain

The bacterial smear was prepared on the slide by passing the slide over the flame. The fixed slide was flooded with the solution of malachite green and placed the slide over boiling water bath for 5 min. After rinsing, the smear was counter stained with safranin.

ii. Voges Proskauser reaction

Culture tubes containing 10 ml sterile VP broth were inoculated with the bacterial isolates and incubated at 37°C for 3 days. To the cultures 0.6 ml of 5 % w/v ethanolic α napthol and 0.2 ml of 40 % w/v aqueous potassium hydroxide were added and kept at room temperature for 1 h for production of pink color indicating positive reaction.

iii. Catalase

Bacterial cultures (24 h old) were flooded with 0.5 ml of 10 % H_2O_2 solution and gas bubbles production indicated the positive reaction.

iv. Urea digestion

Streaks were made on the slants containing urea medium and incubated at 37°C for 3-7 days. The change in color of the medium indicates the presence of urease.

v. Esculine hydrolysis

The slants containing esculine hydrolysis test medium were inoculated with 24 h old bacterial culture and incubated at 30°C for 7 days. Blackening of the medium indicates the hydrolysis of esculine.

vi. Casein hydrolysis

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

vii. Starch hydrolysis

The bacteria were streaked on sterilised starch agar plates (NA + 0.1 % soluble starch) and incubated for 5 days at 37°C. The plates were flooded with Lugol's Iodine solution. The clear zone underneath and around the growth indicates the starch hydrolysis.

viii. Indole Test

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

ix. Reduction of nitrate to nitrite

Sterile nitrate broth was inoculated with test isolates and incubated at 37°C till the medium became turbid. Three drops of nitrate reduction test reagent [Sol A: H_2SO_4 0.8 g, 100 ml 5 N Acetic acid; Sol B: 0.5 g α napthylamine and 100 ml acetic acid. The solutions A and B were mixed in equal volume just before use] was mixed in 1ml of the cultures and observed for the development of red or yellow color indicating the presence of nitrate.

3.5. Plant materials

3.5.1. Tea

Different varieties of tea plant (*Camellia sinensis* (L) O. Kuntze), which were maintained in the Germ Plasm bank at Department of Botany, North Bengal University, were used for the experimental purposes. The plant varieties were originally collected from Darjeeling Tea Research Centre, Darjeeling (K1/1, CP 1, S 449 and HV 39), Tea Experimental Station, Tocklai (TV 20, T 78, TV 18 and T 17) and United Planters Association of South India, Tamil Nadu (UP 26, UP 3 and BSS 2).

Tea plants were propagated by cutting. Soil preparation is the most important in propagation technique and hence, care was taken to prepare the soil well. Sandy soil (75 % sand and 25 % soil) with a pH 4.5 - 4.9 was used. Soil pH was adjusted by applying 2 % aluminium sulphate solution followed by leaching with water to remove excess aluminium sulphate.

Polyethylene sleeves (8"x6") were filled up with the prepared soil and stacked in rows in bed and watered thoroughly. The cuttings of all varieties were allowed for rooting in individual sleeves after dipping them in rooting hormone. Sleeves of each bed were covered with polythene cloche and the whole setup was kept under a green agro house.

In case of young seedlings, manuring (aluminium phosphate - 8 parts by weight, ammonium phosphate- sulphate 16: 20 – 35 parts by weight, magnesium sulphate and zinc phosphate 3 parts by weight) was done after rooting following the method of Ranganathan and Natesan (1987). The mixture was dissolved @ 30 g in 1 L of water and applied @ 50 ml per plant. Tipping was done once in a year to promote lateral branching in young plants but in case of mature plants two year of deep pruning cycle was maintained.

The selected seedlings were transplanted in the 12" earthen pots. The soil mixture was prepared in the ratio 4.5 kg of well rotten dry cattle manure, 30 kg rock phosphate, 30 kg super phosphate and 2.5 g phorate [O,O-diethyl S-(ethylthiomethyl) phosphor dithioate]. At the bottom of the pot little bit of rock phosphate is placed following which the pot was covered with planting soil mixture. Ten month seedlings with well developed shoot and root system were transferred from the sleeves to the pots. These were then maintained in the pots with regular watering.

3.5.2. Chickpea

The seeds of two different genotypes of chickpea *viz.* ICC V2 (Swetha) and ICC C37 (Kranthi) required for experimental purpose were obtained from the seed germplasm bank of International Crop Research Institute for Semi Arids and Tropics, Patancheru, Andhra Pradesh. Seedlings of different genotypes were then

raised from these seeds stock in the experimental garden of Botany Department, North Bengal University.

3.5.3. Orchid

Three different genera of orchid *viz. Phaleonopsis, Oncidium* and *Vanda* were obtained from Janak Nursery, Siliguri. The plants were maintained in pot containing mixture of brick pieces and charcoal (50:50) at greenhouse and watering regularly.

3.6. Fungal culture

3.6.1. Source of culture

Tea root pathogens *Fomes lamaoensis* (Murr.) Sacc. & Trott., *Poria hypobrumea* and *Sphaerostilbe repens* B & Br. were obtained from Immunophyto-pathology laboratory, Dept. of Botany, North Bengal University and were being maintained in the laboratory with regular sub culturing in PDA for subsequent tests. *Sclerotium rolfsii* Sacc. I causing seedling blight in tea, *Sclerotium rolfsii* Sacc. II causing crown rot in orchid and collar and root rot disease in *C. arietinum* and *Sclerotinia sclerotiorum* (lib.) de Bary causing white rot, isolated from *Vanda* were obtained from Immuno-Phytopathological laboratory, Department of Botany, North Bengal University (Plate II).

3.6.2. Assessment of mycelial growth

Mycelial growth was assessed in both on solid media and liquid media for various experimental purposes.

3.6.2.1. Solid media

To assess the growth of fungal cultures in solid media, the fungus was first grown on petridishes, each containing 20 ml of PDA and NA followed by incubation for 5-8 days at 30°C. Agar block (6 mm diameter) containing the mycelia was cut with sterile cork borer from the actively growing region of mycelial mat and transferred to each petridish containing 20 ml of sterilized solid media. The colony diameter was studied at regular interval of time.

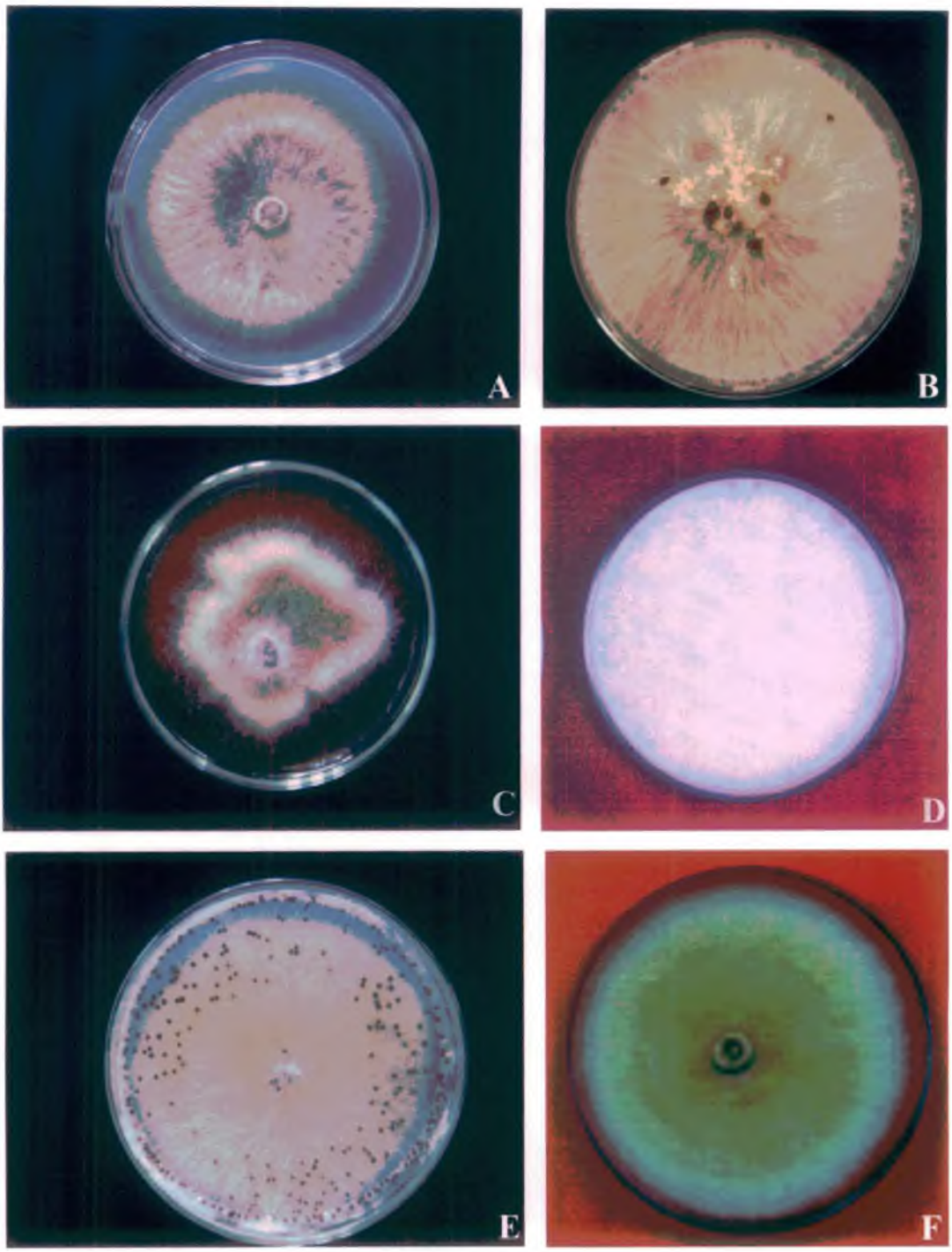


Plate II (A-F): Test fungi growing on PDA plate A: *Fomes lamaoensis*; B: *Sclerotinia sclerotiorum* C: *Sphaerostilbe repens*; D: *Sclerotium rolfsii* I E: *Sclerotium rolfsii* II ; F: *Poria hypobrumea.*

3.6.2.2. Liquid media

The mycelial block from the actively growing region of the fungus in the petriplate was cut with sterilised cork borer and transferred to Ehrlenmeyer flask (250 ml) containing 50 ml of sterilized liquid media (NB or PDB) and incubated for 6 - 8 days with constant stirring at room temperature. After incubation the mycelia were strained through muslin cloth, collected in aluminium foil cup of known weight and dried at 60°C for 96 h, cooled in dessicator and weighed.

3.7. *In vitro* characterization of PGPR activity of selected bacteria

3.7.1. 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. This method was shown to be more sensitive and more specific than the Salkowski based calorimetric technique (Glickmann *et al.*, 1995). 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.

3.7.2. Phosphate solubilisation

The phosphate solubilising test was done in the solid medium. The medium used was Pikovskaya's medium (Pikovskaya, 1948). Agar plates were prepared and the bacterial strains were individually spot inoculated at the centre of the plates followed by incubation for 5 - 6 days. The plates were observed for clear zone around the colony and the diameter of the clearing zone was measured.

3.7.3. Siderophore production

The selected isolates were characterized for iron dependent siderophore production following standard method (Schwyn and Neiland, 1987) using blue indicator dye, chrome azurol S (CAS). For preparing CAS agar, 1 L, 60.5 mg CAS

was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl). 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 (25 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.

The antagonistic bacterial strains were streaked separately into the petriplates containing PDA amended with $FeCl_3$ at concentration of 150 μg ml^{-1}, 300 μg ml^{-1}, and 600 μg ml^{-1}. The actively growing discs of fungal test pathogen *F. lamaoensis* was placed opposite the bacterial streak simultaneously and incubated at 28°C. The zone of inhibition was measured after 7 days of growth. The plate containing the fungal pathogen and the bacteria on PDA without $FeCl_3$ was treated as control. The experiment was done in replicates of three.

3.7.4. HCN production

Production of hydrocyanic acid was determined using the procedure described by Wei *et al.* (1991) with slight modification. Bacteria were grown on NA medium amended with glycine (4.4g L^{-1}.) in a petriplate, and then the strip soaked in picric acid solution was placed in the lid of each petriplate. The plates were sealed with parafilm and incubated for 2-4 days. HCN production is indicated by the change in color of the filter paper strip from yellow to brown to red.

3.7.5. Chitinase production

For detecting the chitinolytic behavior of the bacteria chitinase detection agar (CDA) plates were prepared by mixing 10 g colloidal chitin with 20 g of agar in M9 medium (Na_2HPO_4 0.65 g, KH_2PO_4 1.50 g, NaCl 0.25 g, NH_4Cl 0.50 g, $MgSO_4$ 0.12 g, $CaCl_2$ 0.005 g and distilled water 1 L; pH 6.5).

The CDA plate was spot inoculated with organism followed by incubation at 28°C for 7-10 days. Formation of clear zone around the organism is considered positive reaction (West and Colwell, 1984).

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.

3.7.6. Volatile production

The antagonistic bacteria were grown in petriplates for 12 h. A fungal disc (6 mm) cut from freshly growing mycelia of fungus was placed at the centre of the bottom part of another plate containing PDA medium. The plate inoculated with fungus was placed in an inverted position over the other plate containing the bacterial culture of different age (same as pathogen, 1 day older, 2 days older, 3 days older and 4 days older than the pathogen) and were sealed with parafilm to isolate inside atmosphere and to prevent loss of volatiles formed. Plates were incubated at 25°C for 3-4 days. Radial growth of pathogen was measured after definite time interval. The plate without bacteria was treated as control (Dennis and Webster, 1971.). Each experiment considering a single bacterial isolate was performed in triplicate.

3.8. Extraction of antifungal compounds from bacteria

3.8.1. Cell free culture filtrate

The antagonistic bacteria were grown in NB medium for 96 h at 37°C in shaking condition. Centrifugation was done at 15000 rpm for 20 min and supernatant was pooled and passed through the micro filter (0.22 μm pore size). Portion of this was further used for further bioassays.

The cell free culture filtrate (1000 ml) extracted separately three times with equal volume of acetone, benzene, chloroform, ethylacetate and diethylether. The organic fraction and corresponding aqueous fractions were evaporated to complete dryness in a rotary evaporator at room temperature and residue in each case was dissolved in 2.5 ml of 80 % methanol used for the bioassays.

3.8.2. Whole cells

Extraction of the active principles from the bacteria grown in solid media was carried out with slight modification of the method described by Howell and Stipanovic (1980). The bacterial lawn was prepared by growing in NA plate for 7 days. The agar plates were cut into 1cm squares and extracted in 200 ml of 80% aqueous acetone, filtered through a cheese cloth and the filtrate was centrifuged at 15000 rpm for 10 min. The supernatant was evaporated to remove acetone. To residue (5 g 100 ml^{-1}), NaCl was added and centrifuged at 15000 rpm for 10 min. Each portion of the supernatant was extracted twice in diethyl ether and dried over anhydrous calcium chloride. The compound in a crystalline form was recovered from this residue. This was dissolved in methanol and used for bioassay.

3.9. Bioassay of active principle

Antifungal activity of active principles or cell free culture filtrate was checked by radial growth bioassay, spore germination bioassay and sclerotial germination bioassay.

3.9.1. Spore germination

For spore germination bioassay, a drop of the test solution was placed on a clean, grease free slide and allowed to evaporate following which a drop of spore suspension was placed over it. In another slide, methanol was added in place of the antifungal compound which was treated as solvent control. The slide without any solvent but containing only spore suspension was considered as water control. The slide was incubated in a moist petriplate for 24 hrs at 25°C ± 1°C. Finally one drop of lactophenol – cotton blue was added to each spot to fix the germinated spore. The

slides were observed under microscope and the percentage of germination was determined.

3.9.2. Sclerotial germination

For assessing the effect of the active principle from bacteria or cell free culture filtrate on the sclerotial germination of *S. rolfsii* and *S. sclerotiorum*, the sclerotia were scrapped off from the culture growing in PDA. The sclerotia were then soaked overnight in test solution. The sclerotia soaked in sterile distilled water served as the control. After overnight soaking the sclerotia were transferred asceptically to the petridish containing PDA and incubated at room temperature. The inhibition in the germination of sclerotia was evaluated by counting the number of germinated sclerotia.

3.9.3. Radial growth

The test solution was added to the media (PDA) at different percentage starting from 20 % up to 80 %. The plates were allowed to solidify and then inoculated with pathogen.

3.9.4. Agar cup bioassay

PDA medium was poured in the sterile petriplates and allowed to solidify, then bored with a sterile cork borer (7 mm). Test solution (0.2 ml) was added to each cup and the fungal inoculum (6 mm) was placed at the centre. The plate was incubated at 27°C for desired period. The diameter of the fungal pathogen was noted. The plate containing 0.2 ml of dissolving solvent and plate without any solvent served as solvent control and control respectively. The experiment was performed in replicates of three and was repeated thrice.

3.9.4. Liquid medium

Cell free culture filtrate was added to the liquidmedium (PDB and NB) at different percentage (20–80 %) and inoculated with pathogen.

The whole setup was incubated at 28°C for 6-7 days to observe the growth of pathogen. The media without the cell free culture filtrate was treated as the control.

3.10. Partial characterization of active principle

3.10.1. UV-spectrophotometry

The antifungal compounds were analyzed in UV spectrophotometer (SISCO, model Digispec 200GL) at a range of 200 to 400 nm and maximum absorption was determined.

3.10.2. HPLC

Analysis by HPLC was carried out on a Shimadzu Advanced VP Binary Gradient system with a C-18 ODS column using 50 % acetonitrile as the mobile phase, in isocratic mode. Injection volume of the sample was 20 µl and flow rate 1 ml min^{-1}. Detection was at 236 nm and 207 nm for 2 samples. Total run time was 20 min.

3.11. Application of bacteria

3.11.1. Soil drench

The bacteria were grown in NB for 48 h at 28°C and centrifuged at 15000 rpm for 15 min. The pellet obtained was resuspended in sterile distilled water. The optical density of the suspension was adjusted using a UV-VIS spectrophotometer following method to obtain a final density of $3x10^6$ cfu ml^{-1}.

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

3.11.2. Foliar spray

The bacterial pellet suspended in sterile distilled water at a concentration of $3x10^6$ cfu ml^{-1} after the addition of a few drops of Tween-20 was sprayed until run off on the foliar part of the ten year old bushes after pruning. The spraying was done fortnightly till the new shoots started appearing.

3.11.3. Seed bacterisation

C. arietinum seeds were surface sterilized with 0.1 % mercuric chloride and treated with bacterial culture with a concentration of $2x10^6$ cfu ml^{-1}. The seeds were soaked in bacterial suspension using 0.2 % sterilized carboxymethyl cellulose as an adhesive. The seeds soaked in sterile distilled water served as control. After 24 h the seeds were blotted dry, and sown in plastic pots (6" dia.). Following germination, 10-12 plants were maintained in each pot.

3.12. Determination of plant growth promoting activity

3.12.1. Tea

The experiment was conducted under greenhouse conditions to assess the efficacy of selected bacterial isolates to promote plant growth. The growth promotion by individual bacteria was assessed in seedlings by comparing the increase in height, number of leaves of the treated plants to the untreated control plants under the same environmental and physical conditions (temperature 30-34°C; R.H. 60-80 %; 16 h photoperiod). The experiment consisted of ten replicates in each treatment in completely randomized design. Similarly, the growth promotions in 2 year old plants were also recorded in terms of Leaf Area Index (LAI) of individual plant (Watson, 1947) and number of branches of the treated and untreated control plants. Observations were recorded after 2 and 4 months of final application of bacterium to the soil.

The growth promotion by foliar application of bacterial suspension was assessed by the time of appearance of new shoots, overall growth of canopy and general increase in biomass.

3.12.2. Chickpea

Plant growth promoting activity of bacteria was assessed based on the seedling vigour index. Seed germination percentage of seeds was determined. The root and shoot length of individual seedlings were measured to work out the vigour index using the formula suggested by Baki and Anderson (1973).

Vigour Index = (Mean shoot length + root length) × % germination.

The experiment was carried out in three replicates with 30 seeds in each plate.

3.13. Extraction of enzymes from leaves

3.13.1. β–1,3-glucanase

Extraction of β-1,3- glucanase (EC.3.2.1.39) was done following the method described by Pan *et al.* (1991). Tea leaf sample (1 g) was crushed in liquid nitrogen and extracted using 5 ml of chilled 0.05 M sodium acetate buffer (pH 5.0) by grinding at 4°C using mortar and pestle. The extract was then centrifuged at 10000 rpm for 15 min at 4°C and the supernatant was used as crude enzyme extract.

3.13.2. Chitinase

Extraction of chitinase (EC. 3.2.1.39) was done by following the method described by Boller and Mauch (1988) with modifications. 1g of leaf sample from the tea plants were crushed in liquid nitrogen and extracted using 5 ml of chilled 0.1 M sodium citrate buffer (pH 5.0). The homogenate was centrifuged at 12000 rpm for 10 min, and precipitate was discarded. The supernatant was used as enzyme source.

3.13.3. Phenylalanine ammonia lyase

Extraction of phenylalanine ammonia lyase (EC.4.3.1.5) was done by following the method described by Chakraborty *et al.* (1993) with modifications. Leaf samples were crushed in liquid nitrogen and extracted using 5 ml of 0.1 M sodium borate buffer (pH 8.8) containing 2 mM β–mercaptoethanol in ice. The slurry was centrifuged at 15000 rpm for 20 min at 4°C. The supernatant was collected and after recording its volume, was immediately used for assay or stored at -20°C.

3.13.4. Peroxidase

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

3.13.5. Polyphenol oxidase

Extraction of polyphenol oxidase (EC.1.14.18.1) from the tea leaf tissue was done following the method described by Mahadevan and Sridhar (1982) with modifications. 1g leaf samples were weighed out and cut into pieces of 1-2cm. The sample pieces were crushed in liquid nitrogen and extracted using 5ml (0.1 M) phosphate buffer pH 6.6. The brei was centrifuged at 15000 rpm for 15 min at 4°C. The supernatant was decanted and used as enzyme source for further assay.

3.14. Assay of enzyme activities

3.14.1. β–1,3-glucanase

Estimation of the 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.

3.14.2. Chitinase

Chitinase activity was measured according to the method described by Boller and Mauch (1988). Assay mixture consisted of 10 µl of 1 M sodium acetate buffer, pH 4.0, 0.4 ml of enzyme solution and 0.1 ml of colloidal chitin. Colloidal chitin was prepared as per the method of Roberts and Selitrennikoff (1988). After 2 h of incubation at 37°C, the reaction was stopped by centrifugation at 10000 g for 3 min. An aliquot of supernatant was pipetted into a glass reagent tube containing 30 µl of 1 M potassium phosphate buffer, pH 7.1 and incubated with 20 µl of (3% w/v) desalted snail gut enzyme Helicase (Sigma) for 1h. After 1h, the pH of the reaction mixture was brought to 8.9 by addition of 70 µl of 1 M sodium borate buffer (pH 9.8). The mixture was incubated in boiling water bath for 3 min and then rapidly

cooled in an ice. After addition of 2 ml β-dimethylamino benzaldehyde (DMAB) reagent, the mixture was incubated for 20 min at 37°C

Immediately thereafter the absorbance value at 585 nm was measured using a UV-VIS spectrophotometer. N-acetyl glucosamine (GlcNAc) was used as standard Reissig *et al.*,1959). The Enzyme activity was expressed as μg GlcNAc min^{-1} mg^{-1} fresh tissue.

3.14.3. Phenylalanine ammonia lyase

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

3.14.4. Peroxidase

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

3.14.5. Polyphenol oxidase

Polyphenol oxidase activity was measured by the method of Mahadevan and Sridhar *et al.* (1982). The reaction consists of 1ml of enzyme extract and 2 ml of 0.2 M sodium phosphate buffer (pH 6.0). For reaction 2 ml of 0.2 M sodium

phosphate buffer pH 6.0 containing 0.01M pyrogallol was added. Reading was noted after every 1 minute at 495nm. Activity was expressed as ΔA_{495} $min^{-1}g^{-1}$ fresh wt.

3.15. Isozyme analysis by PAGE

3.15.1. 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 Jayaraman *et al.*, (1987) and used immediately for the isozyme analysis.

i). 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.

ii). 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.

iii). 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.

iv). 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.

v). 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.

3.15.2. Polyphenol oxidase

i). Preparation of gel

For native anionic PAGE, resolving gel 8 % acrylamide concentration and stacking gel 4 % acrylamide concentration were prepared as mentioned above.

i). Electrophoresis

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

ii). Staining

After electrophoresis, gel was equilibrated in 0.1 % p-phenylene diamine in 0.1 M potassium phosphate buffer (pH 7.0) for 30 min. This was followed by addition of 10 mM catechol in same buffer. The gel was shaked in the above buffer. After the appearance of dark brown discrete band, analysis of isozyme was done immediately.

3.16. Extraction and quantification of chlorophyll

Extraction

The chlorophyll was extracted from leaves of the treated and control plants following the method of Harborne (1973) with modifications. Crushing of 1 g leaf tissue was done in a mortar and pestle using 80% acetone in the dark to prevent the photo oxidation of chlorophyll. The extract was filtered through Whatmann No. 1 filter paper, adding sufficient amount of acetone and final volume was made up 25 ml.

Estimation

For the estimation of chlorophyll, O. D. values of the dilution of the crude sample were measured directly at 645 nm and 663 nm in a spectrophotometer. The amount of total chlorophyll, chlorophyll a and chlorophyll b was calculated by the following formula:

Total chlorophyll = $(20.2\ A_{645} + 8.02\ A_{663})$ mg g^{-1} tissue

Chlorophyll a = $(12.7\ A_{663} - 2.69\ A_{645})$ mg g^{-1} tissue

Chlorophyll b = $(22.9\ A_{645} - 4.68\ A_{663})$ mg g^{-1} tissue

3.17. Extraction of soluble proteins

Preparation of stock solution

Soluble protein was extracted from the leaves of bacteria treated and control plants following the method of Chakraborty *et al.* (1995). Plant tissues were frozen in liquid nitrogen and ground in 0.05 M sodium phosphate buffer (pH 7.2) containing 10 mM $Na_2S_2O_5$, 0.5 mM $MgCl_2$ and 2 mM PMSF was added during crushing and centrifuged at 4°C for 20 min at 12000 rpm. The supernatant was used as crude protein extract and total soluble protein content was estimated following the method of Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard .

3.17.1. Estimation of protein content

Soluble proteins were estimated following the method as described by Lowry *et al.* (1951). To 1 ml of protein sample 5 ml of alkaline reagent (1 ml of 1 % $CuSO_4$ and 1 ml of 2 % sodium potassium tartarate, added to 100 ml of 2 % Na_2CO_3 in 0.1 N NaOH) was added. This was incubated for 15 minutes at room temperature and then 0.5 ml of 1 N Folin Ciocalteau reagent was added and again incubated for further 15 minutes following which optical density was measured at 720 nm. Quantity of protein was estimated from the standard curve made with bovine serum albumin (BSA)

3.17.2. 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:

(A) 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 4°C and used within one month.

(B) Sodium Dodecyl Sulphate (SDS)

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

(C) Tris Buffer

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

(D) Ammonium Persulphate (APS)

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

(E) Tris –Glycine electrophoresis buffer

Tris running buffer consists of 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.

(F) 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.

(G) 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

(H). Sample Preparation

Sample (50 µl) was prepared by mixing the sample protein (35 µl) with 1X SDS gel loading buffer (15 µl) in cyclomixture. All the samples were floated in boiling water bath for 3 minutes 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.

(I). 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.

(J). 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.

3.18. Extraction of phenols from leaves

Phenol was extracted from the fresh leaves following the method of Mahadevan and Sridhar (1982). 1 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 passed through two layers of cheese cloth and then 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.

3.18.1. Estimation of phenol contents

3.18.1.1. 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 in a systronic photometric colorimeter Model 101 at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.18.1.2. O-dihydroxy phenol

O-dihydroxy phenol was 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 by systronic photometric colorimeter Model 101 at 515 nm. Quantity of the O- dihydroxy phenol was estimated using caffeic acid as standard.

3.19. Extraction of antifungal phenolics

Free and glycosidically linked phenolics were extracted from control and bacteria treated plants following the method of Daayf *et al.* (1995). 10 g of both bacteria treated and untreated control were taken and boiled in methanol and mixed with of 80 % methanol at 10 ml g^{-1} tissue and homogenized by blending for 1 min. Samples were extracted for 48 h on a rotary shaker at 40 rpm covered with aluminium foil. The homogenate was filtered through Whatman No.1 and concentrated by evaporation of final volume of 20 ml. Concentrates were first portioned against equal volume of anhydrous diethyl ether (Fraction I). The aqueous fraction was portioned secondly against equal volume of ethyl acetate thrice (Fraction II). Acid hydrolysis with 4(N) HCl of the remaining aqueous fraction was done according to the method described by Daayf *et al.* (1997) to yield phenolic

aglycones. Aglycones were recovered by partitioning hydrolysates against equal volume of ethyl acetate thrice (Fraction III). All fractions were evaporated to dryness and finally dissolved in 3 ml of respective solvents.

3.20. Bioassay of antifungal phenolics

3.20.1. Spore germination

For spore germination bioassay, a drop of Fraction I, II and III were placed on a clean, grease free slide and allowed to evaporate following which a drop of spore suspension was placed over it. The slides added with respective solvents was considered as solvent control and slide without any solvent but containing only spore suspension was considered as water control. The slides were incubated in moist petriplate for 24 h at 25°C ± 1°C. Finally one drop of lactophenol – cotton blue was added to each spot to fix the germinated spore. The slides were observed under microscope and the percentage of germination was determined.

3.20.2. Agar cup bioassay

10 ml of PDA was poured in sterile 6 cm petri plate. After solidification of the medium 6 mm wells were made in each plate with sterile cork borer. Aliquots (40 µl) of individual fraction and ethyl acetate (as control) were dispersed at each of the three wells made in each plate. A 5 mm agar slice of test fungus was placed at the centre in equal distance from wells. Observations were made after 7 days of incubation at 30°C.

3.20.3. TLC- plate bioassay

All fractions derived from the extraction was analysed by TLC on silica gelG. All the three fractions were spotted on TLC plate and development of chromatogram was carried out at the room temperature using a ethyl acetate: chloroform (9:11) solvent mixture as suggested by Chakraborty and Saha (1994). After development, chromatogram inhibition assay was performed as devised by Hofmans and Fuchs (1970) using *F. lamaoensis* as the test organism. Spore suspension in 2 % sucrose supplemented with PDB was sprayed on the development TLC plate and incubated in sterile humid chamber at 25°C for 6 days. Fungitoxicity

was ascertained by the appearance of inhibition zone, which was visualized as white spots surrounded by a deep black background of mycelia. Diameter of inhibition zone and R_f values were noted.

3.21. Extraction of catechins

Catechin was extracted from tea leaf tissues following the method of Obanda 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. Water 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).

3.22. HPLC analysis of catechins

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 a total min of 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.

3.23. Inoculation technique

The mass culture of fungal pathogens was prepared in sterilized sand maize meal media containing maize, washed sterile sand and water in the ratio 1:9:1.5 (Biswas and Sen, 2000), which was inoculated with mycelial bits of pathogen taken from the margin of actively growing culture and incubated at 25°C ±1°C for 14 days. The two weeks old cultures were used for inoculating the soil.

The potted plants were inoculated with the pathogen inoculum prepared in sand maize meal media. The rhizosphere of each plant was inoculated with 100 g of pathogen inoculum. Regular watering of the plants was done to assure the successful establishment of the pathogen.

3.24. Disease assessment

The rhizosphere of tea plants pre-treated with the antagonists or without treatment was inoculated with pathogen. In pre-treated plants pathogen inoculation was done 3 days after application of antagonist. 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 - 5 scale as adopted by Mathew and Gupta (1996).

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.

3.25. Preparation of talc-based formulation with bacteria and its application in the soil

For development of bio-formulation 10 g of CMC was mixed with 1 kg of talcum powder and pH was adjusted to 7.0 by adding calcium carbonate. It was then sterilized for 30 min in two consecutive days. To this sterilized talcum powder 400 ml of bacterial inoculum containing $3x10^9$ cfu ml^{-1} was added and mixed well under sterile condition. The talc mix was dried under shade to bring moisture to less than 20 %. The formulation was packed in milky white color polythene bags, sealed and stored at room temperature for future use.

The talcum based formulation was applied in the field at the rate of 100 g per pot. The experiment was conducted to assess the effect on shoot growth, increase in number of leaves etc. due to the application of talc based formulation.

3.26. Preparation of antigens

3.26.3. Bacterial antigens

Soluble protein was obtained by strong sonication of bacterial cells in 0.05 M sodium phosphate buffer (pH 7.2) and centrifuged at 4°C for 20 minutes at 10.000 rpm. The supernatant was used as antigen for the preparation of antibodies against it.

The samples were also subjected to one-dimensional SDS-PAGE by using 12 % resolving and 5 % stacking gels. After standard electrophoresis, the gels were stained with Coomassie Brilliant Blue R-250, as mentioned earlier.

3.26.4. Fungal antigens

The fungal mycelia were grown in PDA medium. 1 g mycelia were taken in 2 ml 0.05 M sodium-bicarbonate buffer (pH 9.6) in a chilled mortar and pestle. The mycelia were crushed followed by centrifugation at 10000 rpm. The supernatant was collected and used as antigen.

3.26.5. Soil antigens

1 g soil was taken in 2 ml of 0.05 M sodium-bicarbonate buffer (pH 9.6) in a mortar and pestle and kept overnight at 4°C. Next day, centrifugation was done at 10000 rpm for 10 min. The supernatant was collected and used as antigen.

3.27. Raising of polyclonal antibodies against bacterial antigens

New Zealand white male rabbits were used to raise antisera. The body weights were recorded and observed for at least one week inside the cages before starting the immunization schedule. They were regularly fed with green grass. soaked gram, carrot and green vegetables etc. twice a day. After each bleeding they were given saline water for 3 consecutive days and cages were cleaned everyday in the morning for better hygienic conditions.

A specified antibody response was elicited in rabbit injecting 1 ml of antigen (protein extracted from bacteria) in Freund's complete adjuvant (Genei) for first two injection followed by additional subcutaneous injection of incomplete adjuvant upto 12 weeks at a regular interval of 7 days. Following the method of Alba and Devay (1985), before immunization, normal sera were collected from rabbit.

The rabbits were bled by marginal ear vein puncture, 3 days after first six injections and then subsequently seven times more every fortnight.

During bleeding the rabbit was fixed at 60° angles, hairs were removed from upper side of the ear followed by disinfection with alcohol. The ear vein was irritated by the application of xylene and incision was made with sharp sterile blade and 5-10 ml of blood sample was collected in sterile graduated glass tube. After collection precautionary measures were taken to stop the flow of the blood from the puncture. The blood sample was kept at 37°C for 1 h for clotting. The clot was loosened with a sterile needle. Serum was clarified by centrifugation (5000 rpm for 10 min at room temperature) and distributed in 1 ml vial and stored at -20°C. The serum was used for double diffusion analysis, dot blot analysis and Enzyme Linked Immunosorbent Assay (ELISA).

3.28. Purification of IgG

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.

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 0.005 M 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 30 cm height) and allowed to settle for 2 h. After the column material had settled, 25 ml of buffer (0.02 M sodium phosphate, pH 8.0) washing was given to the column material.

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 column. Another connection above the

surface of buffer (1) was connected to another flask with buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to column, buffer (2) was soaked into buffer (1) thereby producing a continuous raise in molarity. Ultimately, 40 fractions each of 5 ml were collected and the optical density values were recorded at 280 nm using UV-Vis spectrophotometer (DIGISPEC-200GL).

3.29. IgG against *F. lamaoensis*

IgG against *F. lamaoensis* was obtained from Antisera Reserves of Phytopathogens, Department of Botany, University of North Bengal.

3.30. Immunodiffusion

Glass slides (6 cm x 6 cm) were degreased in 90 % v/v ethanol; ethanol: diethyl ether (1:1 v/v) and ether, then dried in hot air oven. After drying the plates were sterilized inside the petridish each containing one plate. Agarose gel is prepared in 0.05 M Tris-barbiturate buffer (pH 8.6). The buffer is heated within a conical flask placed in a boiling water bath. 0.9 % agarose is mixed to the hot buffer and boiled for the next 15 min. The flask is repeatedly shaken thoroughly in order to prepare clean molten agarose and 0.1 % (w/v) sodium azide is added into it. For the preparation of agarose gel, the molten agarose is poured on the sterilized glass slides (10 ml $slide^{-1}$) in laminar flow chamber and kept 15 min for solidification. After that 3-7 wells are cut out with a sterilized cork borer (6 mm diameter) at a distance of 1.5 cm- 2 cm away from central well and 2.0 - 2.5 cm from well to well (peripheral).

i). Diffusion

Agar gel double diffusion tests were carried out using antigen and antiserum following the method of Ouchterlony (1976). The antigen and undiluted antisera (50 µl $well^{-1}$) were pipetted directly into the appropriate well in a laminar chamber. The diffusion was allowed to continue in a moist chamber for 72 h at 25°C. Precipitation reaction was observed in the agar gel only in cases where common antigen was present.

ii). 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 slides were stained with Coomassie brilliant blue (R250, Sigma: 0.25 g Coomassie blue, 45 ml methanol, 45 ml distilled water and 10 ml glacial acetic acid) for 10 minutes at room temperature. After staining, the slides were washed in destaining solution (methanol: distilled water: acetic acid in 45:45:10 ratio) with changes until the background become clear. Finally slides were washed with distilled water and dried in hot air oven for 3 h at 50°C.

3.31. Determination of bacterial sustainability in soil

The sustainability of bacteria in the rhizosphere soil was determined by immunological techniques.

3.31.1. ELISA

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

3.31.2. Dot-blot

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

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

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

III. Blocking solution 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)

IV. Alkaline phosphatase buffer (100 mM Tris HCl, 100 mM NaCl, 5 mM $MgCl_2$.

Assay

Nitrocellulose membrane (Millipore, 7 cm x 10 cm, pore size 0.45µm, Millipore corporation, Bedford) was cut carefully into the required size and placed inside the template. 4 µl of coating buffer was loaded into each well of the template over the NCM and was kept for 30 minutes to dry. Following this 5 µl of test samples (antigen) was loaded into the template wells over the NCM and was kept for 1 h at room temperature. Template was removed and blocking of NCM was done with 10 % non fat dry milk (casein hydrolysate, SRL) prepared in TBST for 30 - 60 minutes on a shaker, respective polygonal antibody (IgG 1:500) prepared against that antigen was added directly in the blocking solution and further incubated at 4°C for overnight. The membrane was then washed gently with running tap for 3 minutes, following three times 5 minutes washes 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 2 h at 37°C. The membrane was washed as before. 10 ml of the NBT/BCIP substrate (Genei) was added next and color development was stopped by washing the NCM with distilled water and color development was categorized on the intensity of dots.

3.31.3. Bacterial colony transfer

The soil suspension was prepared by using bacteria inoculated rhizosphere soil and plated on NA medium in sterile petriplates. The plates were allowed to grow for 24 h. After 24 h the colony was transferred to NCM and probed with antibody. The presence of purple dot in the NCM shows the presence of bacteria in the soil.

3.32. Determination of pathogen in soil by immunological methods

For detecting the presence of pathogen in the soil, the protein samples from pathogen inoculated soil, pathogen and *B. megaterium* inoculated soil as well as uninoculated sterile soil was extracted. For extraction of protein, 5 g of soil was taken in 10 ml of protein extraction buffer [0.05 M sodium phosphate buffer (pH 7.2)]. The soil was stirred for 24 h followed by crushing and centrifugation as previously mentioned. The protein samples collected from different soil samples were used for immunological detection as mentioned below.

3.32.1. ELISA

The protein extracted from pathogen inoculated soil samples were analysed by ELISA technique as mentioned before.

3.32.2. Dot-blot

The determination of presence of pathogen in soil inoculated with pathogen as well as *B. megaterium* and pathogen inoculated soil was done by Dot-blot technique using antigen prepared from the soil as mentioned above.

EXPERIMENTAL

4.1. Screening of microorganisms from tea rhizosphere and selection of antagonistic bacteria

The soil samples collected from the rhizosphere of the healthy tea plants of different age growing in different regions of the foothills of Darjeeling Himalayas were brought to the laboratory and the microorganisms isolated as described under materials and methods. The numbers of bacterial and fungal colonies were counted in the plates and the microbial population determined in soils from different rhizospheres. Microbial populations, determined as cfu g^{-1} soil, ranged between $12x10^6$ – $50x10^8$cfu. Results revealed that the maximum population was observed in the rhizosphere of 40 year old bushes of the Terai, and minimum in 10 year old bushes of NBU Experimental Garden. The rhizosphere of the very old as well as young tea bushes had much lesser microbial population (Table 1). Population did not show any consistent difference with

Table 1: Microbial population and number of isolates from tea rhizosphere

Soil sample	*Age years*	*Microbial population (cfu g^{-1} soil)*	*No of bacterial isolates*
Hansqua Tea Estate, Terai	90	$14x10^7$	10
Falakata Tea Estate, Dooars	50	$16x10^7$	15
Margaret's Tea Estate, Darjeeling	75	$25x10^8$	12
Chandmani Tea Estate, Siliguri	80	$10x10^8$	14
Nagrakata Tea Research station, Dooars	40	$50x10^8$	46
Tea Experimental Garden, NBU	10	$12x10^6$	10

the variety. The maximum microbial population was obtained in the hot humid months, which decreased during winter.

The soil samples from each site were also tested for their pH. The soils were found to be acidic ranging from pH 4.2 – 5.1 (Table 2).

Table 2: pH of the soil samples collected from different tea plantations

Source	Soil pH
Hansqua Tea Estate, Terai	4.38
Falakata Tea Estate, Dooars	4.8
Margaret's Tea Estate, Darjeeling	5.2
Chandmani Tea Estate, Siliguri	5.1
Nagrakata Tea Research station, Dooars	4.9
Tea Experimental Garden NBU	4.95

All isolated bacteria which were grown in NB and labeled were tested for their antagonistic activity against certain root pathogens - *Fomes lamaoensis*, *Sphaerostilbe repens*, *Poria hypobrumea*, *Sclerotium rolfsii* I & II and *Sclerotinia sclerotiorum in vitro* by dual pairing tests. Their interactions were categorized into the types - A: Homogenous; B: Overgrowth; C: Inhibition and D: Cessation at line of contact (Table 3).

Among all tested microorganisms, it was observed that four of the bacterial isolates *viz.* HRW_{10}, HRW_9, $HSoil_1$ and $NT16_4$ showed antagonistic activity against the soil borne pathogens. Among these four antagonists, HRW_9 and $NT16_4$ were selected for further investigations due to their higher potential to check the growth of the pathogen in dual culture.

The selected antagonistic bacterial strains were morphologically and bio-chemically characterized in the laboratory (Table 4, Plate III & IV).

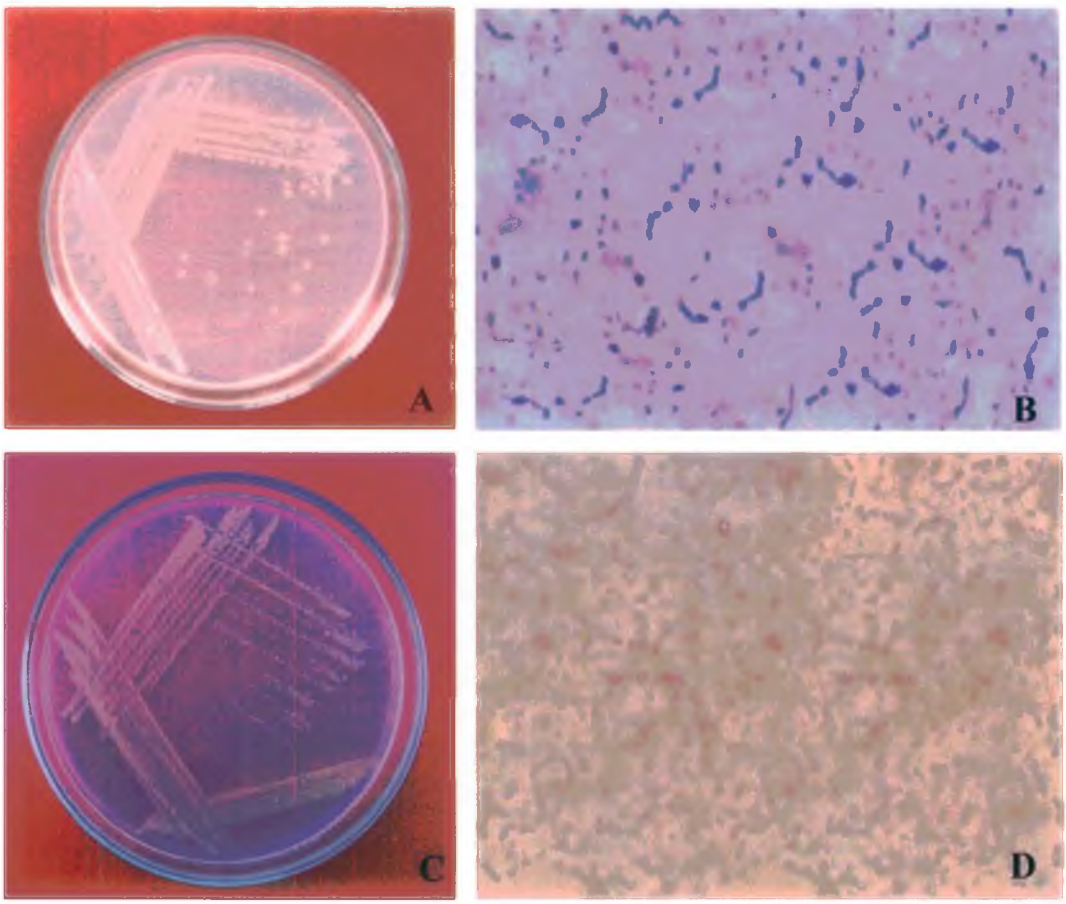
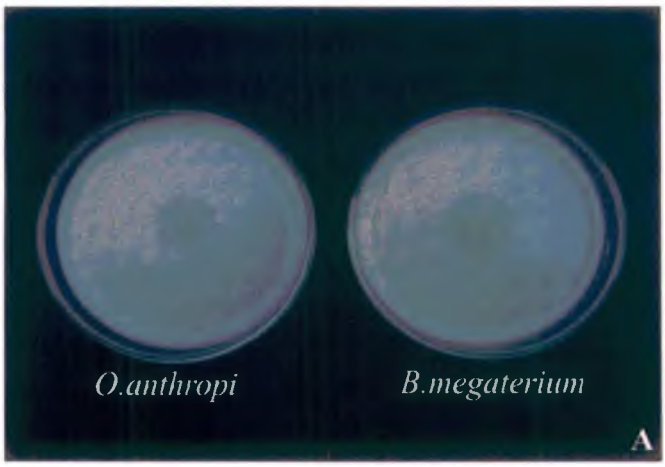
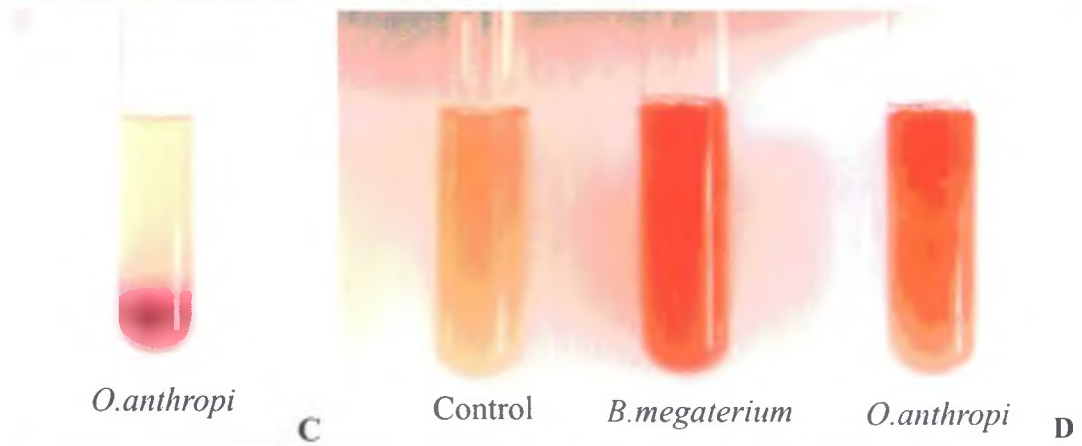


Plate III (A-B): **A** :*Bacillus megaterium* grown in NA medium; **B**: Microscopic view of *Bacillus megaterium* **C:** *Ochrobactrum anthropi* grown in NA medium **D:** Microscopic view of *Ochrobactrum anthropi*





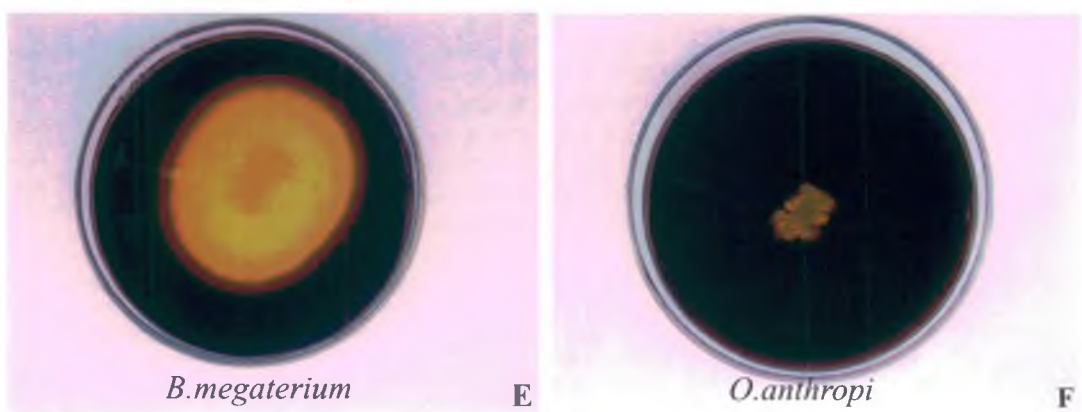


Plate IV (A-F): showing results of biochemical tests performed on antagonistic bacterial isolates.
A: Casein digestion; B & C: Indole production; D: Voges-Proskauer Test; E & F: Starch Hydrolysis

Table 3: Interaction of soil isolates in pairing tests with test fungi

Sl. No.	Source of soil	Isolate NO.	Type of reaction in tests against				
			S. rolfsii	*S. sclerotiorum*	*F. lamaoensis*	*P. hypobrumea*	*S. repens*
1	Hansqua Tea Estate	HS 1	C	C	C	C	C
2		HS 2	B	B	B	B	B
3		HS 3	B	B	B	B	B
4		HSl 4	B	B	B	B	B
5		HRW 1	B	B	B	B	B
6		HRW 2	B	B	B	B	B
7		HRW 3	B	B	B	B	B
8		HRW 4	B	B	B	B	B
9		HRW 7	B	B	B	B	B
10		HRW 8	B	B	B	B	B
11		HRW 9	B	B	C	C	C
12		HRW 10	D	B	C	C	C
13	Nagrakata Tea Research Station TV-16	NT16 1	B	B	B	B	B
14		NT16 2	B	B	B	B	B
15		NT16 3	B	B	ND	partial inhibition	ND
16		NT16 4	C	C	C	C	C
17		NT16 5	B	B	B	B	B
18		NT16 6	B	B	B	B	B
19		NT16 7	B	B	B	B	B
20		NT16 8	B	B	B	B	B
21		NT16 9	B	B	B	B	B
22		NT16 10	B	B	B	B	B
23		NT16 14	B	B	B	B	B
24	S_3A_1	NJ 1	B	B	B	B	B
25		NJ 2	B	B	B	B	B
26		NJ 3	B	B	B	B	B
27		NJ 4	B	B	ND	ND	ND
28		NJ 5	B	B	B	B	B
29		NJ 6	B	B	B	B	B
30	T-7/1/154	Te 1	B	B	B	B	B
31		Te 2	B	B	B	B	B
32		Te 3	B	B	ND	ND	ND
33		Te 4	B	B	B	B	B
34		Te 5	B	B	B	B	B
35		Te 6	B	B	B	B	B
36	TV-1	NT1 1	B	B	B	B	B

37		NT1 $_2$	B	B	B	B	B
38		NT1 $_3$	B	B	B	B	B
39	TV-23	NT23 $_1$	B	B	B	B	B
40		NT23 $_2$	B	B	B	B	B
41		NT23 $_3$	B	B	B	B	B
42	TV –9	NT9 $_1$	B	B	B	B	B
43		NT9 $_2$	B	B	B	B	B
44		NT9 $_3$	B	B	B	B	B
45		NT9 $_4$	B	B	B	B	B
46		NT9 $_5$	B	B	B	B	B
47		NT9 $_6$	B	B	B	B	B
48		NT9 $_7$	B	B	B	B	B
49		NT9 $_8$	B	B	ND	ND	ND
50	TV-28	NT28 $_1$	B	B	ND	ND	ND
51		NT28 $_2$	B	B	ND	ND	ND
52		NT28 $_3$	B	B	B	B	B
53	Chandmani T.E. TV-18	TR/B$_C$ 1	B	B	B	B	B
54		TR/B$_C$ 2	B	B	B	B	B
55		TR/B$_C$ 3	B	B	B	B	B
56		TR/B$_C$ 4	B	B	B	B	B
57		TR/B$_C$ 5	B	B	B	B	B
58		TR/B$_C$ 6	B	B	B	B	B
59		TR/B$_C$ 7	B	B	B	B	B
60		TR/B$_C$ 8	B	B	B	B	B
61		TR/B$_C$ 9	B	B	ND	ND	ND
62		TR/B$_C$ 10	B	B	ND	B	ND
63	NBU Expt. Garden	TR/B$_P$-1	B	B	B	B	B
64		TR/B$_P$-2	B	B	B	B	B
65		TR/B$_P$-3	B	B	B	B	B
66		TR/B$_P$-4	B	B	B	B	B
67		TR/B$_P$-5	B	B	B	B	B
68		TR/B$_P$-6	B	B	B	B	B
69		TR/B$_P$-7	B	B	B	B	B
70		TR/B$_P$-8	B	B	B	B	B
71		TR/B$_P$-9	B	B	C	C	ND
72		TR/B$_P$10	B	B	B	B	B

A: Homogenous; B: Overgrowth; C: Inhibition & D: Cessation at line of contact; ND: Not done.

Based on their morphology and biochemical tests, the bacteria HRW_9 and $NT16_4$ were tentatively identified in the laboratory and their identity was confirmed at Diagonistic and Advisory Service, CABI Bioscience, U.K. These were identified as:

HRW_9 *Ochrobactrum anthropi* TRS-2

$NT16_4$ *Bacillus megaterium* de BaryTRS-3

Table 4: Basic identification results of rhizobacterial isolates

Characteristics	*O. anthropi*	*B. megaterium*
Morphological		
Shape	Coccus	Rod
Gram reaction	+ ve	+ ve
Endospore	-ve	+ ve
Margin	Smooth	Wavy
Surface	Smooth	Rough
Pigment	-	-
Density	Opaque	Opaque
Biochemical		
V. P. reaction	+ ve	+ ve
Catalase production	+ ve	+ ve
Urea digestion	+ ve	- ve
Esculine hydrolysis	- ve	+ ve
Casein hydrolysis	+ ve	+ ve
Starch hydrolysis	- ve	+ ve
Indole test	+ ve	+ ve
Nitrate reduction	- ve	- ve
Phenol Red Tartarate	+ve	- ve

4.2. *In vitro* antagonistic studies of *B. megaterium* and *O. anthropi* against test fungi

After identification, the inhibitory effect of these isolates *viz. B. megaterium* and *O. anthropi* were confirmed by dual culture methods both in solid and liquid media.

4.2.1. Solid media

In solid medium, inhibition of the growth of different fungal pathogens by the antagonistic bacteria and the zone of inhibition were recorded. The results revealed that *B. megaterium* inhibited the growth of all test pathogens *viz. F. lamaoensis, S. repens, P. hypobrumea, S. rolfsii* I, *S. rolfsii* II and *S. sclerotiorum* effectively (Plate V & VI). *O. anthropi* checked the growth of *F. lamaoensis, S. repens* and *P. hypobrumea* efficiently (Plate VII) but was not effective against *S. rolfsii* I, *S. rolfsii* II and *S. sclerotiorum* (Table 5; Fig. 1).

Table 5: *In vitro* antagonistic effect of rhizobacterial isolates against test pathogens

Test pathogens	Diameter of inhibition zone (cm)*	
	B. megaterium	*O. anthropi*
F. lamaoensis	2.2 ± 0.05	1.2 ± 0.04
P. hypobrumea	1.6 ± 0.03	1.5 ± 0.11
S. repens	2.0 ± 0.07	0.6 ± 0.01
S. rolfsii I	3.0 ± 0.06	0.00
S. rolfsii II	0.6 ± 0.02	0.00
S. sclerotiorum	1.5 ± 0.05	0.00

± = Standard error; *Average of 3 replicates.

4.2.2. Liquid media

The bacteria were also tested for their inhibitory activity against the test fungi in liquid medium. After 8 days of growth, mycelia were harvested, dried and mycelial dry weigh was taken. Results revealed similar observation as in

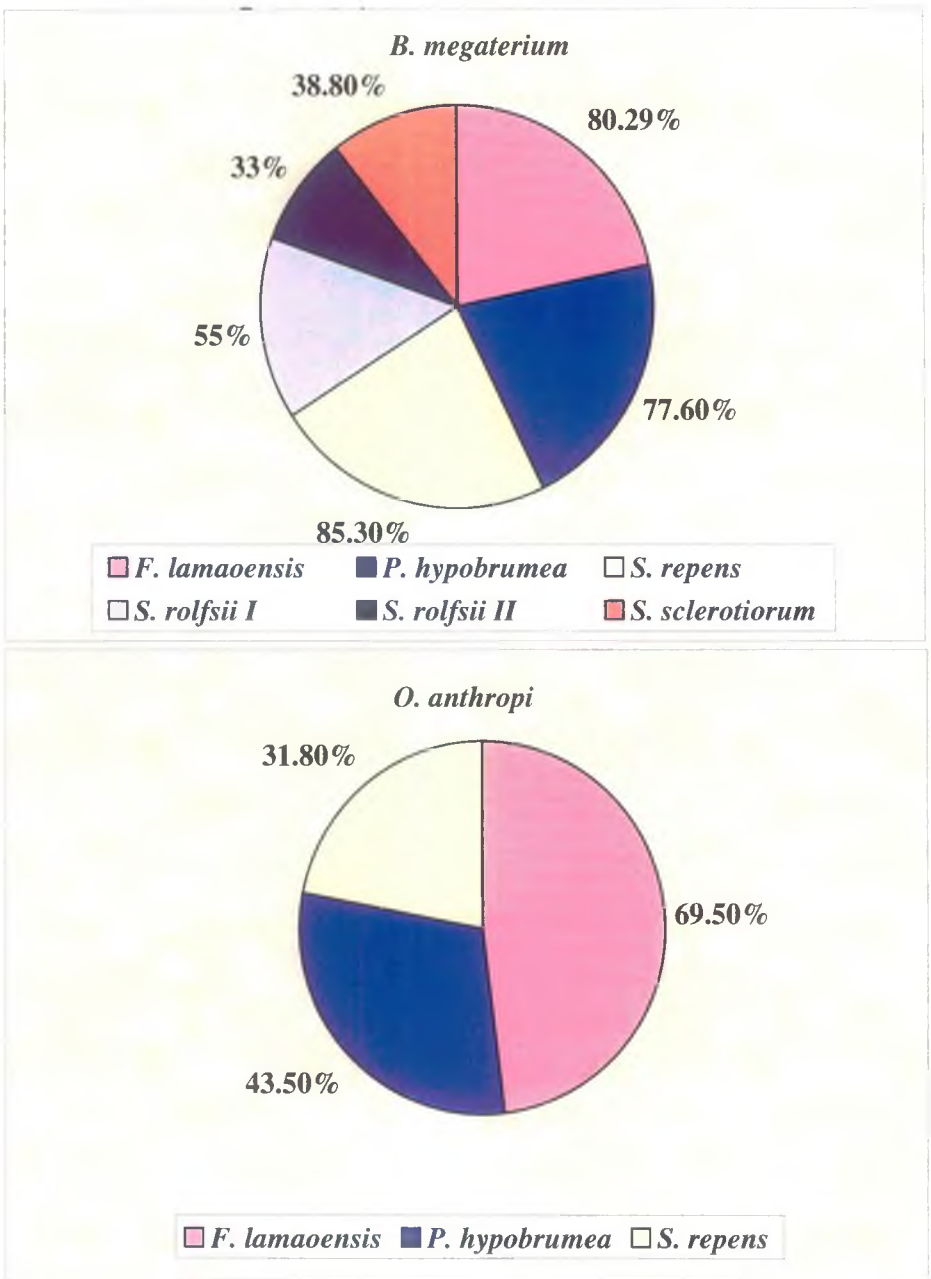


Fig. 1: Inhibition of growth of test fungi by *B. megaterium and O. anthropi.*

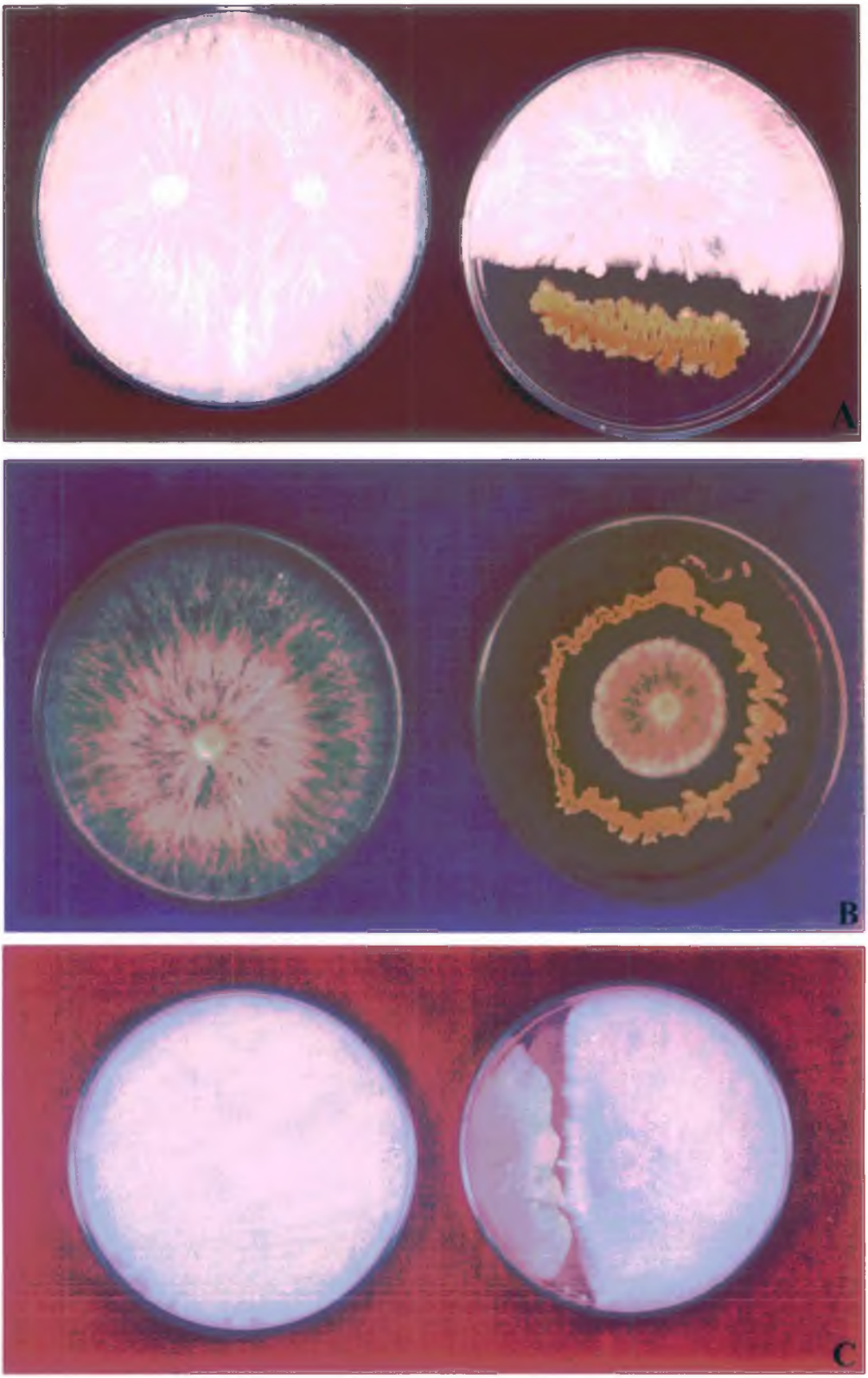


Plate V(A-C): *In vitro* antagonism of *B. megaterium* against *Sclerotinia sclerotiorum* (A) *Sclerotium rolfsii* I (B) and *Sclerotium rolfsii* II (C)

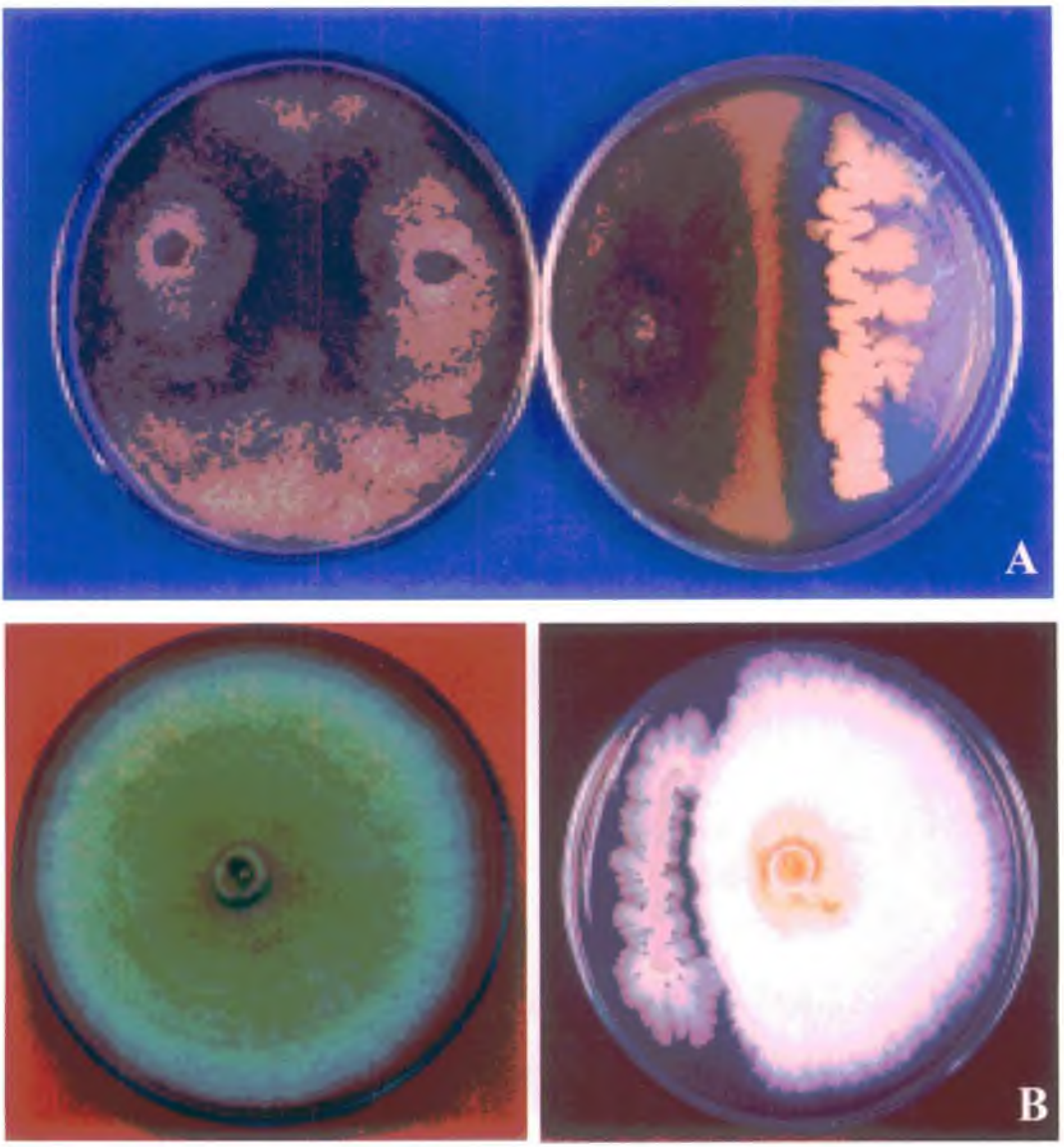


Plate VI (A-B): *In vitro* antagonism of *B. megaterium* against *Fomes lamaoensis* (A)and *Poria hypobremea* (B)

solid media (Table 6). Reduction of the growth by *B. megaterium* varied from 33.7 % (*S. repens*) to 69.5 % (*S. rolfsii* II) while *O. anthropi* did not inhibit *S. rolfsii* I and II and *S. sclerotiorum.*

4.3. Studies on cultural characteristics of *B. megaterium* and *O. anthropi*

Before further work was carried out with the two isolated antagonists, their cultural characteristics were determined in order to obtain information about their optimal growth conditions. Effects of different pH, temperature and media on growth of the two bacteria were determined.

Table 6: Effect of antagonistic rhizobacterial isolates on mycelial fresh weight of test pathogens

	B. megaterium		*O. anthropi*	
	MDW*(g)	Reduction** (%)	MDW*(g)	Reduction** (%)
F. lamaoensis	0.098	62.6 ± 1.7	0. 112	56.8 ± 1.9
P. hypobrumea	0.105	62.4 ± 2.4	0.122	56.3 ± 2.2
S. repens	0.351	33.7 ± 1.4	0.327	38.2 ± 1.3
S. rolfsii I	0.191	59.2 ± 2.6	0.468	0.00
S. rolfsii II	0.120	69.5 ± 3.0	0.394	0.00
S. sclerotiorum	0.107	51.1 ± 1.6	0.219	0.00

* Average mycelial dry weight of three replicates **Reduction per cent over control after 8 days of growth in PDB

4.3.1. pH

B. megaterium and *O. anthropi* grew best at pH 6.0 and almost did not grow at pH 2.0 and pH 10.0 (Fig. 2A).

4.3.2. Temperature

The growth of these bacteria was observed at different temperatures ranging from 20 to 50°C. NB medium was inoculated with the bacteria and the

flasks were incubated at 20, 30, 40 and 50°C. Both the bacteria grew well within these ranges of temperature but grew best at around 40°C (Fig. 2B).

4.3.3. Media

Five different media were selected for testing the growth of the bacteria. These were Potato Dextrose broth (PDB - Potato 400 g, Dextrose 20 g and 1 L dist. water, pH 7.3 ± 0.2), Nutrient Broth (NB: Peptone 5 g, NaCl 5 g, Beef extract 3 g, Yeast extract 3 g and 1 L dist. water, pH 7.4 ± 0.2), King's B (KB – Peptone 20 g, K_2HPO_4, anhydrous 1.5 g, $MgSO_4$. $7H_2O$ 1.5 g, Glycerol 15 ml in 1 L dist. Water, pH 7.4 ± 0.2), Luria broth (LB – Peptone 10 g, Yeast extract 5 g, NaCl 5 g in 1 L dist. Water pH 7.2 ± 0.2) and Succinic acid medium (SA – Succinic acid 4 g, $(NH_2)_4$ SO_4 1 g, KH_2PO_4 3 g, K_2HPO_4 0.1 g, $MgSO_4$. $7H_2O$. 0.2 g in 1 L dist. water, pH 7) *B. megaterium* and *O. anthropi* were grown in different media and both the bacteria could grow in all the tested media but the growth was recorded best in NB media (Fig. 2C).

4.4. *In vitro* determination of mechanism of action of selected antagonists

The plant growth promoting rhizobacteria are known to act by several means- i.e., phosphate solubilization, chitinolytic activity, production of HCN, IAA, volatiles, siderophores etc. Experiments were conducted to determine the mechanism of action of the two selected antagonists isolated from tea rhizosphere results of which have been summarized in Table 7.

4.4.1. IAA

Both the bacterial strains were assessed for their ability to produce indole acetic acid by growing them in Tripticase soya agar supplemented with tryptophane (0.1 mM). *B. megaterium* recorded the IAA production of 50 mg L^{-1}. *O. anthropi* was found to produce 22 mg L^{-1}.

4.4.2. Phosphate solubilisation

The phosphate solubilising ability of the two antagonists was assessed in terms of the diameter of clear zone formed around the colony grown in

Pikovskaya's medium. In Pikovskaya's medium both the bacterial antagonists *B. megaterium* and *O. anthropi* produced clear zone of diameter 17 mm and 20 mm respectively after 3 days of incubation, indicating that both the isolates could solubilise the insoluble phosphate into simpler substances (Plate VIII A & B).

4.4.3. Siderophore production

Siderophore production by the antagonistic bacterial strains was detected by growing the bacteria individually in chrome azurol S agar plate. The plates were observed for 10-15 days after inoculation with bacteria. The appearance of yellow halo region was observed around both *B. megaterium* and *O. anthropi* which indicated that both the bacterial isolates were able to chelate Fe^{3+} from chrome azurol S agar. The diameters of halo region were 1.8 cm and 1.9 cm for *B. megaterium* and *O. anthropi* respectively after 12 days of incubation (Plate VIII C & D).

Production of siderophore was further confirmed by the Fe^{+} chelate test. Both *B. megaterium* and *O. anthropi* bacteria showed reduction in their ability to inhibit the mycelial growth of *F. lamaoensis* with increasing concentration of iron supplemented in the medium. The inhibition was maximum in case of 150 μg ml^{-1} and minimum in case of 600 μg ml^{-1} iron concentration in case of both the antagonists (Plate VIII E). Hence the inhibition of pathogen was curtailed in presence of iron possibly due to reduction of siderophore production.

4.4.4. HCN production

To test the ability of *B. megaterium* and *O. anthropi* to produce hydrocyanic acid (HCN) the bacteria were grown in medium amended with glycine. Filter paper strip soaked in picric acid was placed on the inner side of the lid of each petriplate and sealed properly with parafilm. Results were observed after 4-7 days. Both *B. megaterium* and *O. anthropi* were found to be non-cyanogenic in nature. This suggests that compounds other than HCN may be associated with the antagonistic nature of the rhizobacteria.

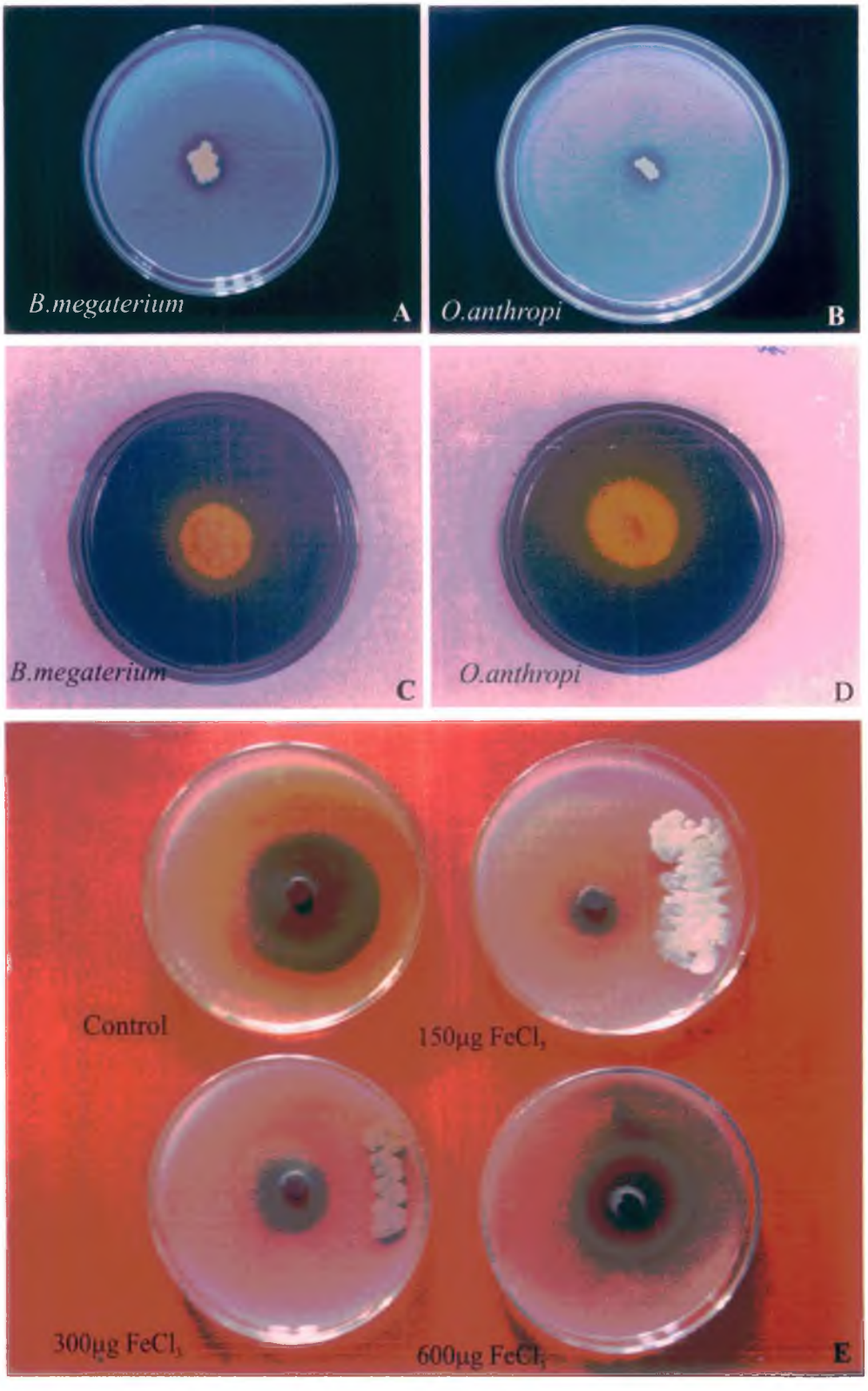


Plate VIII(A-E): *In vitro* characterisation of antagonistic bacterial isolates. A &B: solubilisation of phosphates; C & D: production of siderophore; E: Effect of $FeCl_3$ on the level of antagonism

4.4.5. Chitinase production

The bacteria were spot inoculated in the 5 % colloidal chitin amended minimal medium and when incubated at 27°C for 6-7 days. It was observed that no extracellular chitinase was secreted by *O. anthropi* and *B. megaterium* even when grown on chitin amended media.

4.4.6. Volatile production

The efficacy of the volatile compound produced by the antagonists was determined by the inhibition of the mycelial growth of the test pathogen in comparison to the mycelial growth in control plate containing only the pathogen as described under materials and methods.

Results indicated that both the antagonists produced volatile antibiotics, the maximum inhibition being exhibited by *B. megaterium* which retarded the growth of *F. lamaoensis* by 81.25 %, *S. rolfsii* I by 61.11 % and *S. rolfsii* II by 56.67 %, followed by *O. anthropi* which inhibited *F. lamaoensis* by 75 % (Plate IX).

Further, when the effect of age of bacteria on the growth inhibition of pathogen was compared maximum inhibition was observed on the first day of incubation by fresh *B. megaterium* inoculum (0 day) compared to 1, 2 and 3 days old inoculum (Fig. 3) but as the incubation days increased, inhibition was also increased in all cases. Maximum inhibition was obtained on 4^{th} day irrespective of the age of antagonists. They produced considerable amount of volatile antibiotics to restrict the growth completely. The cumulative growth was significantly reduced by antagonists of all age. The comparison revealed that though on the first day of incubation, maximum efficiency was shown when the antagonists and pathogens were of the same age, the age of the antagonists did not have significant effect on the growth inhibition of the pathogen.

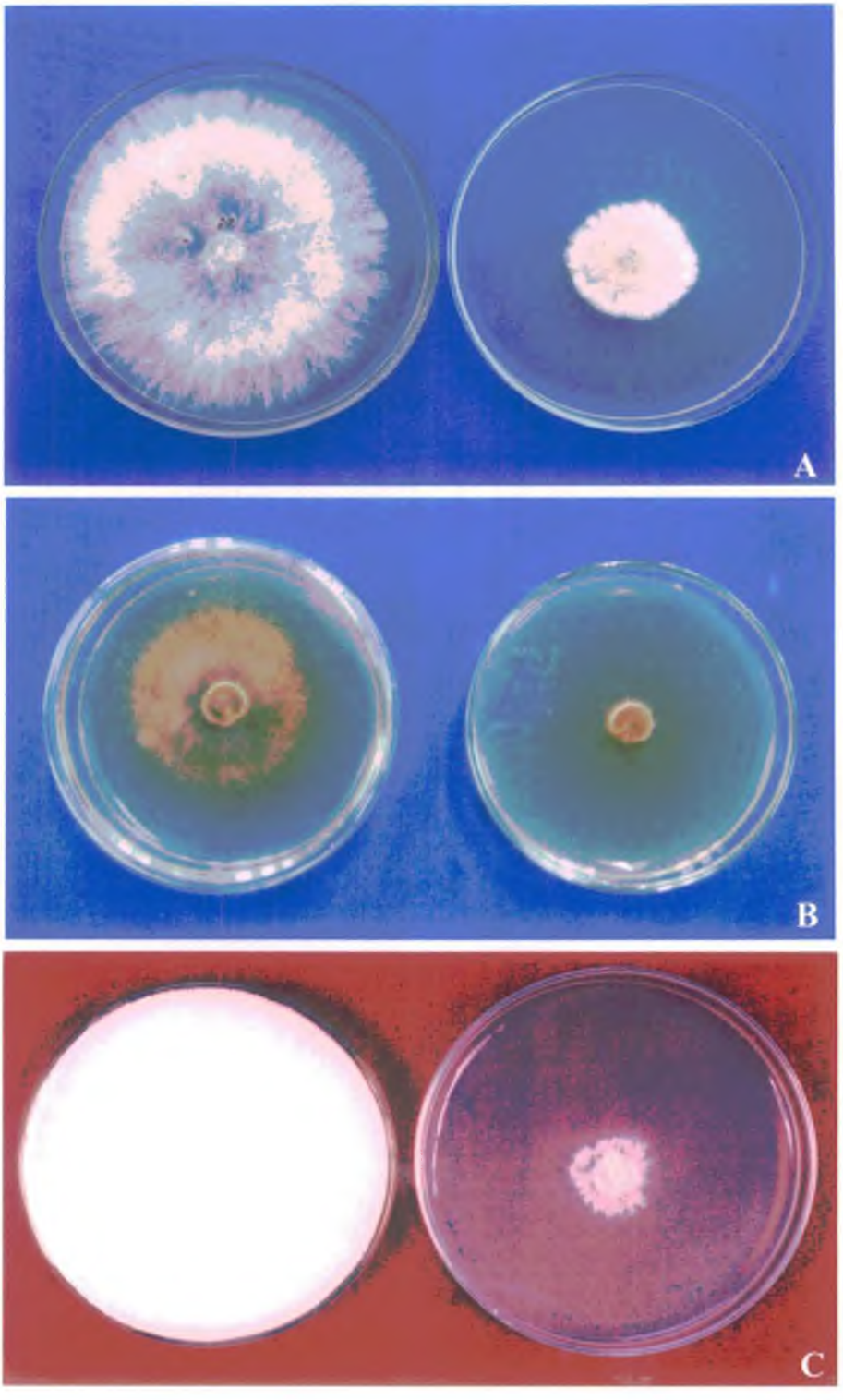


Plate IX (A-C): Inhibition of *S. rolfsii* II (A); *F. lamaoensis* (B); and *S. rolfsii* I (C) by the volatile produced by *B. megaterium*

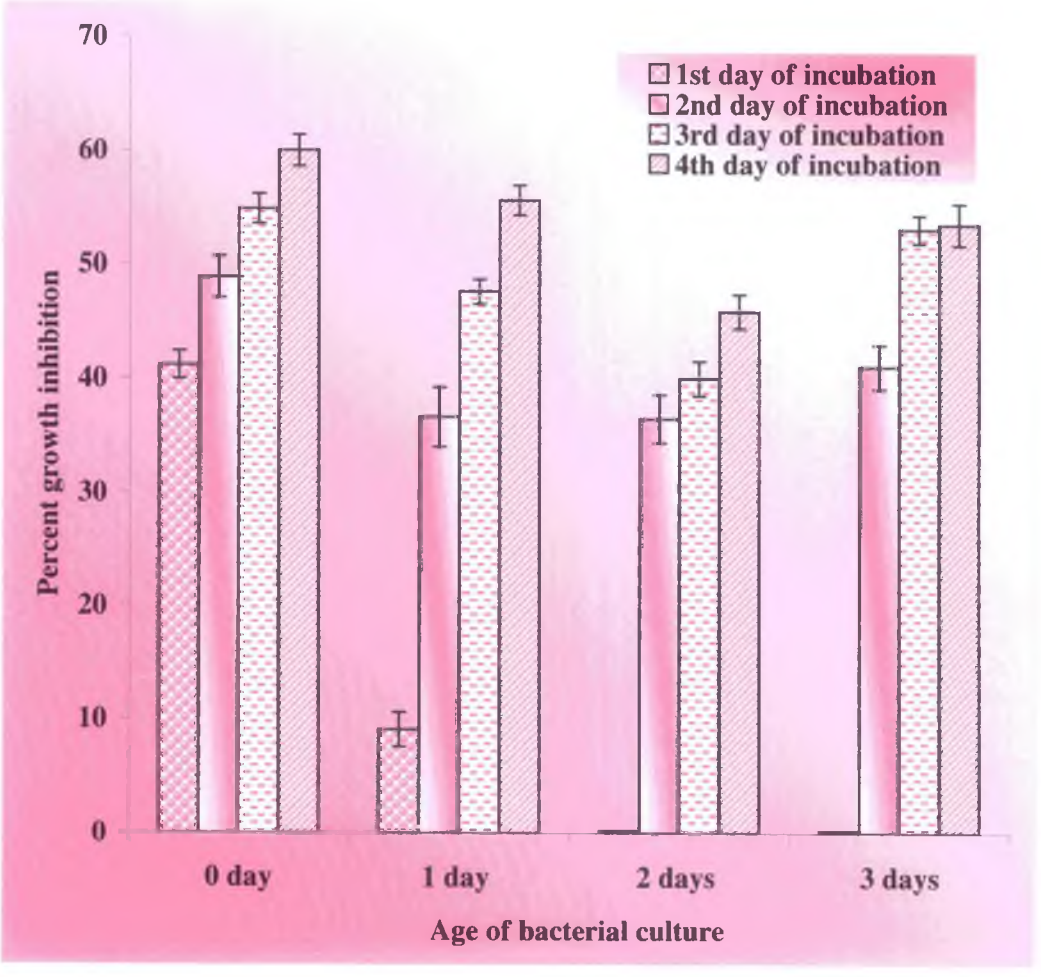


Fig. 3: Growth inhibition of *S. rolfsii* by volatile compound released by *B. megaterium* culture of different ages

Table 7: Mechanism of action of selected antagonists

	B. megaterium	*O. anthropi*
IAA	+	+
P solubilisation	+	+
Siderophore production	+	+
Volatile production	+	+
HCN production	-	-
Chitinase production	-	-

Average of three replicates.

4.5. Bioassay of active principle from *B. megaterium* and *O. anthropi* against test fungi

4.5.1. Cell free culture filtrate

Cell free culture filtrate of both the bacterial strains *B. megaterium* and *O. anthropi* significantly restricted the growth of *F. lamaoensis in vitro* while *S. rolfsii* I was inhibited by *B. megaterium* and not by *O. anthropi*. As the ratio of culture filtrate in broth increased, a marked decrease in mycelial growth of the pathogen was recorded (Fig. 4A). When the sclerotia from *S. rolfsii* I and *S. sclerotiorum* were soaked overnight in culture filtrate and grown on the plate containing media, the germination of sclerotia was completely inhibited.

The active principle present in the culture filtrate of *B. megaterium* was found to be heat stable. Autoclaved culture filtrate retained full inhibitory activity against mycelial growth of the pathogens. There was hardly any difference in the percentage of inhibition of pathogen growth due to the autoclaved culture filtrate and filter sterilized culture filtrate (Fig. 4B).

4.5.2. Solvent extracts of culture filtrate

Results of previous experiment showed that both the selected antagonistic bacteria secreted antifungal metabolites in the medium. For further analysis of these metabolites, cell free culture filtrates were extracted separately with five

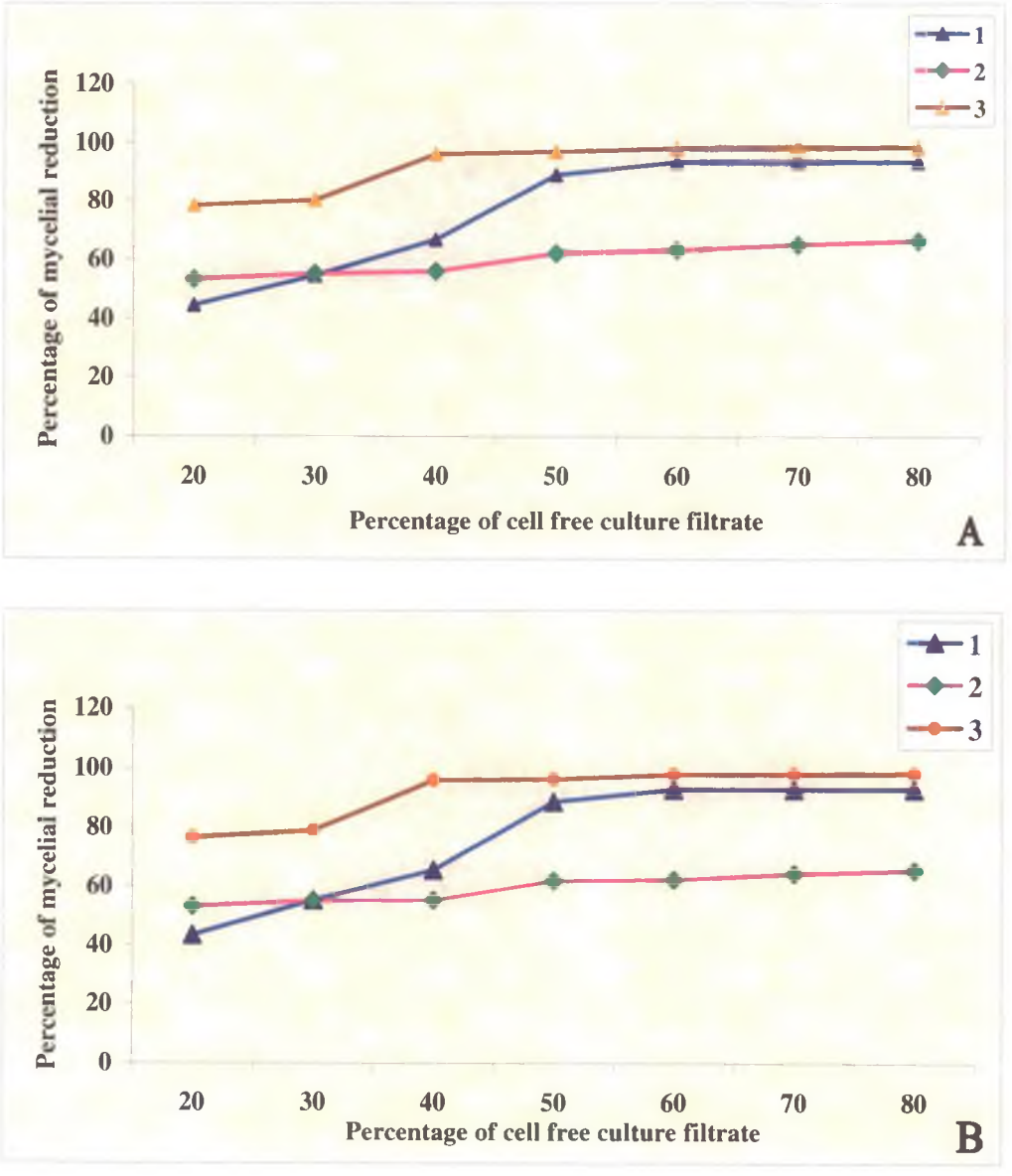


Fig. 4: Mycelial growth inhibition of pathogens by cell free culture filtrate of *B. megaterium* and *O. anthropi*

A: Autoclaved culture filtrate and B: cold sterilised culture filtrate

[Inhibition of *S. rolfsii* (1) and *F. lamaoensis* (2) by *B. megaterium*; *F. lamaoensis* (3) by *O. anthropi*

solvents- acetone, benzene, chloroform, diethyl ether or ethyl acetate. The solvent fractions were dried and were finally dissolved in ethanol.

The ability of the solvent fractions extracted from cell free culture filtrate of *B. megaterium* to inhibit test fungi *F. lamaoensis* was confirmed by growing the pathogen in medium containing the solvent fractions. When 0.2 ml of individual active principle was added to each cup in petriplate containing media, it significantly inhibited *F. lamaoensis* growth in comparison to water and solvent control. Similarly it was observed that active principle extracted from culture filtrate of *O. anthropi* could efficiently inhibit the growth of the pathogen *F. lamaoensis in vitro* (Table 8; Plate X).

The different solvent extracts were also bioassayed by spore germination tests against *S. repens*. For spore germination bioassay, a drop of each solvent extract was placed on a clean, grease free glass slide and allowed to evaporate following which a drop of spore suspension of *S. repens* was placed over it and the percentage of germinated spores was recorded after 24 h. Spores with only solvent served as control. It was observed that all the solvent extracts from both the bacteria inhibited spore germination. The result showed that diethyl ether fraction completely inhibited the spore germination. Almost 80 % of inhibition in spore germination was observed with other fractions like acetone, chloroform and ethyl acetate compared to 80-85 % germination in control spores (Table 9).

4.5.3. Whole cells

The ability of the compound extracted from *B. megaterium* to inhibit test fungi *F. lamaoensis* and *S. rolfsii* I was confirmed by growing the pathogen in medium amended with compound extracted from the bacteria. When 0.2 ml of extracted compound was added to each cup in petriplates containing media, it inhibited *F. lamaoensis* by 53.9 % in comparison to water and solvent control and when the same extract was added to the media in 1:5 ratio, it inhibited the growth of the test pathogen *S. rolfsii* I completely (Plate XI). Similarly it was observed that compound extracted from *O. anthropi* could efficiently inhibit the growth of the pathogen *F. lamaoensis in vitro* by 81.42 % compared to control plate when

0.2 ml of the extracted compound was added to each cup and fungal inoculum (6 mm) was placed at the centre (Plate X C). The compound extracted from both the bacteria completely inhibited the spore germination of *S. repens* compared to germination in water control samples.

Table 8: Inhibition of mycelial growth of *F. lamaoensis* by solvents extracts of cell free culture filtrate.

Solvent fraction	*B. megaterium*		*O. anthropi*	
	Mycelial diameter (cm) *	Percent reduction of mycelial growth	Mycelial diameter (cm)*	Percent reduction of mycelial growth
Control	6.0 ± 0.17	-	6.0 ± 0.00	-
Acetone	2.3 ± 0.35	61.5 ± 3.04	2.5 ± 0.11	58.5 ± 2.09
Benzene	2.5 ± 0.23	58.0 ± 5.07	2.7 ± 0.33	54.7 ± 0.03
Chloroform	2.1 ± 0.17	64.8 ± 3.91	2.9 ± 0.23	51.9 ± 4.05
Diethyl ether	1.7 ± 0.06	71.6 ± 1.79	2.1 ± 0.11	65.2 ± 1.75
Ethyl acetate	1.9 ± 0.03	68.9 ± 0.49	2.3 ± 0.07	62.9 ± 1.02

* After 7 days of incubation; Average of three replicates; ±= Standard error

Table 9: Effect of solvent extracts of cell free culture filtrates of *B. megaterium* and *O. anthropi* on spore germination of *Sphaerostilbe repens* extracted from culture filtrate of antagonistic bacterial isolates

Solvent fraction	% spore germination	
	*B. megaterium**	*O. anthropi**
Control	81.0 ± 2.08	83.0 ± 1.16
Acetone	21.4 ± 0.98	20.0 ± 1.11
Benzene	23.5 ± 0.46	23.8 ± 0.21
Chloroform	16.7 ± 0.52	19.2 ± 0.35
Diethyl ether	14.3 ± 0.47	10.0 ± 1.05
Ethyl acetate	17.4 ± 0.40	23.5 ± 0.80

* Average of 200 spores; ± = Standard error; Difference of all tests with control significant at P= 0.01 as tested by Student's 't' test

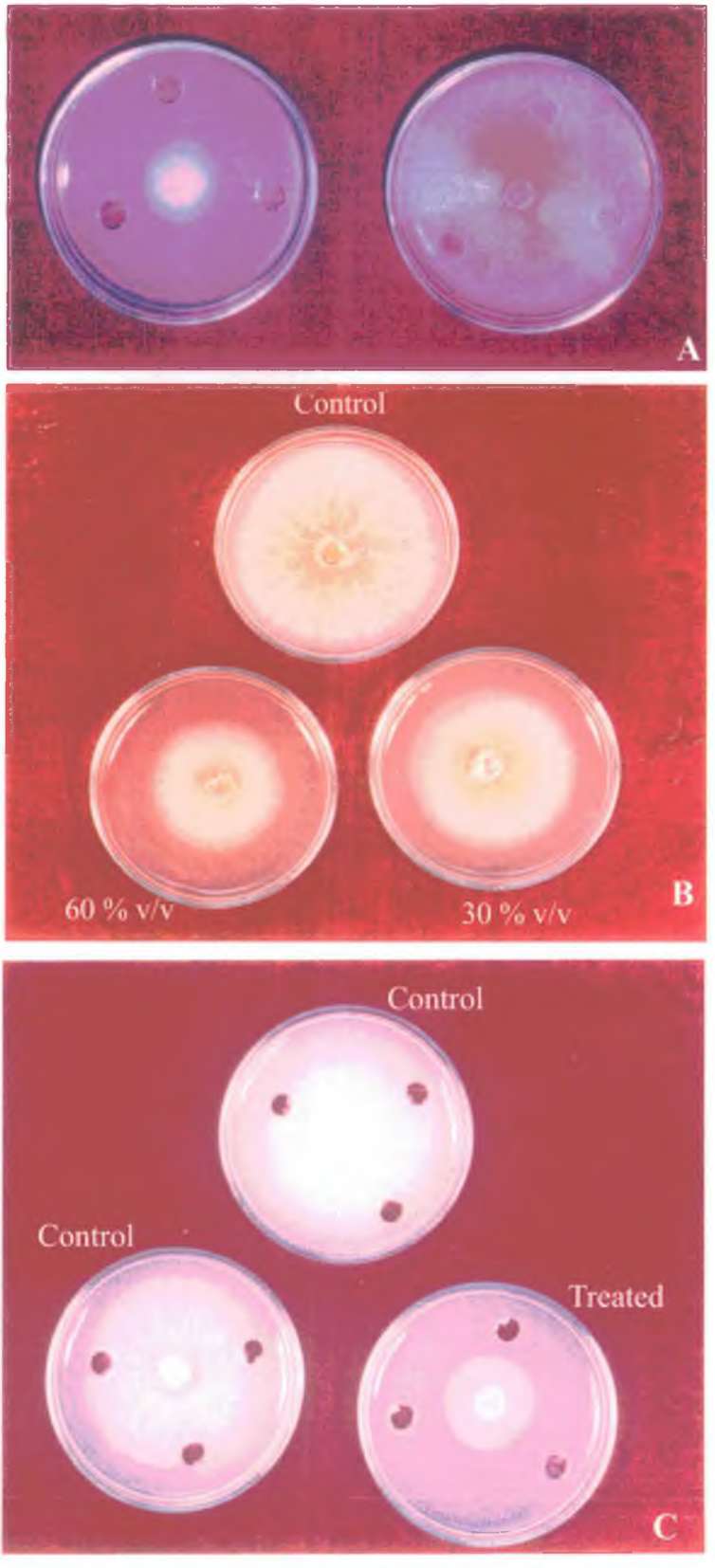


Plate X (A-C): A: Bioassay of diethyl ether fraction; B: different percentage of culture filtrate and C: active principle of *O. anthropi* against *F. lamaoensis*

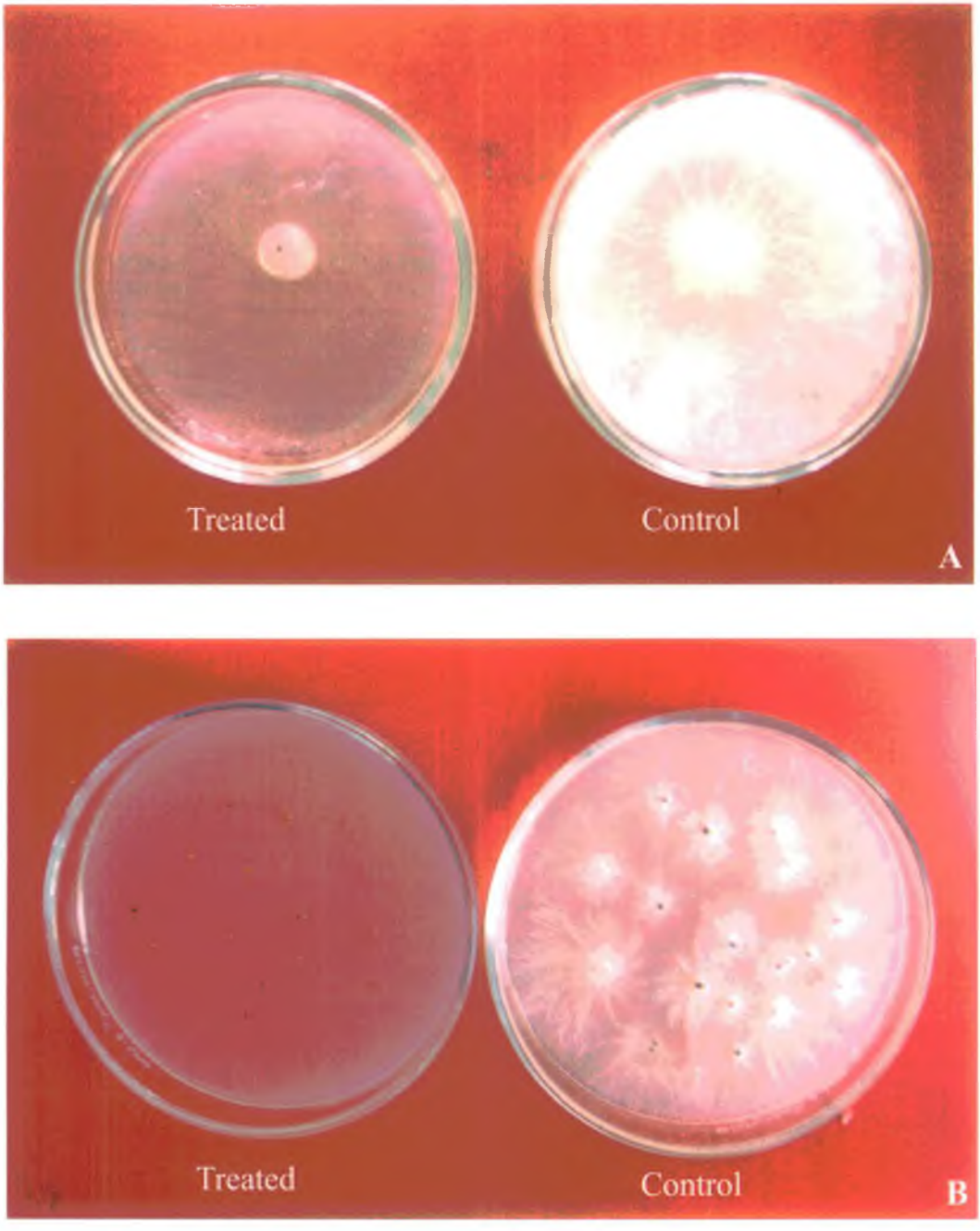


Plate XI (A-B): Bioassay of active principle from *B. megaterium* against *S. rolfsii* I (A) and against sclerotia of *S. rolfsii* I (B)

4.6. Partial characterization of active principles from *B. megaterium* and *O. anthropi*

4.6.1. UV-Spectrophotometry

UV-spectrophotometric analysis showed that antifungal compound from *B. megaterium* and *O. anthropi* cells showed the maximum absorbance at 236 nm and 230 nm respectively.

4.6.2. HPLC

HPLC analysis of the sample was done as described in materials and methods. Detection was done at 236 nm and 230 nm for *B. megaterium* and *O. anthropi* respectively. One peak with a retention time of 2.5 min. was obtained (Fig. 5) for both the bacterial isolates.

4.7. Effect of application of bacteria on growth of plants

4.7.1. Tea

4.7.1.1.Seedlings

Single Application

The growth promotion of different varieties of tea seedlings was observed in terms of increase in height of seedlings, number of shoots and number of leaves. It was observed that treatment with both the bacteria increased the rate of growth of the seedlings in relation to untreated control (Plate XII). Percentage increase in height of plants as well as number of leaves after two and four months of application of bacteria to the soil were calculated (Table 10). The result showed that both *B. megaterium* and *O. anthropi* efficiently promoted growth in tea plants irrespective of their variety.

Joint application

Since both the selected bacterial strains showed plant growth promoting activity, it was decided to co-inoculate the bacteria to determine if they show any synergistic growth promoting activity. Accordingly, bacteria grown in broth were

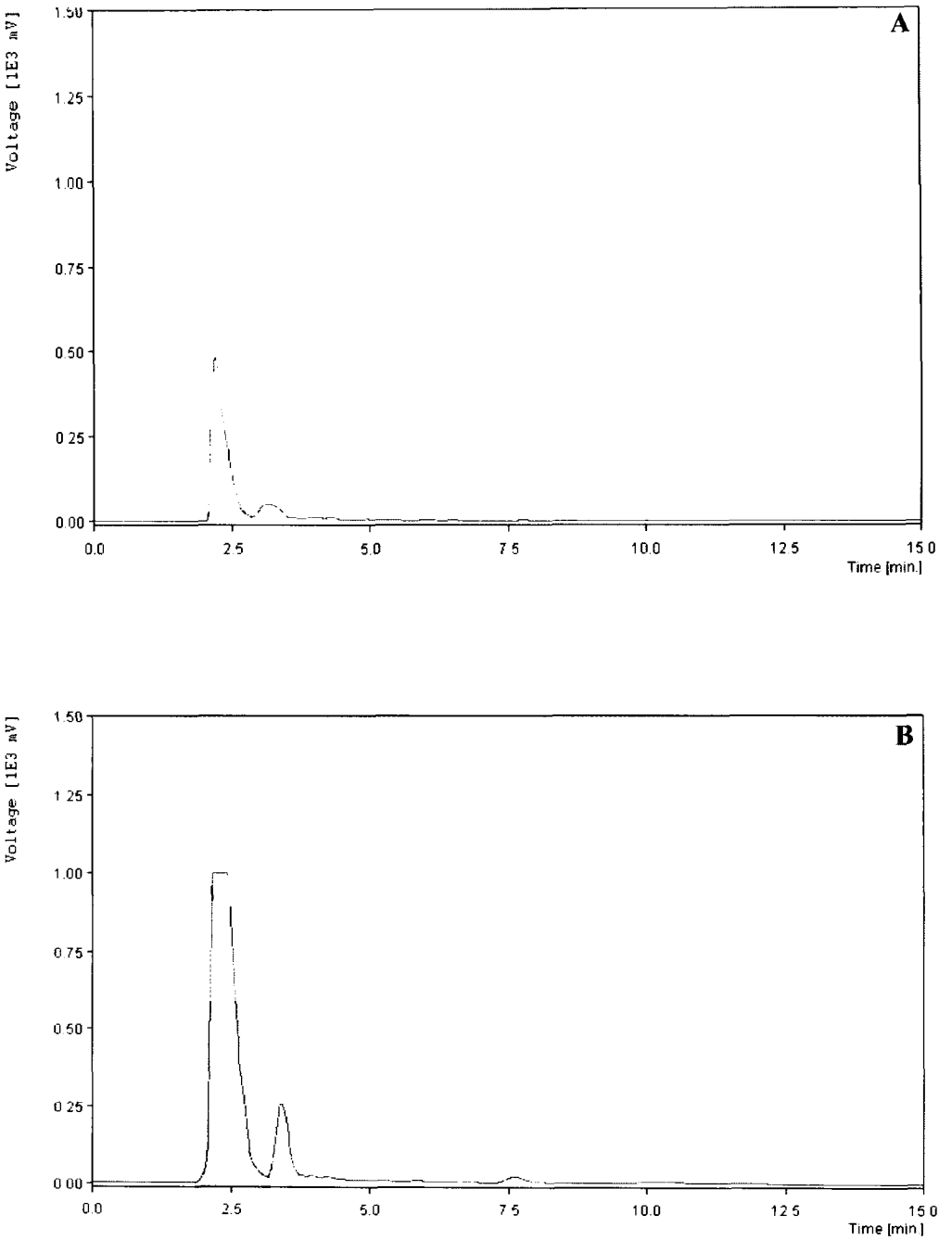


Fig. 5: HPLC analysis of antifungal extracts from bacterial lawn.
A: *B. megaterium* B: *O. anthropi*

Plate XII : Growth promotion of different varieties of tea seedlings following Inoculation with *B. megaterium* and *O. anthropi*

applied individually as well as in different combinations to the young seedlings of four varieties of tea (T 17, K1/1, TV 18 and UP 26). The growth of the seedlings was noted in each case and increase in growth rate was computed. It was observed that in relation to control, tea plants subjected to all treatments showed increased growth rate. It was further observed that percentage increase in height and number of leaves was greater when two bacteria were applied together in comparison to individual application (Table 11 and Fig. 6). The growth was 2-5 folds higher than in control plants (Plate XIII).

Table 10: Effect of rhizobacteria on growth of tea seedlings

Tea varie	Treatment	2 months after treatment		4 months after treatment	
		% increase in height	% increase in no. of leaves	% increase in height	% increase in no. of leaves
CP 1	Control	6.6 ± 1.20	25 ± 1 .71	26.6 ± 1.21	75.0 ± 1.95
	O. anthropi	56.6 ± 1.32	100 ± 6.01	70.6 ± 2.46	200.0 ± 1.00
	B. megaterium	60.4 ± 1.85	120 ± 1.97	67.5 ± 1.31	180.0 ± 1.61
TV 20	Control	8.6 ± 1.02	50 ± 1.92	15.7 ± 2.33	51.0 ± 2.05
	O. anthropi	57.9 ± 1.99	100 ± 1.64	103.5 ± 7.59	183.3 ± 1.93
	B. megaterium	63.5 ± 1.54	125 ± 1.64	126.9 ± 1.55	225.0 ± 3.32
UP 26	Control	12.0 ± 0.29	21.0 ± 2.31	25.3 ± 1.39	60.0 ± 2.31
	O. anthropi	45.2 ± 1.32	38.0 ± 1.73	98.5 ± 1.33	158.0 ± 1.16
	B. megaterium	51.3 ± 1.04	31.0 ± 1.16	104.2 ± 1.91	128.0 ± 5.20
T 17	Control	10.4 ± 0.87	14.3 ±1.46	23.2 ± 1.56	52.0 ± 1.73
	O. anthropi	58.0 ± 0.88*	33.3 ± 0.88	110.2 ± 0.92	127.3 ± 1.73
	B. megaterium	60.0 ± 0.81	42.9 ± 1.21	121.3 ± 0.92	138.6 ± 1.68
K1/1	Control	13.0 ± 1.16	20.0 ± 1.73	26.5 ± 0.75	60.5 ± 2.02
	O. anthropi	25.0 ± 1.73	60.0 ± 1.16	86.6 ±1.91	190.0 ± 2.61
	B. megaterium	30.0 ± 2.31	50.0 ± 2.31	90.6 ± 2.72	187.0 ± 5.51

Ten plants per treatment; * Difference of all tests with control significant at P= 0.05 rest significant at P= 0.01 as tested by Student's 't' test.

Plate XIII (A-B): Growth promotion in tea seedlings following application of PGPR isolates

1: *O. anthropi* 2: *O. anthropi* + *B. megaterium* 3: *B. megaterium* treated and 4: Control plants

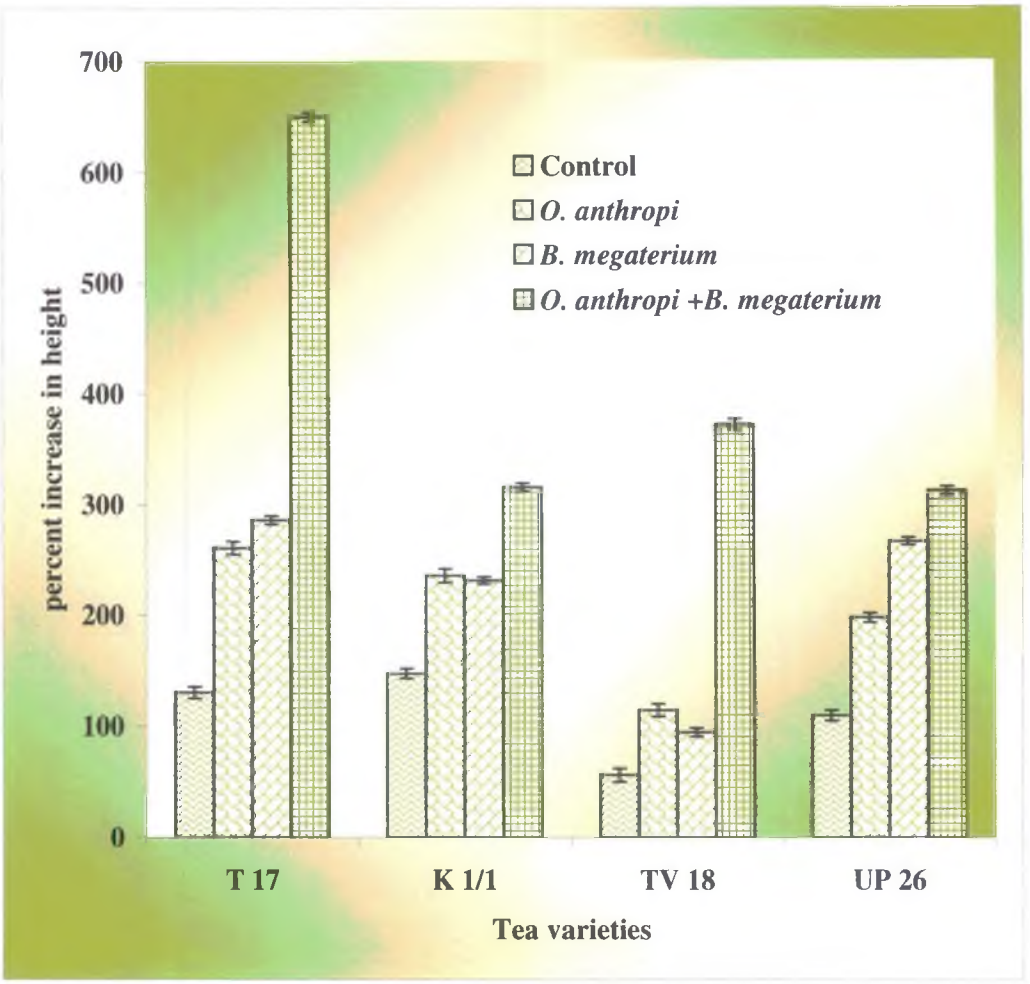


Fig. 6: Effect of joint application of *B. megaterium* and *O. anthropi* on growth of tea seedlings

4.7.1.2. Two year old plants

When *B. megaterium* and *O. anthropi* were applied to the rhizosphere of two year old plants in potted conditions at a regular interval of 20-25 days and growth promotion by individual bacteria was compared to that of the untreated control plants under same environmental and physical conditions, it was observed that in all six varieties bacterial application led to significant increase in height of plants, as well as number of lateral branches, leaves and leaf area index (Plate XIV, Table 12 and Figs. 7 & 8).

4.7.1.3. Ten year old bushes

In order to confirm the ability of bacteria to promote growth, aqueous suspensions of these bacteria were made and sprayed on tea bushes after pruning. It was observed that spraying with the suspension led to significantly better growth of the shoots. More luxuriant growth was obtained. Canopy growth was greater and more spread out, indicating the appearance of more shoots and leaves (Plate XV). It was also observed that certain infections which normally occur in natural conditions were delayed following spraying with the bacterial suspension.

Table 11: Effect of bacterial consortia on the growth of tea seedlings

Treatment	% increment in shoot length over initial *			
	T 17	K 1/1	TV 18	UP 26
Control	130.0 ± 2.65	147.2 ± 1.05	56.0 ± 1.16	109.1 ± 2.08
O. anthropi	260.5 ± 1.26	235.5 ± 0.76	114.0 ± 2.09	197.4 ± 0.46
B. megaterium	286.8 ± 1.42	231.2 ± 2.05	94.1 ± 2.37	266.6 ± 2.43
O. anthropi + *B. megaterium*	650.0 ± 5.06	315.8 ± 0.71	372.0 ± 2.08	312.7 ± 1.85

*Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded 10 months following the bacterial inoculation. Difference of all tests with control significant at P= 0.01 as tested by Student's 't' test

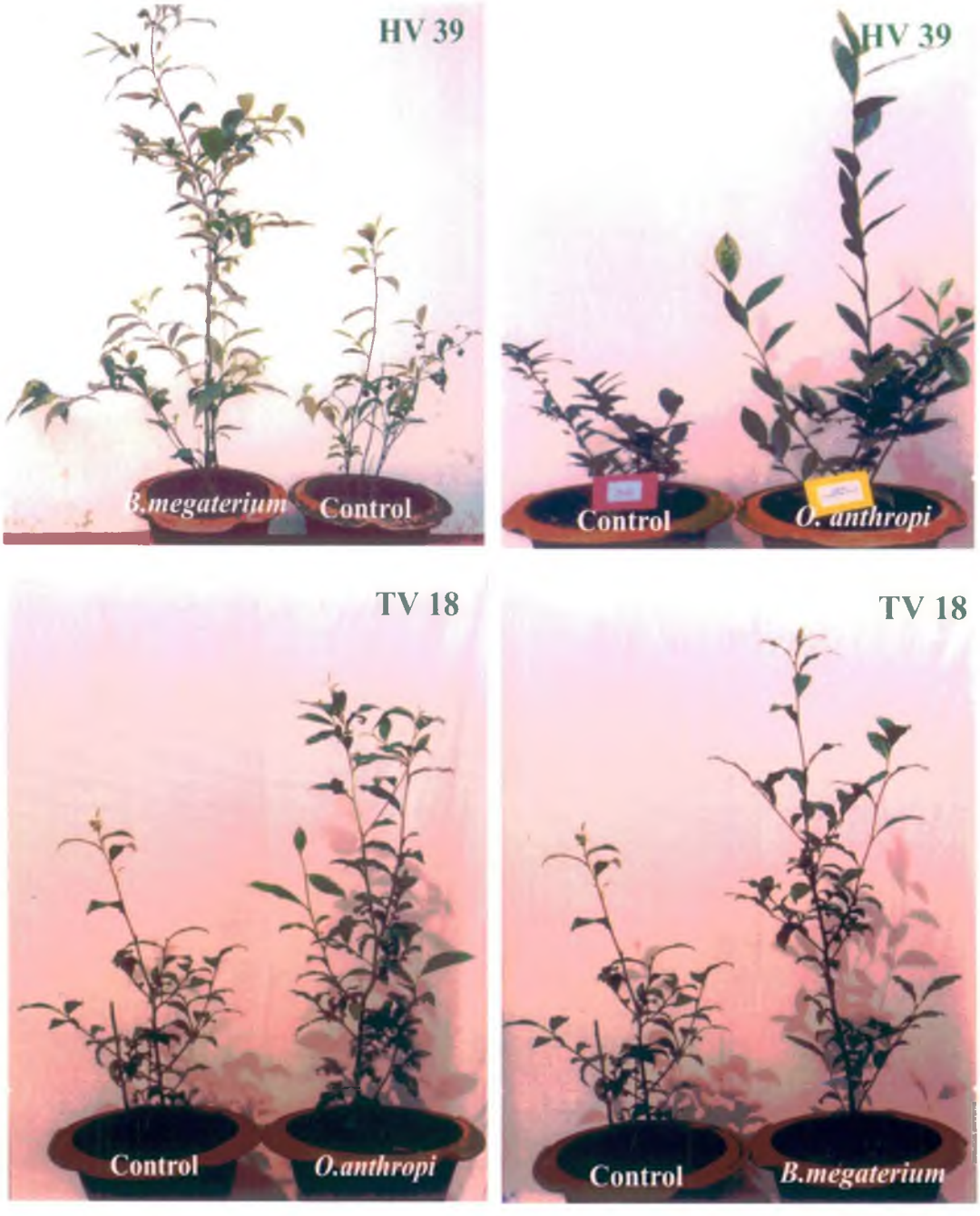
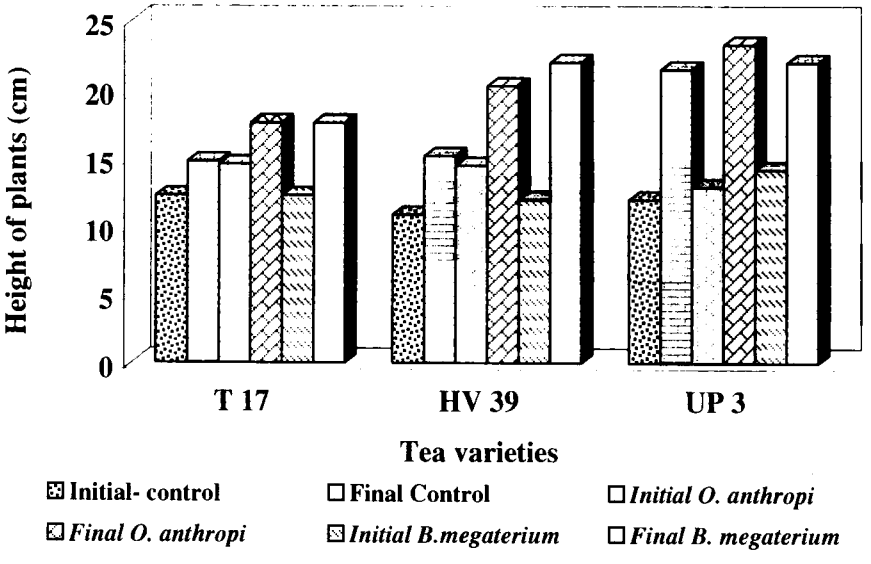


Plate XIV : Growth promotion of two year old tea plants following treatment with *B. megaterium* and *O. anthropi*



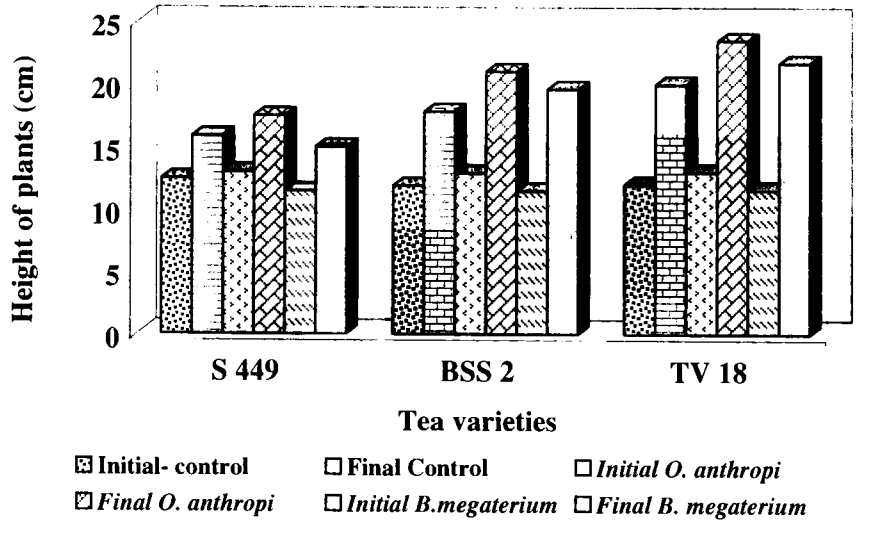


Fig. 7: Effect of *B. megaterium* and *O. anthropi* on shoot height of 2 year old plants of tea.

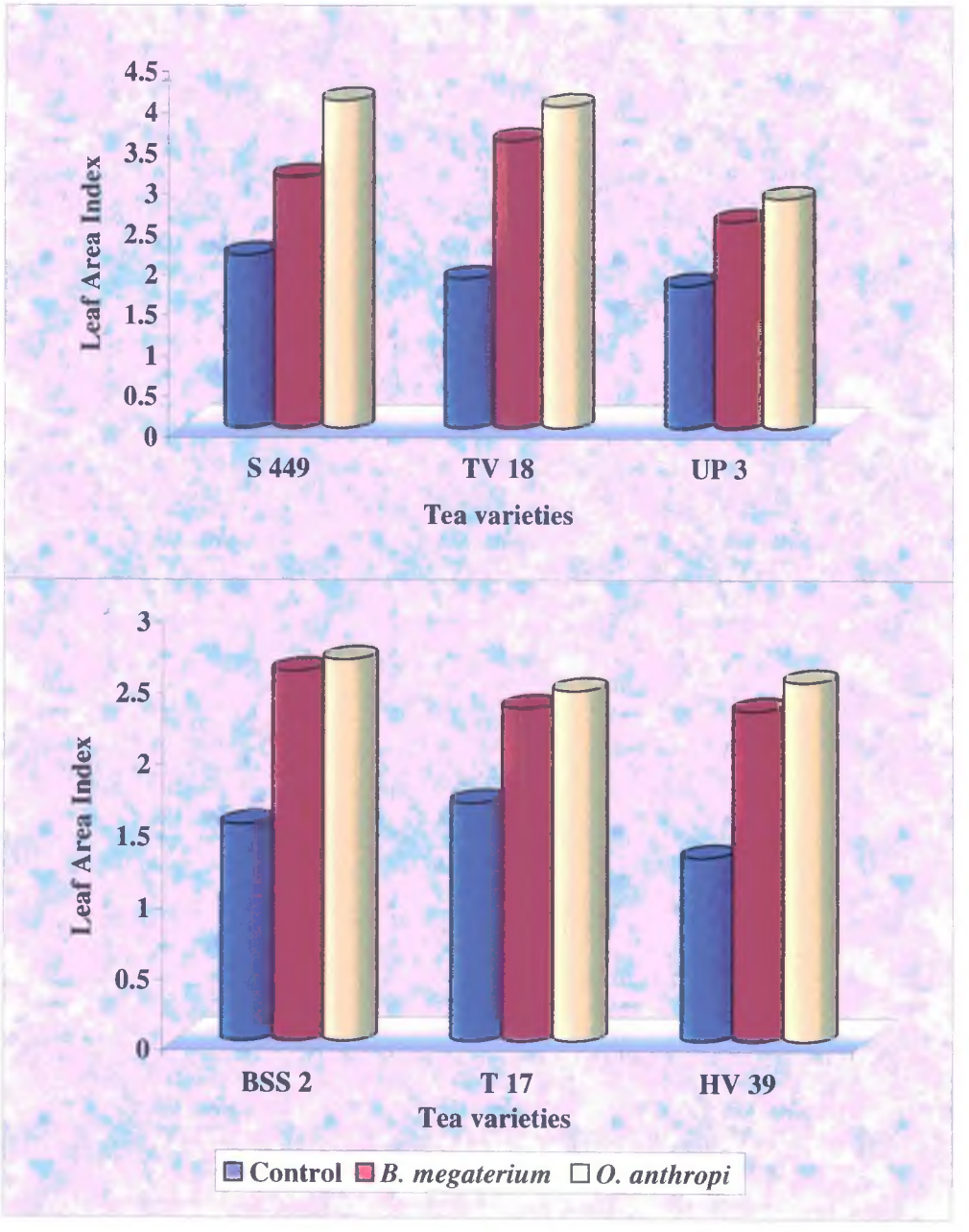


Fig. 8: Effect of *B. megaterium* and *O. anthropi* on Leaf Area Index. of tea plants.

Plate XV (A-D): Foliar application of *B. megaterium* and *O. anthropi* after pruning in tea bushes of BSS 2

A: After pruning at the time of spray; B-D : 2 months after spraying.

B: Control. C & D: PGPR sprayed

Table 12: Growth promotion of 2 year old tea plants after treatment with bacterial isolates

Tea variety	Treatment	Percent increase in No. of lateral branches	Percent increase No. of leaves
T-17	Control	28.4 ± 2.5	12.8 ± 1.4
	O. anthropi	48.0 ± 5.7[a]	26.1 ± 1.8[a]
	B. megaterium	41.4 ± 3.3[b]	28.5 ± 0.4
HV-39	Control	12.2 ± 0.9	20.5 ±3.9
	O. anthropi	21.3 ± 3.7[a]	32.9 ± 2.0[b]
	B. megaterium	51.7 ± 5.2[a]	43.5 ± 0.9[a]
UP-3	Control	10.7 ± 6.4	11.6 ± 1.6
	O. anthropi	52.7 ± 0.32[a]	30.8 ± 7.7[b]
	B. megaterium	33.7 ± 6.2[b]	27.6 ± 2.1[b]
S 449	Control	15.7 ± 1.1	26.9 ± 3.4
	O. anthropi	42.5 ± 9.8	43.4 ± 1.9[b]
	B. megaterium	33.0 ± 4.4[b]	40.6 ± 0.69[a]
BSS 2	Control	18.9 ± 3.2	23.6 ± 1.4
	O. anthropi	39.4 ± 3.2[a]	38.7 ± 2.7[a]
	B. megaterium	42.7 ± 5.7[a]	48.8 ± 1.5[b]
TV 18	Control	31.7 ± 9.3	33.2 ± 7.3
	O. anthropi	46.3 ± 11.8[b]	46.9 ± 7.8[b]
	B. megaterium	42.9 ± 3.7[a]	46.9 ± 7.8[b]

Ten plants per treatment; ± = Standard error; [a] Difference with control significant at P= 0.05; [b] insignificant and the rest significant at P=0.01, done by Student's 't' test

4.7.2. Chickpea

Plant growth activities of the selected bacteria were also tested in a different crop – chickpea which is an annual plant, while tea is a perennial. For this purpose, two varieties of chickpea seeds were bacterized and sown. The germination percentage and vigour index of the plants were calculated.

Both the bacterial isolates enhanced the germination percentage and vigour index significantly as compared to non-inoculated control seeds

(Tables 13 & 14). Overall growth of the plants was also greatly increased in the field by application of the bacteria (Plate XVI). Further the total yield was found to be almost 32-40 % more in PGPR treated plants in comparison to untreated control plants after harvesting. Bacterization of seeds also resulted in increased biomass as compared to control.

Table 13: Effects of *B. megaterium* and *O. anthropi* on germination of chickpea seeds.

Day	Percentage of germination**					
	(ICC V2)			(ICC C37)		
	Control	*B.megaterium* treated	*O.anthropi* treated	Control	*B.megaterium* treated	*O.anthropi* treated
1	44±4.37	94±1.67*	56±2.89	51±2.31	91±1.16	63±3.47
2	61±1.73	94±2.31	75±1.73	80±1.73	91±1.16*	91±2.31*
3	71±4.62	100±0.00*	88±2.31	83±1.16	94±1.73*	95±1.73*

*= Difference with control significant at P= 0.05, rest significant at P= 0.01 as done by Student's 't' test; **Average of 50 seeds; ± = Standard error

Table 14: Effect of seed bacterization with rhizobacteria on Vigour Index of seedlings

Treatment	% Germination	Mean Root + mean shoot Length (cm)	Vigour Index*
***C. arietinum* ICC V_2**			
Control	71 ± 4.62	11.0	781.0
O. anthropi	88 ± 2.31	20.5	1804.0
B. megaterium	100 ± 0.00	20.0	2000.0
***C. arietinum* ICC C37**			
Control	83 ± 1.16	10.5	871.5
O. anthropi	94 ± 1.73	25.0	2350.0
B. megaterium	95 ± 1.73	32.5	3087.0

Results show the mean of 10 plants; Difference with control significant at P= 0.01 as done by Student's 't' test

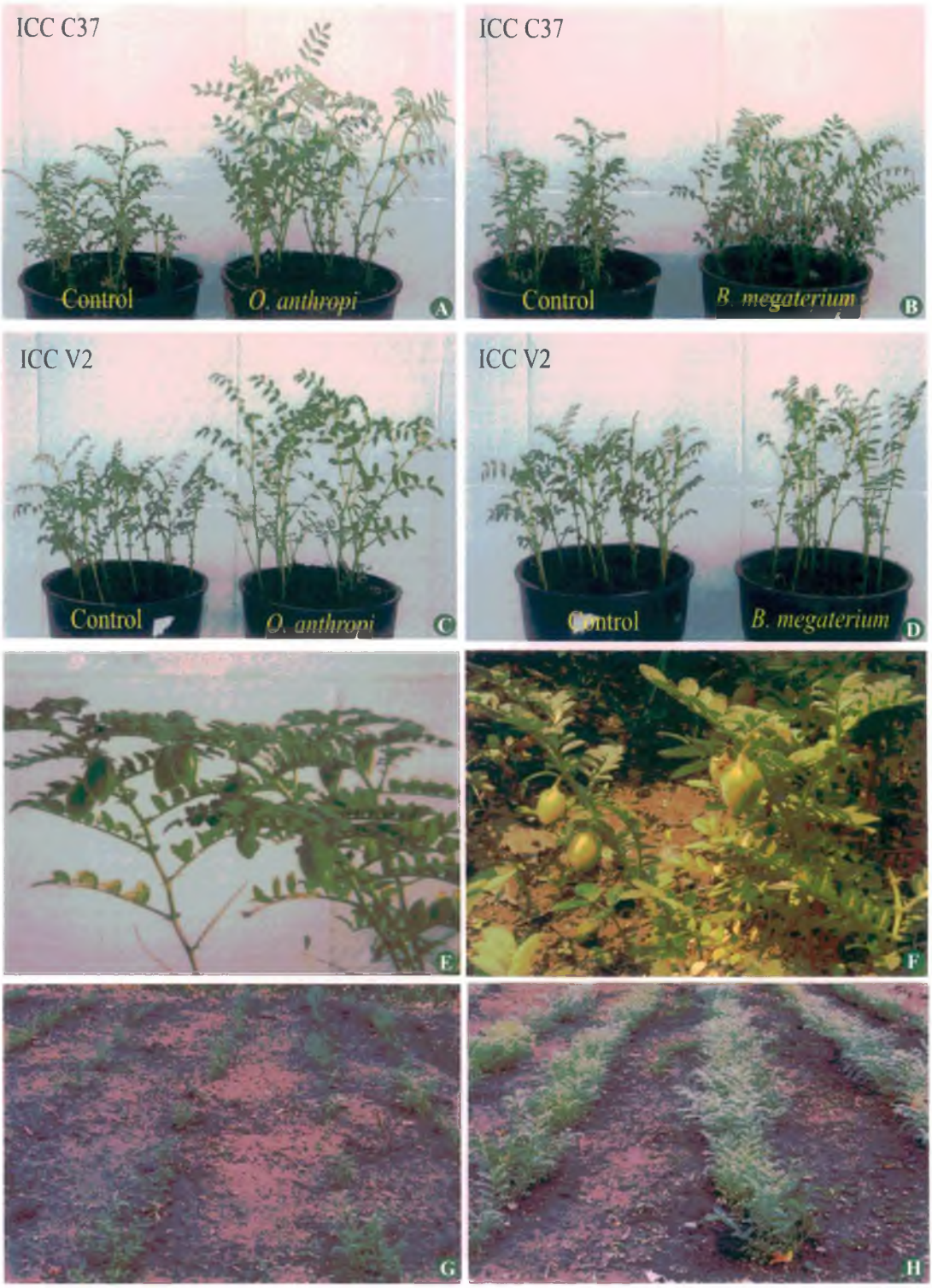


Plate XVI (A-H): Growth promotion **of** chick pea by *B. megaterium* and *O. Anthropi* A-D: Different genotypes of potted plants. E&F: Close up view of fruiting plants treated with *O. anthropi* (E) and *B. megaterium* (F) G&H: Field grown chickpea plants. Control (G) and *B. megaterium* treated (H)

4.8. Biochemical changes in tea leaves induced by PGPRs

Experiments were also conducted to assess the effect of the different bacteria on the biochemical components of tea plants. Activities of some of the enzymes which are involved in phenol metabolism as well as in defense- i.e., peroxidase, phenylalanine ammonia lyase, chitinase and β-1,3-glucanase were changed. Polyphenols are major constituents of tea leaves and hence the effect of bacteria on the enzyme polyphenol oidase (PPO) was also determined. Besides, major biochemical components of tea leaves such as protein and chlorophylls were also analysed. Catechins, the flavonoid flavour component of tea leaves are extremely important and changes in these components by the application of PGPRs were also analysed by HPLC.

4.8.1. Enzymes

Activities of the different enzymes were analyzed in the six tea varieties, as described under materials and methods.

4.8.1.1. β-1,3-glucanase

Constitutive β–1,3-glucanase activity was observed in all the six varieties of plants. However, there was increase in the activity of β–1,3-glucanase in bacteria treated plants and activity was lower in untreated control plants (Table 15). In most of the varieties *O. anthropi* induced enzyme activity was more than in *B. megaterium* induced.

4.8.1.2. Chitinase

When the chitinase activity was assayed after the application of PGPR in the rhizosphere of tea plants, the increase in the activity of chitinase enzyme was observed in all the six varieties. *B. megaterium* elicited highest production of chitinase in TV 18 and *O. anthropi* in BSS 2 (Table 15). In all the varieties, induction of chitinase was higher by *B. megaterium* than by *O. anthropi*.

4.8.1.3. Phenylalanine ammonia lyase

Application of *B. megaterium* and *O. anthropi* significantly increased the PAL enzyme activity in all the six varieties of tea in comparison to control plants (Table 15).

Table 15: Enzyme activities in tea leaves of different varieties following application of *B. megaterium* and *O. anthropi* in the rhizosphere

Varieties	Treatment	Enzyme activities			
		PO[a]	PAL[b]	CHT[c]	GLU[d]
T-17	Control	3.7±0.2	65±0.4	12.8±1.1	448±3.9
	B. megaterium	5.2±0.3	95±0.7	19.4±1.0	544±3.8
	O. anthropi	5.9±0.2	100±0.9	17.8± 2.1	496±3.2
HV-39	Control	1.7±0.1	96±0.2	14.4±0.6	336±2.0
	B. megaterium	2.3±0.3	135±0.6	24.4±1.8	464±3.7
	O. anthropi	3.2±1.2	115±1.2	18.3±1.1	512±1.9
UP-3	Control	5.3±0.1	59±0.4	18.3±1.7	464±4.1
	B. megaterium	7.8±0.3	73±0.5	24.4±2.3	496±3.9
	O. anthropi	7.5±0.5	85±0.7	21.1±2.0	482±2.9
TV 18	Control	4.3±0.4	65±1.7	18.1±1.3	768±1.0
	B. megaterium	5.7±0.3	85±1.4	32.8±2.1	928±0.9
	O. anthropi	6.3±0.6	80±2.0	20.0±1.9	896±1.3
S 449	Control	4.5±0.3	85±1.4	13.3±1.2	368±1.8
	B. megaterium	7.5±0.6	105±1.9	25.5±1.5	464±2.4
	O. anthropi	7.0±0.5	185±2.1	20.0±1.9	496±2.1
BSS 2	Control	3.9±0.2	65±1.9	18.3±1.2	416±2.1
	B. megaterium	6.2±0.3	95±2.8	29.4±1.9	480±1.3
	O. anthropi	5.8±0.5	110±2.3	32.2±1.8	544±1.9

a- PO activity assayed as Δ A_{465} $min^{-1}g$ $tissue^{-1}$; b- PAL activity assayed as µg cinnamic acid produced by enzyme from 1 g tissue min^{-1}; c- CHT activity assayed as µg N-Acetyl glucosamine released by enzyme from 1 g tissue $min^{-1}$and d- β1,3-GLU activity assayed as µg glucose released by enzyme from 1 g tissue min^{-1}

4.8.1.4. Peroxidase

PO activities were assayed from control and bacteria inoculated tea leaves.

Application of bacterial suspension in the rhizosphere of tea plants resulted in an increase in peroxidase activity in treated plants compared to untreated control plants (Table 15).

4.8.1.4.1. Isozyme analysis of peroxidase

Native PAGE analysis of PO showed the existence of five isoforms in all the varieties of tea with R_m values of 0.13, 0.31, 0.38, 0.56 and 0.66. 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 (Plate XVII A). Maximum intensity of bands was noticed in the treated plants.

4.8.1.5. Polyphenol oxidase

PPO is an enzyme involved in the phenol metabolism. PPO activity increased significantly on application of *O. anthropi* and *B. megaterium* in the rhizosphere of the tea plants in comparison to untreated control plants (Fig. 9).

4.8.1.5.1. Isozyme analysis of polyphenol oxidase

Native PAGE analysis of PPO showed the existence of two isoforms in all the varieties of tea. All the isoforms were present in untreated control plants, however there was clear distinction between the treated and untreated control plants as far as intensities were considered (Plate XVII B). Maximum intensity of bands was noticed in the treated plants. PAGE analysis of the enzyme extracted from leaves showed isoforms having R_m 0.19 and 0.36.

4.8.2. Chlorophyll

The quantitative analysis revealed that the chlorophyll content in *B. megaterium* or *O. anthropi* treated tea plants increased significantly in comparison to untreated control plants but not very significantly (Table 16).

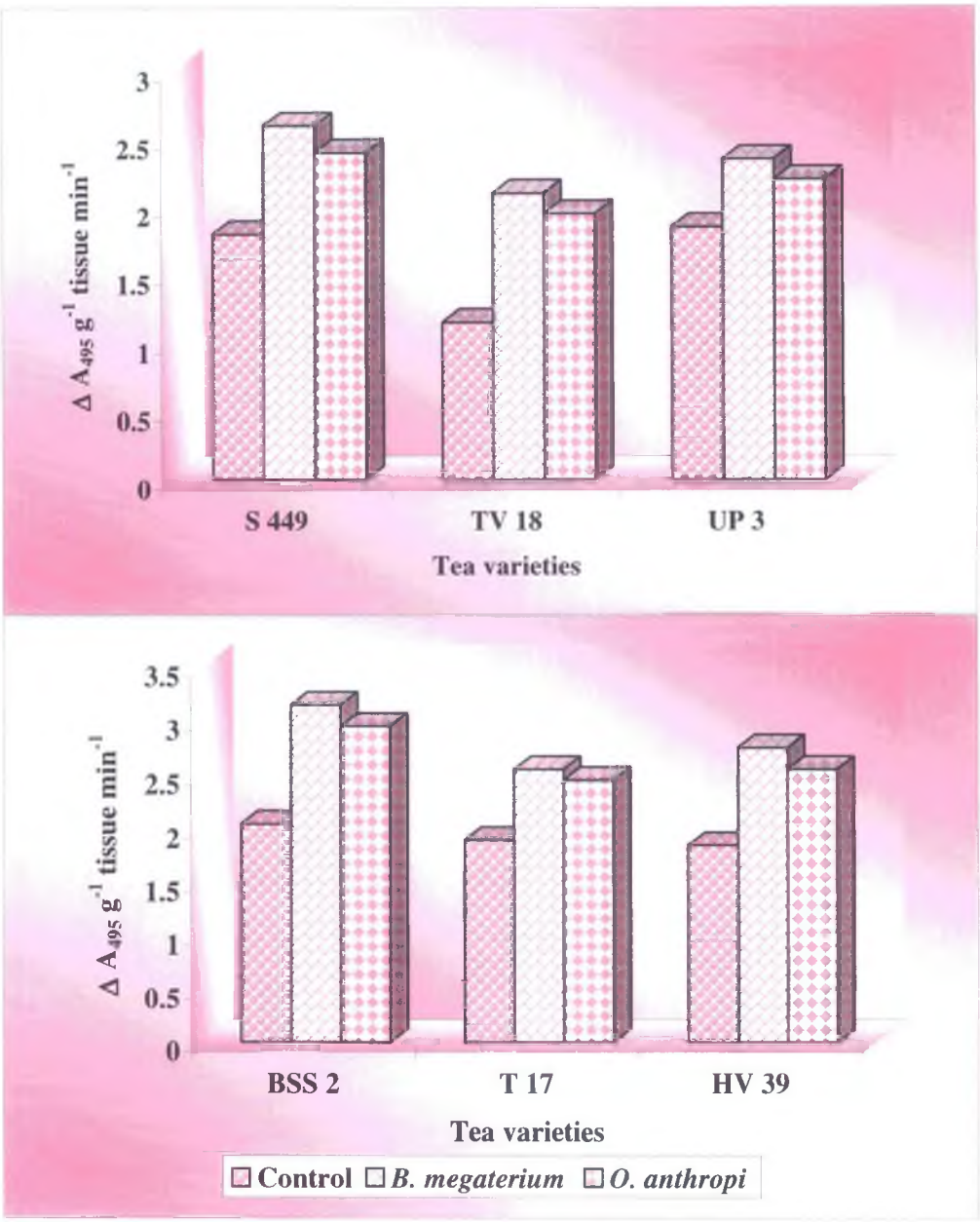


Fig. 9: Changes in polyphenol oxidase activity in tea leaves following inoculation with *B. megaterium* and *O. anthropi*

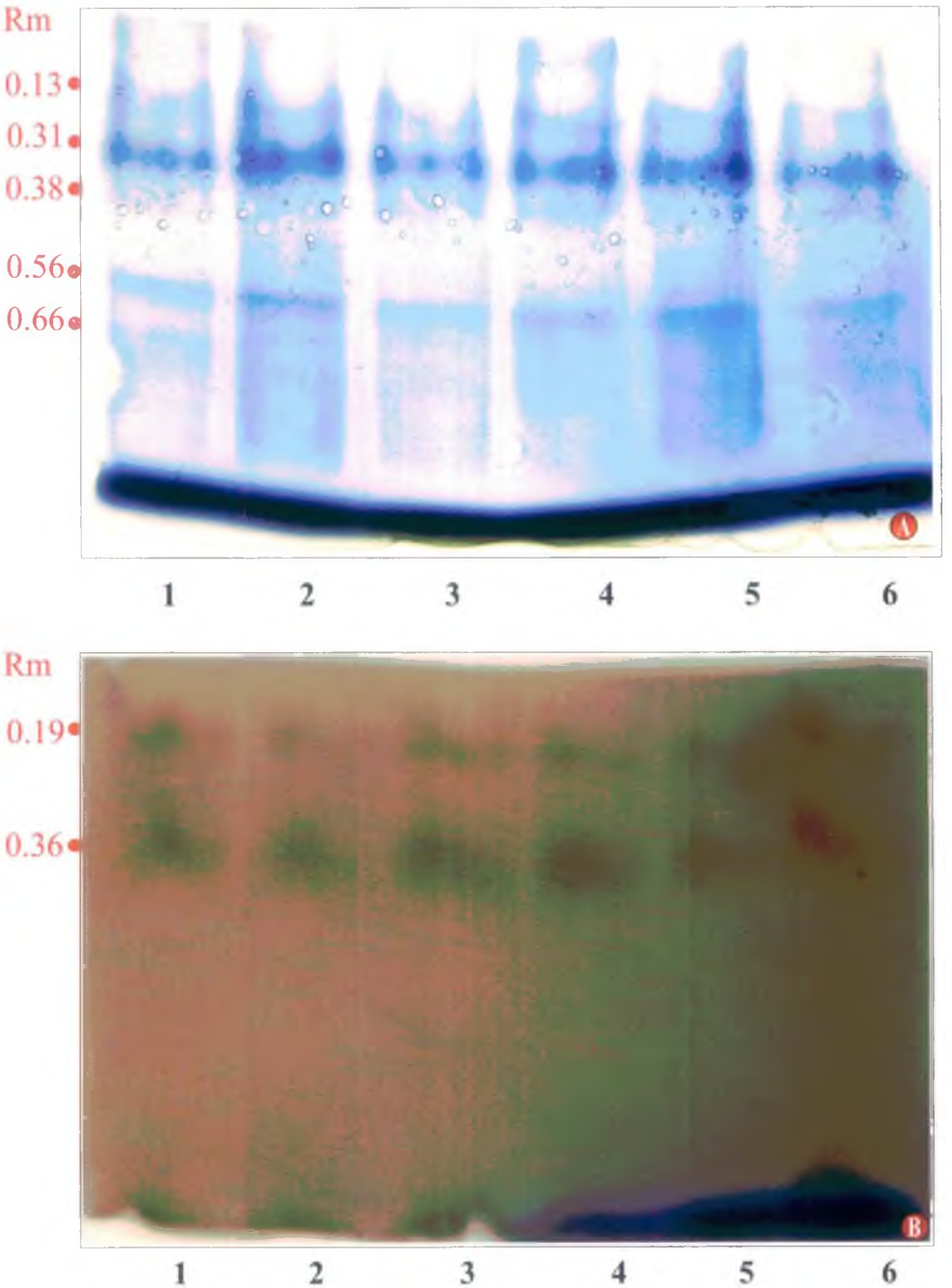


Plate XVII (A-B): Isozyme analysis of enzymes from tea plants treated with PGPR A:Peroxidase isoform profile; B: Polyphenol oxidase isoform profile of control plants (Lane 1 & 4); *B. megaterium* treated plants (Lane 2 & 5); *O. anthropi* treated plants (Lane 3 & 6)

4.8.3. Proteins

4.8.3.1.Protein content

Soluble protein extracted from control and bacteria treated plants from 6 different tea varieties were assayed. Results indicated that soil application of *B. megaterium* and *O. anthropi* resulted in significant increase in the protein content of the plant (Fig. 10)

Table 16: The effect of PGPRs on the chlorophyll contents of tea leaves.

Variety	Treatment	Chlorophyll content (mg g^{-1} tissue)		
		Total Chl.	Chl. a	Chl. b
HV 39	*Control*	1.493±0.06	0.582±0.02	0.923±0.08
	O. anthropi	1.591±0.16[b]	0.613±0.01[b]	0.978±0.15
	B. megaterium	1.572±0.10[b]	0.712±0.01[b]	0.860±0.10
T 17	*Control*	1.233±0.03	0.604±0.04	0.606±0.08
	O. anthropi	1.319±0.02[b]	0.687±0.04[b]	0.632±0.03[b]
	B. megaterium	1.986±0.06	0.807±0.01[b]	1.179±0.06
S 449	*Control*	0.820±0.03	0.395±0.01	0.425±0.02
	O. anthropi	1.242±0.03	0.592±0.05[b]	0.650±0.03
	B. megaterium	1.297±0.01	0.598±0.01	0.699±0.01[a]
BSS 2	*Control*	1.270±0.03	0.474±0.02	0.797±0.01
	O. anthropi	1.290±0.06[b]	0.456±0.01[b]	0.834±0.05
	B. megaterium	1.380±0.02[b]	0.494±0.01[b]	0.886±0.05[a]
UP 3	*Control*	0.838±0.05	0.385±0.07	0.565±0.05
	O. anthropi	1.227±0.05	0.469±0.02	0.758±0.01[a]
	B. megaterium	1.105±0.02	0.596±0.10[a]	0.709±0.04[b]
TV 18	*Control*	1.069±0.29	0.485±0.01	0.554±0.03
	O. anthropi	1.202±0.02[b]	0.509±0.02[b]	0.692±0.01[a]
	B. megaterium	1.532±0.02[a]	0.568±0.00[b]	0.854±0.18[b]

Ten plants per treatment; ± = Standard error; [a] Difference with control significant at P= 0.05; [b]insignificant and the rest significant at P=0.01, done by Student's 't' test

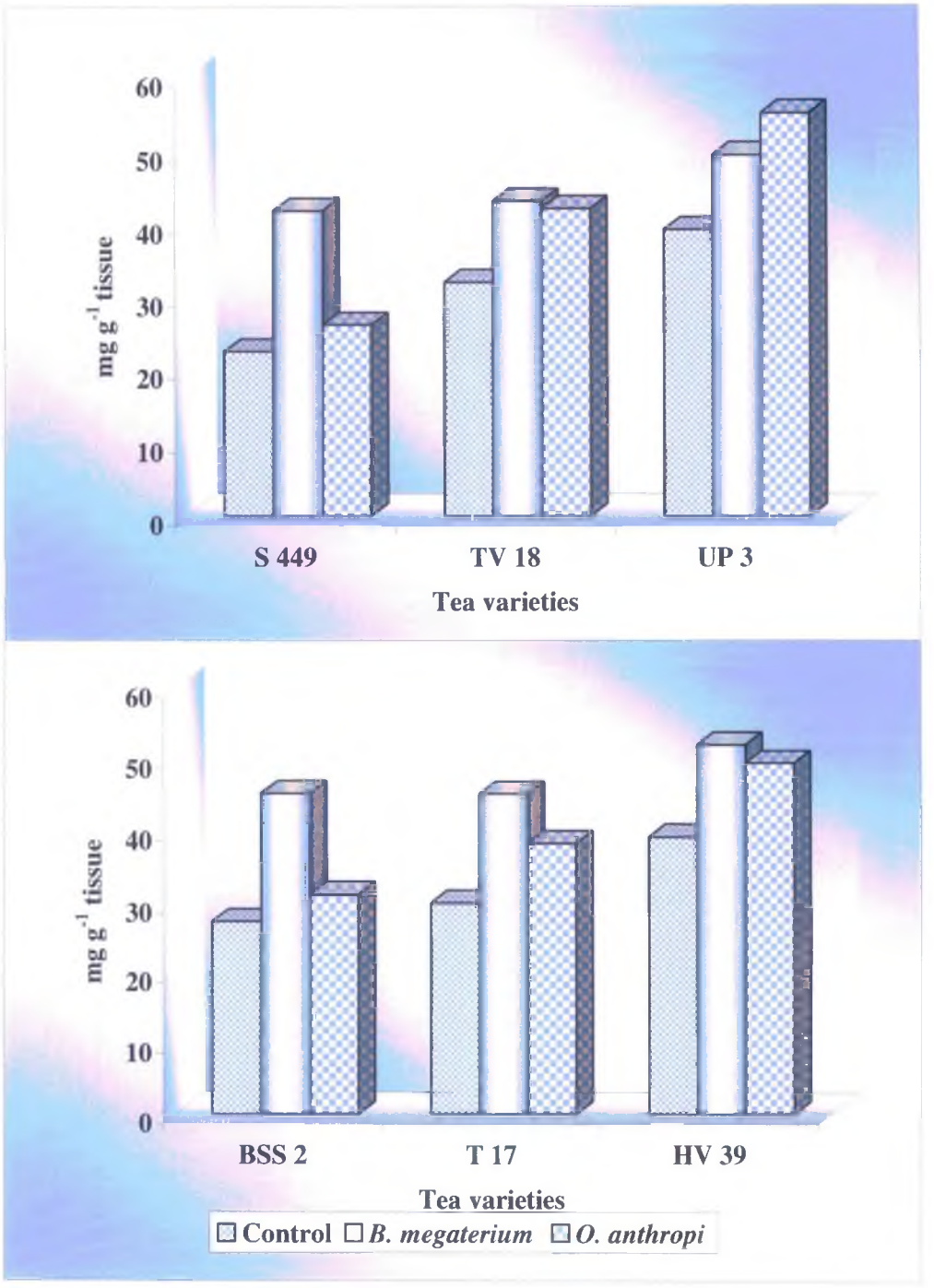


Fig. 10: Changes in protein content in tea following inoculation with *B. megaterium* and *O. anthropi*

4.8.3.1.1. Protein profile

The soluble proteins extracted from control and bacteria treated tea plants were analysed by SDS-PAGE. Upon electrophoretic comparison, proteins from control and treated plants revealed a number of protein bands on staining with coomassie brilliant blue having molecular masses of ca. 29.8, 37.5, 44.6, 47.3. 53.0, 63.0, 89.1, 98.0 and 112.25 KDa. Almost all the proteins detected as bands were constitutively present, however, the level of expressions of some proteins were higher in bacteria treated plants (Plate XVIII).

4.8.4. Phenols

4.8.4.1. Phenol content

Both the total and O-dihydroxy phenol contents of the tea leaves were significantly higher following treatment with *B. megaterium* or *O. anthropi* as compared to non-treated control plants in different varieties of tea (Fig. 11). *B. megaterium* was found to increase phenol contents more than *O. anthropi* in five out of six varieties tested.

4.8.4.2. Bioassay of antifungal phenolics

Since phenols are known to have some antifungal properties, in this study phenols were extracted from tea leaves and bioassayed for antifungal activity. Radial growth bioassay and TLC plate bioassay were conducted to ascertain the fungitoxicity of the antifungal phenolics fractions extracted from PGPR treated tea plants.

4.8.4.2.1. Radial growth

When the aliquots of different fractions and diethyl ether (as solvent control) were dispersed in the wells of petriplates for bioassay and *F. lamaoensis* being inoculated at the centre in equal distance from the wells and incubated for 8 days, the result showed that fraction I and III showed no inhibition. The fraction II from *B. megaterium* treated tea plants showed 30 % more inhibition and that

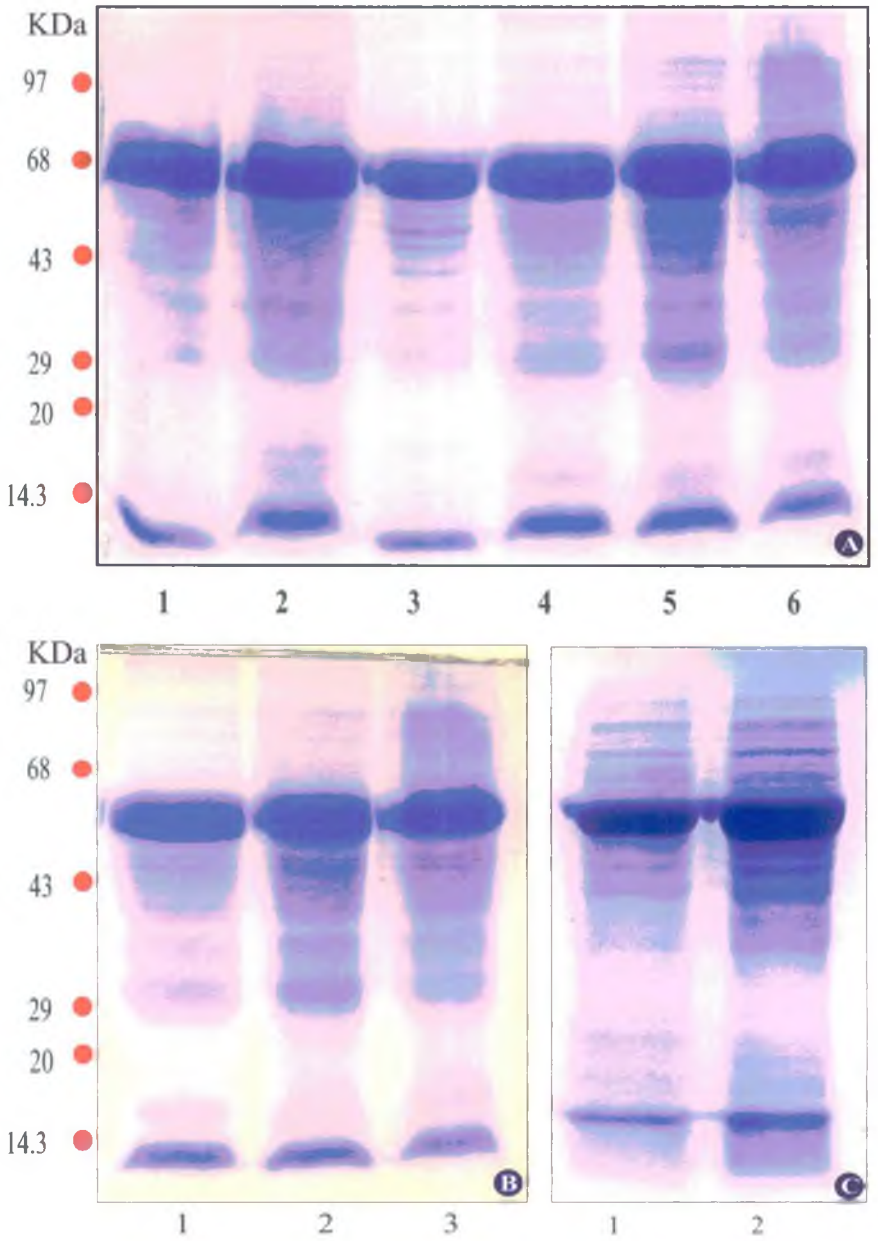


Plate XVIII (A-C): SDS PAGE analysis of proteins from tea leaves treated with PGPR isolates.
A: UP 3 (Lanes 1-3; Lanes 4-6: S449) : B: BSS 2; C: TV 18: Lanes 1 & 4: Control; Lanes 2 & 5 *B. megaterium* treated; Lanes 3 & 6: *O. anthropi* treated;

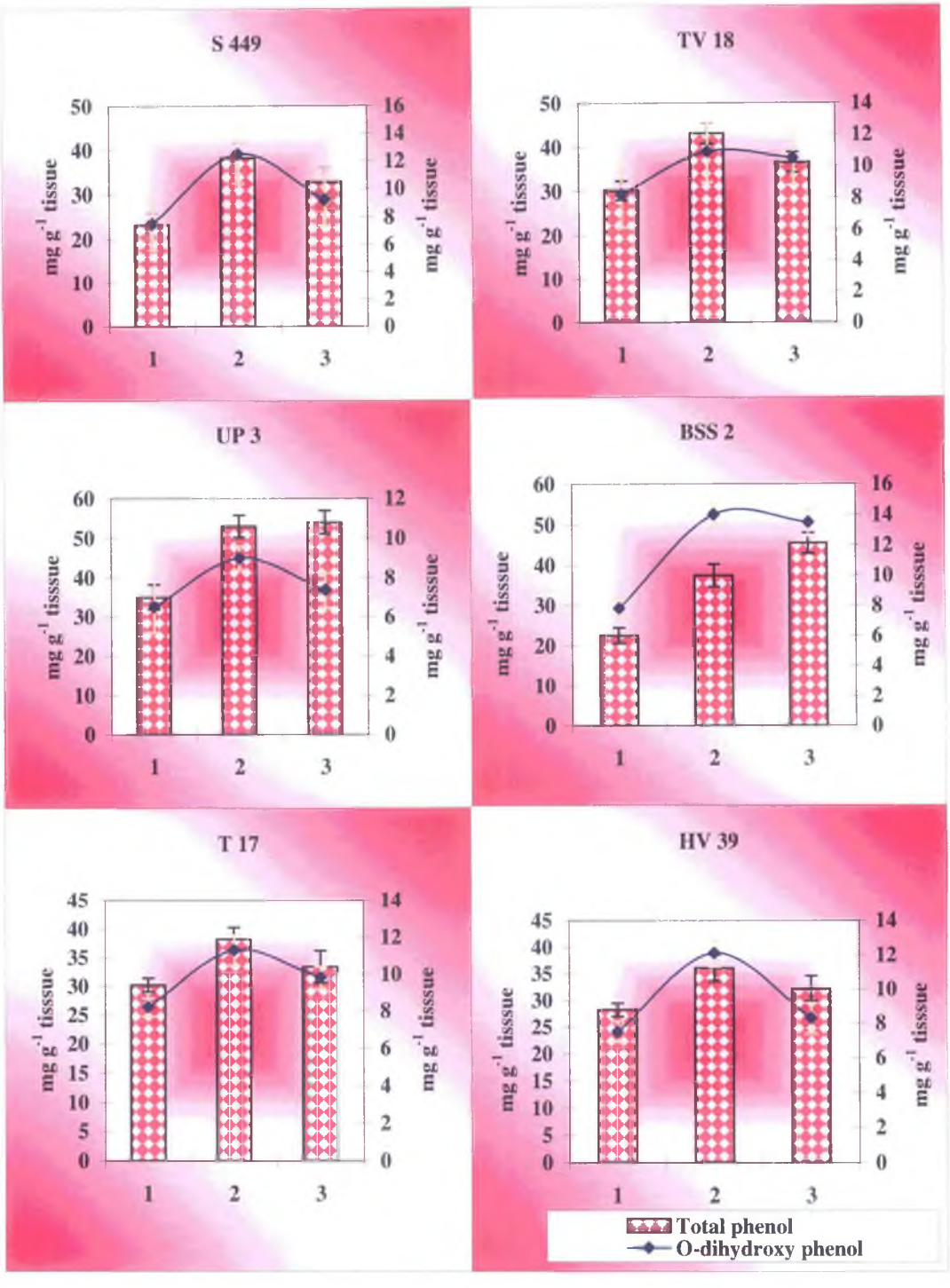


Fig. 11: Effect of *B. megaterium* and *O. anthropi* on the phenol content of tea leaves [1: Control; 2: *B. megaterium*; 3: *O. anthropi* treated].

from *O. anthropi* treated plants showed 40 % more inhibition compared to inhibition by fraction II from untreated control tea plants.

4.8.4.2.2. Thin Layer Chromatography

When all the fractions analysed by TLC on silica gel G, the inhibition zone of diameter 5.5 cm and 6 cm were observed for fraction II obtained from *B. megaterium* and *O. anthropi* treated plants whereas no inhibition was recorded from other fractions. The R_f value of both the inhibition zone was found to be 0.69.

4.9. Catechins

Catechins derived from leaves of the plants whose rhizosphere was soil drenched with bacteria were analysed in HPLC. Results revealed that there were no major changes in the peaks following bacterization but observed appearance of a few new peaks indicating isomers (Fig. 12 & 13; Table 17, 18).

4.10. Biochemical changes in tea leaves following foliar spray of bacteria

Foliar spraying of tea plant with *B. megaterium* or *O. anthropi* individually or in combination resulted in some quantitative biochemical changes within the plant as compared to control plants. Higher enzyme activities were recorded in plants sprayed with the PGPRs in combination than in individually sprayed plants (Table 19). Total protein content was significantly higher in plants when *B. megaterium* and *O. anthropi* were sprayed in combination than individually applied plants (Table 19). Similar observation was recorded when the phenol content of the treated plants was assayed (Table 20). The chlorophyll content was higher in plants treated with *B. megaterium* and *O. anthropi* in combination than in individually applied plants (Table 20).

Interestingly, when catechins were analysed following bacterial spray on the leaves, several new peaks appeared, especially when *O. anthropi* was sprayed. Result of analysis following joint spray was even more significant (Fig. 14 and Table 21).

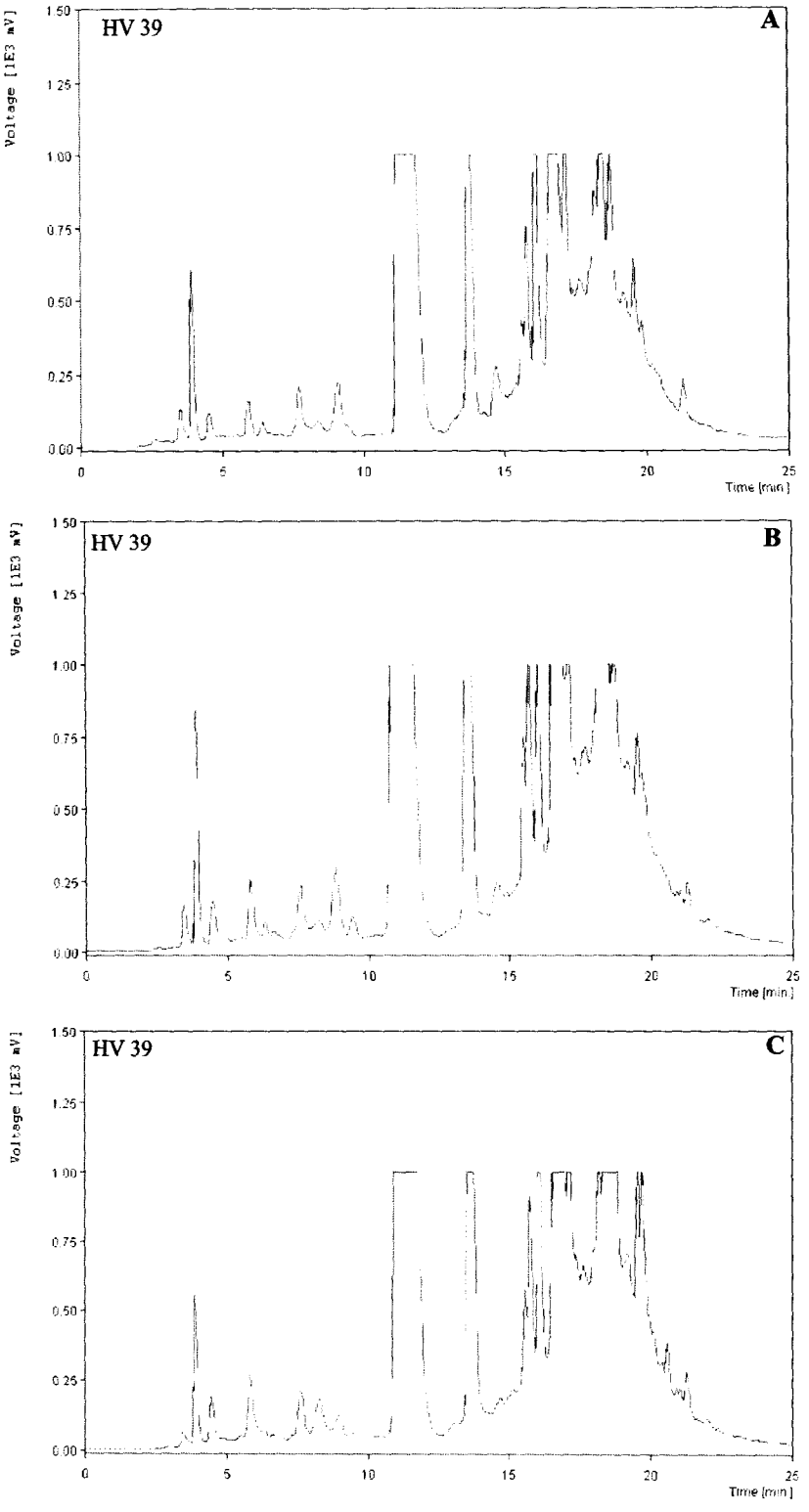


Fig. 12 : HPLC profile of catechins extracted from tea leaves treated with PGPRs

A: Control; B: *B. megaterium* treated; C: *O. anthropi* treated.

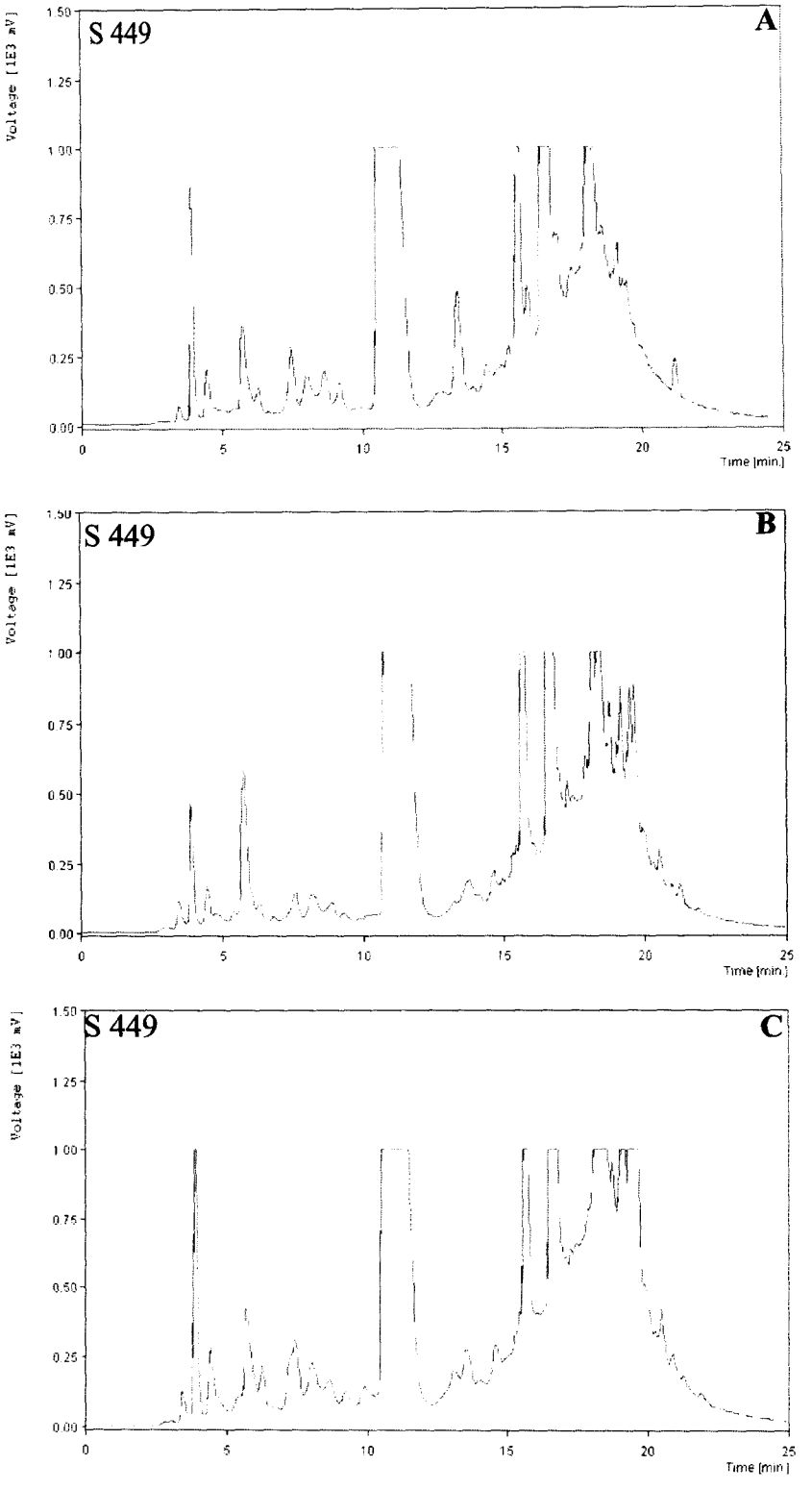


Fig. 13: HPLC profile of catechins extracted from tea leaves treated with PGPRs
A: Control; B: *B. megaterium* treated; C: *O. anthropi* treated.

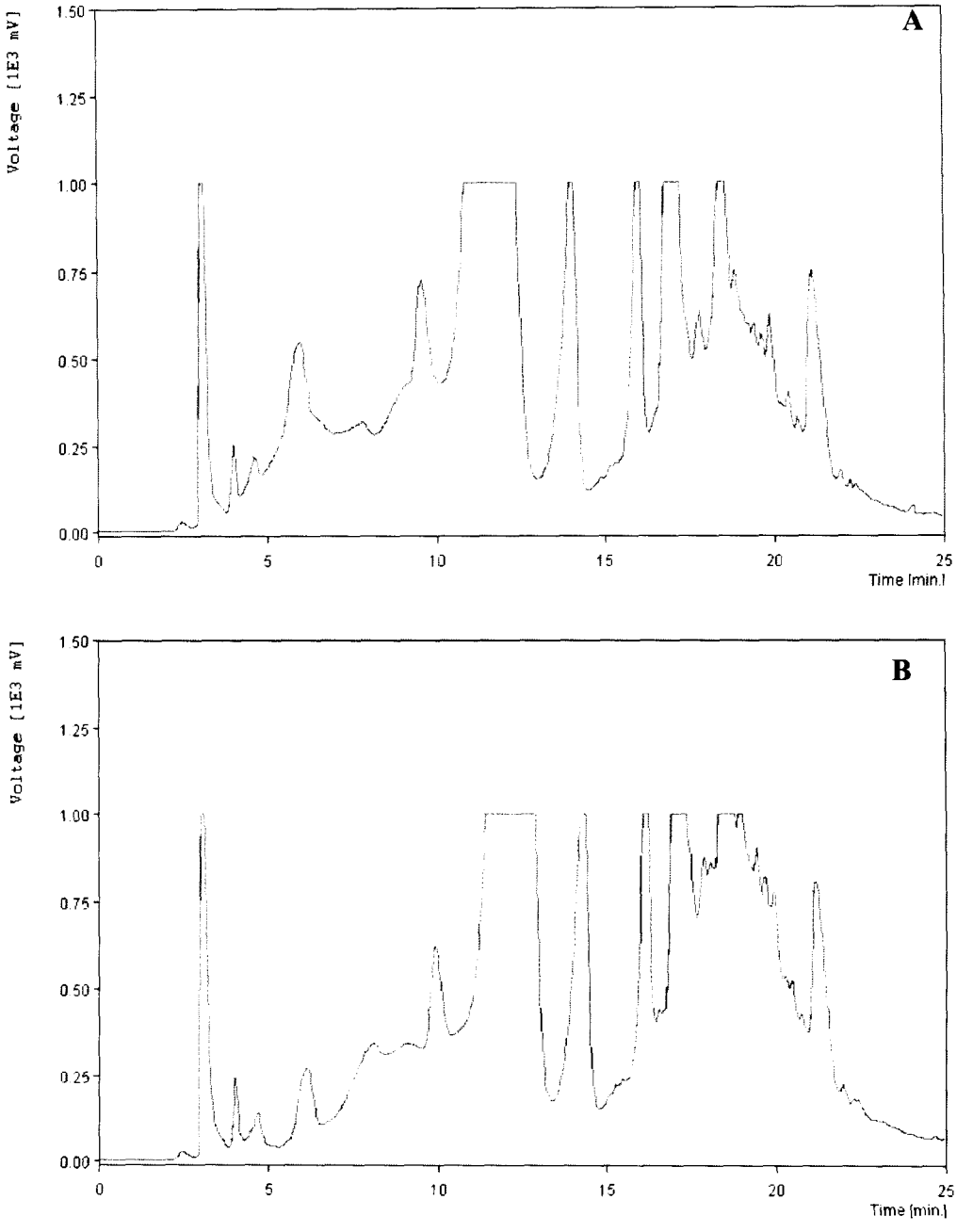


Fig. 14: HPLC analysis of catechin extract from tea leaves sprayed with PGPRs; A: Control; B: *B. megaterium*

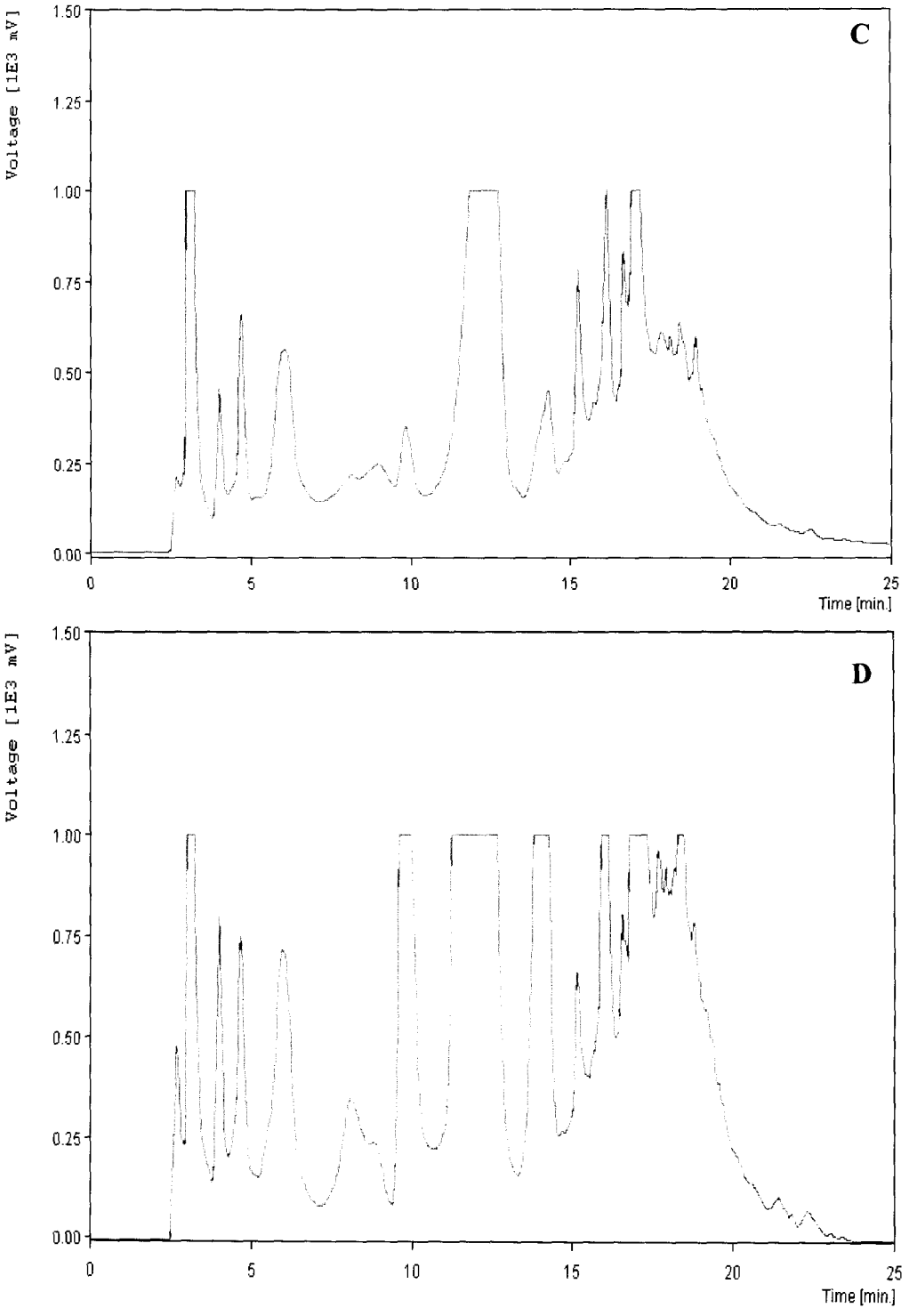


Fig. 14: HPLC analysis of catechin extract from tea leaves sprayed with PGPRs
C: *O. anthropi* and D: *B. megaterium* and *O. anthropi*

4.11. Biochemical changes in chickpea induced by *B. megaterium*

Experiments were also conducted to assess the effect of the different bacteria on the biochemical components of chickpea plants. Activities of some of the enzymes which are involved defense- i.e., peroxidase, phenylalanine ammonia lyase were assayed. In order to determine the effect on photosynthetic apparatus changes in chlorophyll content were also analyzed.

The result showed that the application of *B. megaterium* and *O. anthropi* significantly increased the activities of defense enzymes like PAL and PO, chitinase and β-1,3-glucanase enzyme activity in chick pea in comparison to control plants (Fig. 15 & 16). On quantitative estimation, the protein content of plants was also found to increase following inoculation of plant with PGPR (Fig. 17). When the protein extract from PGPR treated plants were analysed by SDS-PAGE, all the proteins having molecular mass of ca. 16.7, 22.3, 23.7, 29.8, 35.4, 44.6, 50.1, 53.0, 70.7, 89.1, 94.4, 100, 125.8, 126, 133.3 KDa were found to be present constitutively but their intensity was found to increase in PGPR treated plants (Plate XIX B). Treatment of *C. arietinum* plants with *B. megaterium* increased the phenol content of the plants (Fig. 18). The application of PGPR to the plants led to significant increase in chlorophyll content in *C. arietinum* (Table 22).

Table 17 a: Peak result of HPLC analysis of catechin extracts from untreated control plants (cv. HV 39)

Peak No.	R. time	Area (m V.s)	Height (mV)	Area %	Height %
1	3.890	7656.5542	601.786	2.447	9.095
2	4.500	2747.9053	111.081	0.878	1.679
3	5.890	5808.0080	154.650	1.856	2.337
4	7.680	4301.7337	198.085	1.375	2.994
5	9.060	5868.1514	210.666	1.875	3.184
6	11.790	58392.0233	986.706	18.660	14.912
7	13.790	24063.2510	984.191	7.690	14.874
8	14.690	6993.7403	258.686	2.235	3.910
9	16.120	32019.2394	981.295	10.232	14.830
10	16.920	50890.1126	980.351	16.263	14.816
11	18.480	109406.4298	978.423	34.962	14.786

Table 17 b: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *O. anthropi* (cv. HV 39)

Peak No.	R. time	Area (m V.s)	Height (mV)	Area %	Height %
1	3.870	5319.4546	549.829	1.424	5.955
2	4.440	3310.0187	192.227	0.886	2.082
3	5.820	4472.5534	259.086	1.197	2.806
4	7.620	4217.9694	208.247	1.129	2.255
5	8.250	4609.8822	170.802	1.234	1.850
6	8.960	2863.3873	110.483	0.766	1.197
7	11.710	65346.4324	989.793	17.489	10.720
8	13.760	27818.7746	987.748	7.445	10.698
9	14.710	4373.8208	170.359	1.171	1.845
10	15.170	4505.0671	198.190	1.206	2.147
11	16.110	34809.7979	985.410	9.316	10.673
12	17.200	68802.6960	984.374	18.414	10.661
13	18.190	20827.4988	983.367	5.574	10.651
14	18.830	40308.7311	982.770	10.788	10.644
15	19.720	71487.4182	981.851	19.134	10.635

Table 17 c: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *B. megaterium* (cv. HV 39)

Peak No.	R. time	Area (m V.s)	Height (mV)	Area %	Height %
1	3.440	2115.3560	164.355	0.553	2.165
2	3.870	7864.6209	843.042	2.055	11.103
3	4.450	3646.4159	178.746	0.953	2.354
4	5.800	3994.9508	247.191	1.044	3.256
5	7.570	5151.3450	228.163	1.346	3.005
6	8.830	6334.6169	285.763	1.655	3.764
7	9.430	2328.0327	113.894	0.608	1.500
8	11.570	67016.0301	988.717	17.508	13.022
9	13.650	30105.9403	986.383	7.865	12.991
10	14.590	5767.8035	227.254	1.507	2.993
11	15.730	42151.7482	984.054	11.012	12.960
12	17.150	57111.8193	982.522	14.920	12.940
13	18.530	139862.3620	980.991	36.536	12.921

Table 18 a: Peak result of HPLC analysis of catechin extracts from untreated control plants (cv. S 449)

Peak No.	R. time	Area (m V.s)	Height (mV)	Area %	Height %
1	3.870	7682.3199	857.601	2.331	10.523
2	4.420	4259.0464	197.280	1.292	2.421
3	5.710	6447.4943	358.672	1.956	4.401
4	7.450	5257.1728	271.776	1.595	3.335
5	8.630	4563.9448	188.224	1.385	2.309
6	11.360	67655.9305	990.888	20.525	12.158
7	13.400	12530.5400	474.985	3.801	5.828
8	14.460	4986.1522	202.144	1.513	2.480
9	15.220	8726.9136	269.448	2.648	3.306
10	15.630	26869.0730	988.138	8.151	12.124
11	16.720	46211.7512	987.483	14.020	12.116
12	17.490	10799.8614	551.024	3.276	6.761
13	18.280	103569.5928	986.499	31.420	12.104

Table 18 b: Peak result of HPLC analysis of catechins from leaves of tea plants treated with *B. megaterium* (cv. S 449)

Peak No.	R. time	Area (m V.s)	Height (mV)	Area %	Height %
1	3.440	2115.3560	164.355	0.553	2.165
2	3.870	7864.6209	843.042	2.055	11.103
3	4.450	3646.4159	178.746	0.953	2.354
4	5.800	3994.9508	247.191	1.044	3.256
5	7.570	5151.3450	228.163	1.346	3.005
6	8.170	2895.5662	105.343	0.756	1.387
7	8.830	6334.6169	285.763	1.655	3.764
8	9.430	2328.0327	113.894	0.608	1.500
9	11.570	67016.0301	988.717	17.508	13.022
10	13.650	30105.9403	986.383	7.865	12.991
11	14.590	5767.8035	227.254	1.507	2.993
12	15.730	42151.7482	984.054	11.012	12.960
13	17.150	57111.8193	982.522	14.920	12.940
14	18.530	139862.3620	980.991	36.536	12.921

Table 18 c: Peak result of HPLC analysis of catechins from leaves of tea plants treated with *O. anthropi* (cv. S 449)

Peak No.	R. time	Area (m V.s)	Height (mV)	Area %	Height %
1	3.890	11530.2041	997.564	2.803	10.589
2	4.430	5862.8759	271.858	1.425	2.886
3	5.700	13832.6784	420.895	3.362	4.468
4	7.440	10195.4215	307.693	2.478	3.266
5	8.030	6400.7602	221.321	1.556	2.349
6	8.650	4383.2660	160.907	1.065	1.708
7	9.890	4728.3504	136.623	1.149	1.450
8	11.460	73756.0447	990.996	17.928	10.519
9	13.100	7584.3482	191.310	1.844	2.031
10	13.540	10279.1342	261.967	2.499	2.781
11	14.590	7564.1273	279.627	1.839	2.968
12	15.740	36131.9046	987.201	8.783	10.479
13	16.810	43482.1144	986.273	10.569	10.469
14	18.510	84322.0670	984.774	20.496	10.453
16	19.220	19721.5130	984.145	4.794	10.447
17	19.670	66434.0315	983.750	16.149	10.442

Table 19: Effect of foliar spray of PGPRs on the enzyme activity of leaves of tea

Treatment	Peroxidase[a]	Phenylalanine ammonia lyase[b]	Chitinase[c]	β-1,3-glucanase[d]
Control	2.1	105	17.31	336
O. anthropi	3.9	115	33.22	480
B. megaterium	3.5	115	29.42	416
O. anthropi + *B. megaterium*	4.6	130	36.77	544

Difference of all tests with control significant at P=0.01 as tested by Student's 't' test; [a]- PO activity assayed as Δ A_{465} $min^{-1}g$ $tissue^{-1}$; [b]- PAL activity assayed as µg cinnamic acid produced by enzyme from 1 g tissue min^{-1}; [c]- CHT activity assayed as µg N-Acetyl glucosamine released by enzyme from 1 g tissue $min^{-1}$and [d]- β1,3-GLU activity assayed as µg glucose released by enzyme from 1 g tissue min^{-1}

Table 20: Effect of foliar spray of PGPRs on chlorophyll content of tea plant leaves

Biochemical components (mg g^{-1} tlssue)	Treatment			
	Control	*B.megaterium*	*O.anthropi*	*B. megaterium* + *O. anthropi*
Protein	30	47.5	60	72
Phenol				
Total Phenol	12.5	31.25	31.25	56.25
O-dihydroxy phenol	2.5	3.75	6.25	5.625
Chlorophyll				
Chl. a	0.596*	0.693	0.694*	0.731*
Chl. b	0.284*	0.429*	0.417	0.503*
Total Chl.	0.884	1.112	1.121	1.462

* Difference with control significant at P= 0.05, rest significant at P= 0.01 as tested by Student's 't' test.

Table 21a: Peak result of HPLC analysis of catechins from leaves of untreated control tea plants (cv. BSS 2)

Peak No.	R. time	Area (m V.s)	Height (mV)	Area %	Height %
1	3.120	17218.8853	997.074	3.228	12.577
2	3.990	3604.7871	260.989	0.676	3.292
3	4.610	5859.0140	212.786	1.098	2.684
4	5.950	41722.2667	541.317	7.822	6.828
5	7.800	20455.8179	305.019	3.835	3.847
6	9.550	51121.9293	712.538	9.584	8.988
7	12.370	128895.6883	980.621	24.164	12.369
8	14.070	37264.4918	977.556	6.986	12.330
9	16.050	34571.3428	974.012	6.481	12.286
10	17.220	52108.0618	971.946	9.769	12.260
11	18.550	140120.2215	969.566	26.268	12.228

Table 21b: Peak result of HPLC analysis of catechins from leaves of tea plants treated with *B. megaterium* (cv. BSS 2).

Peak No.	R. time	Area (m V.s)	Height (mV)	Area %	Height %
1	3.090	16697.6173	995.983	3.134	12.920
2	4.010	3015.0561	237.876	0.566	3.086
3	6.130	9965.8655	257.734	1.870	3.343
4	8.090	23730.2048	324.983	4.454	4.216
5	9.090	20410.1307	321.923	3.830	4.176
6	9.920	22955.6127	602.131	4.308	7.811
7	12.640	126144.0876	971.571	23.674	12.604
8	14.390	37803.8319	967.066	7.095	12.545
9	16.230	35605.7352	962.335	6.682	12.484
10	17.350	54448.0965	959.481	10.219	12.447
11	18.970	177737.9804	955.334	33.357	12.394

Table 21c: Peak result of HPLC analysis of catechins from leaves of tea plants treated with *O. anthropi* (cv. BSS 2).

Peak No.	R. time	Area (m V.s)	Height (mV)	Area %	Height %
1	2.650	2650.4936	210.737	0.630	3.013
2	3.200	27581.1691	998.716	6.556	14.277
3	4.000	7088.3529	452.605	1.685	6.470
4	4.680	13495.2171	657.439	3.208	9.398
5	6.050	32233.3921	557.267	7.662	7.966
6	8.140	12733.1531	209.459	3.027	2.994
7	8.960	14362.1206	237.471	3.414	3.395
8	9.830	12954.6604	341.692	3.079	4.885
9	12.670	99159.6396	988.778	23.570	14.135
10	14.320	18854.5266	437.034	4.482	6.247
11	15.260	20947.1252	768.958	4.979	10.992
12	17.210	156222.3539	983.985	37.135	14.066

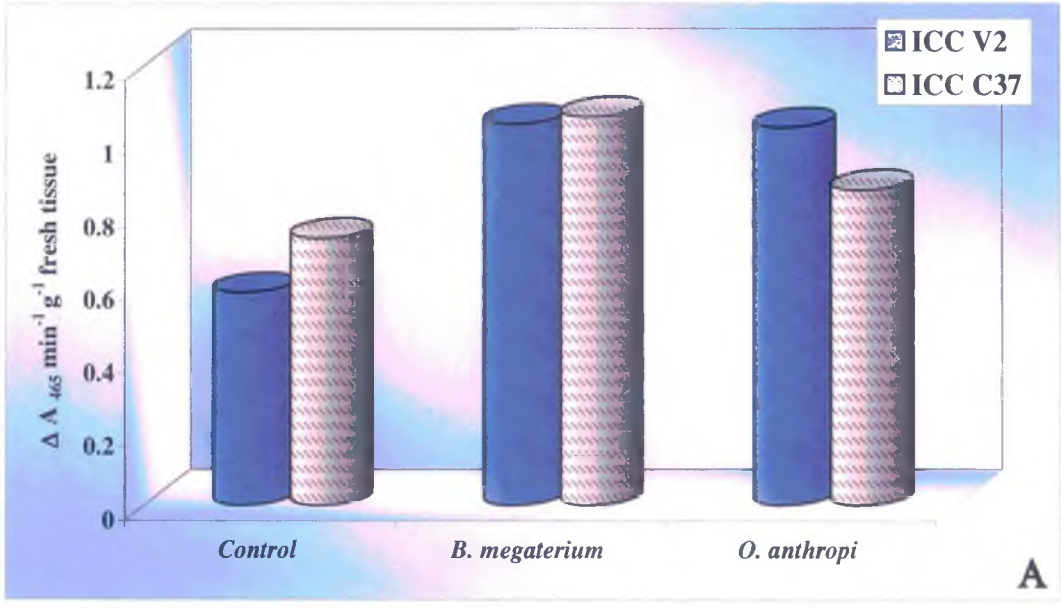
Table 21d: Peak result of HPLC analysis of catechin from leaves of tea plants treated with *B. megaterium* + *O. anthropi* (cv. BSS 2)

Peak No.	R. time	Area (m V.s)	Height (mV)	Area %	Height %
1	2.670	7407.4233	489.167	1.286	4.116
2	3.210	27736.1567	1010.497	4.815	8.502
3	3.990	12624.2015	815.239	2.191	6.859
4	4.660	18660.2908	757.009	3.239	6.369
5	5.970	36453.8598	724.284	6.328	6.094
6	8.100	28498.9312	356.698	4.947	3.001
7	9.990	44294.4975	1010.505	7.689	8.502
8	12.300	111905.3246	1010.515	19.425	8.502
9	14.270	49684.1016	1010.507	8.625	8.502
10	15.170	22163.9595	674.444	3.847	5.675
11	16.150	35359.2355	1010.493	6.138	8.502
12	16.580	15119.9324	813.446	2.625	6.844
13	17.360	47621.1448	1010.508	8.266	8.502
14	18.480	111191.3929	1010.510	19.301	8.502
15	21.440	4422.2603	108.655	0.768	0.914

Table 22: Effect of *B. megaterium* and *O. anthropi* on the chlorophyll content of chickpea

Variety	Treatment	Chlorophyll content ($mg\ g^{-1}$ tissue)		
		Total Chl	Chl a.	Chl. b
ICC V2	Control	2.03	3.89	1.86
	O. anthropi	6.95	4.59	2.35
	B. megaterium	7.59	5.31	2.26
ICC C37	Control	6.01	3.64	2.37
	O. anthropi	10.21	7.03	3.18
	B. megaterium	11.52	7.95	3.57

Difference with control significant at P= 0.05 as done by Student's 't' test.



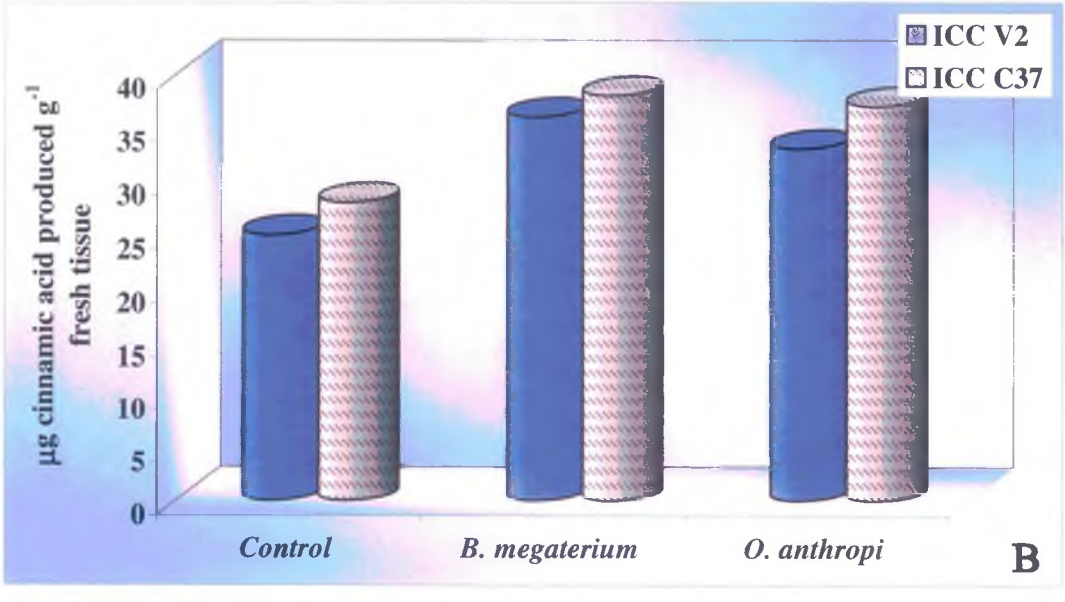


Fig. 15: Effect of *B. megaterium* and *O. anthropi* on the peroxidase (A) and phenylalanine ammonia lyase (B) activities in chickpea

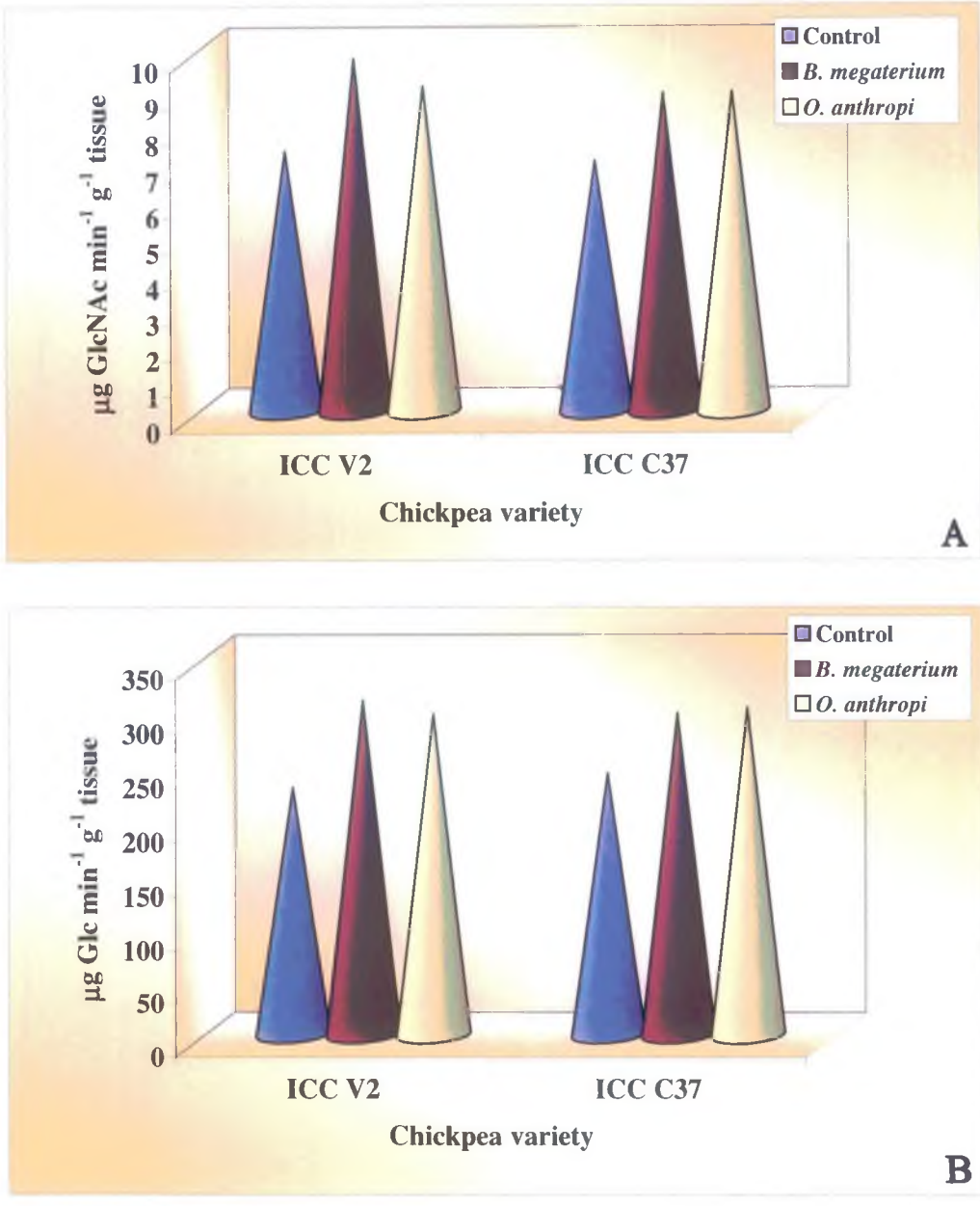


Fig. 16: Effect of *B. megaterium* and *O. anthropi* on the chitinase (A) and β–1,3-glucanase (B) activities in chickpea.

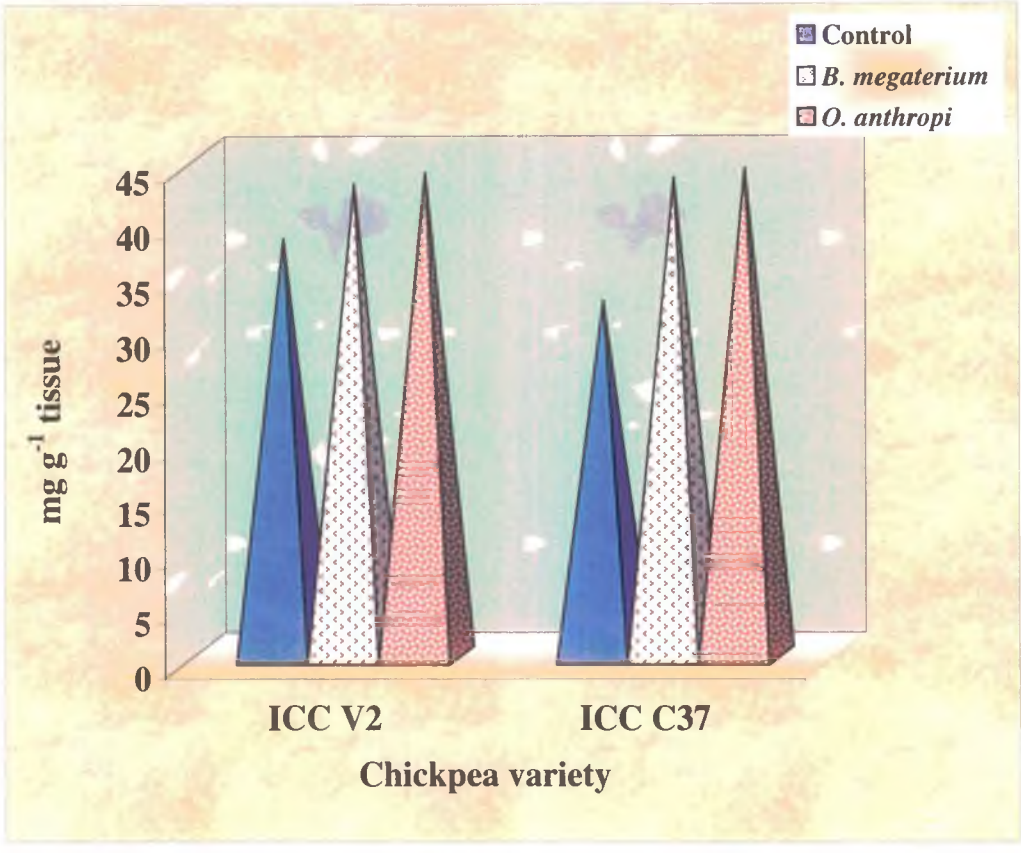


Fig. 17: Changes in protein content in chickpea following treatment with *B. megaterium* and *O. anthropi.*

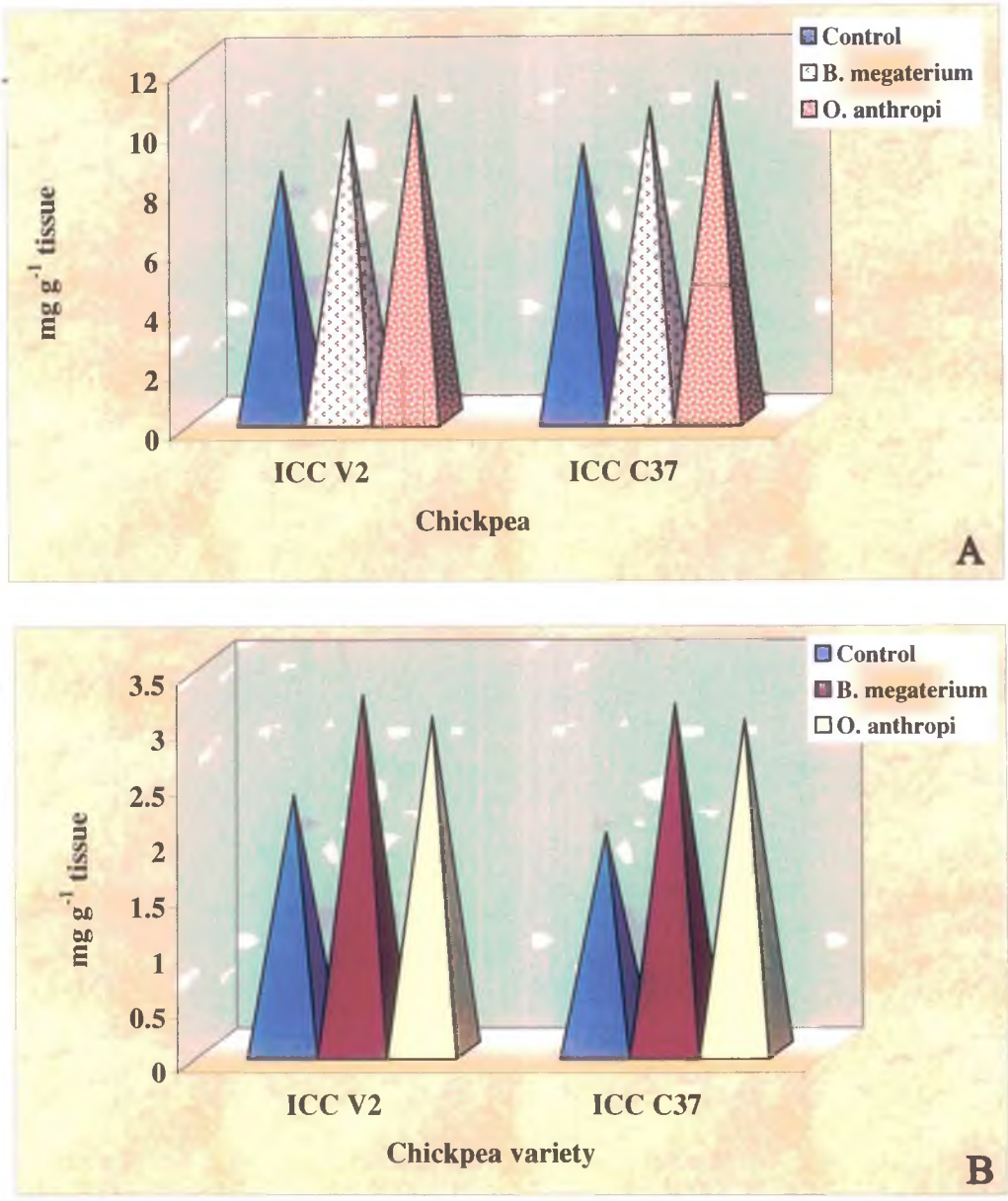


Fig. 18: Effect of *B. megaterium* and *O. anthropi* in the phenol content of chickpea [Total phenol (A) and O-dihydroxy phenol (B)].

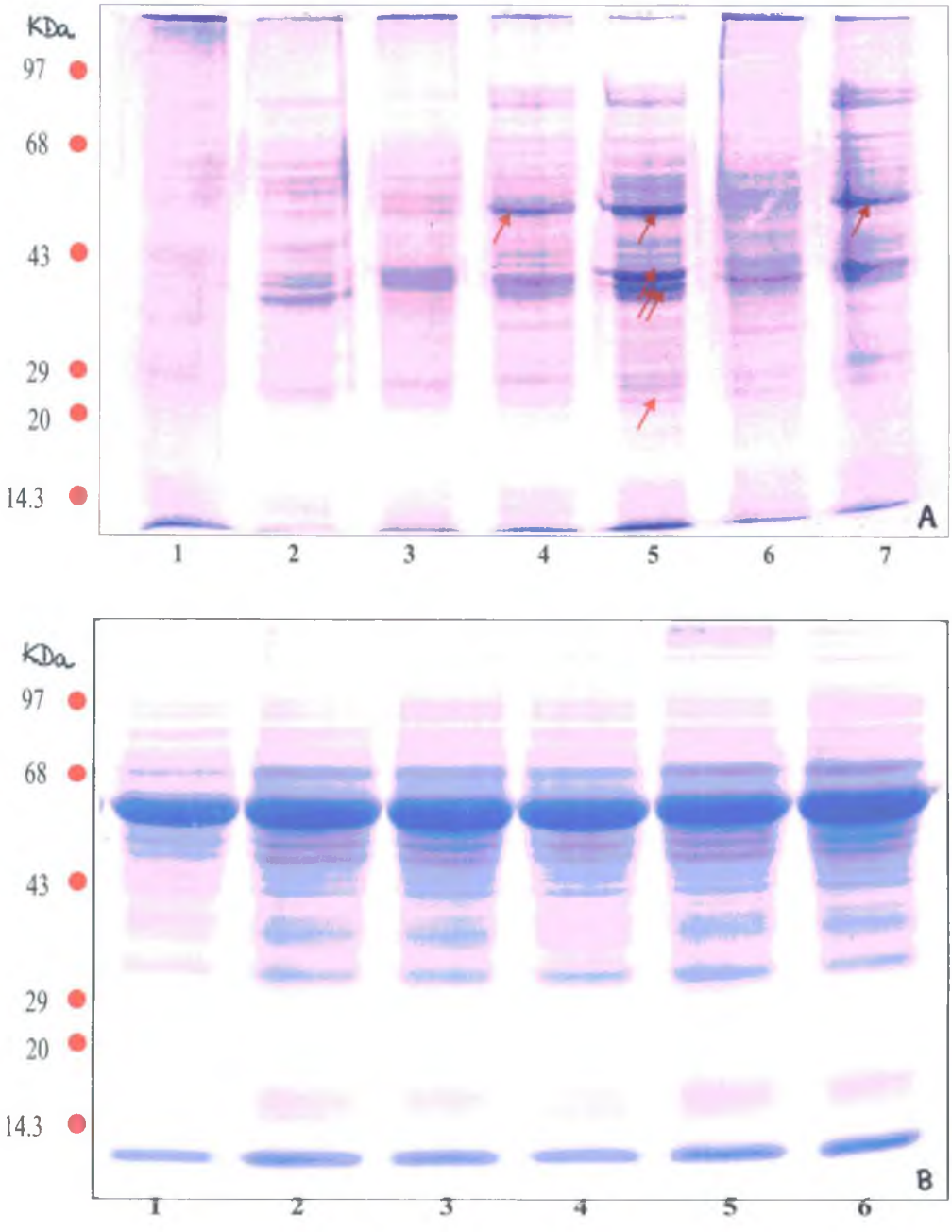


Plate XIX (A-B): SDS PAGE analysis of proteins from orchid (A) and chick pea (B)
A: *Vanda* (Lanes 1, 4 & 7); *Phaleonopsis* (Lanes 2 & 5); *Oncidium* (Lanes 3 & 6)
Lane 1, 2 & 3: Control; Lane 4, 5, 6 & 7: *B. megaterium* treated;
B: ICC V2:(Lanes 1-3) and ICC C37 (lanes 4-6) Lane 1 & 4: control; Lane 2 & 5- *B. megaterium* treated; Lane 3 & 6: *O. anthropi* treated

4.12. Effect of *B. megaterium* and *O. anthropi* on root rot development

Since the bacteria showed antagonistic activity *in vitro*, experiments were further conducted to determine whether these could also control diseases caused by some of the pathogens. *In vitro* tests were conducted on tea, chick pea, as well as the orchids which were highly susceptible to rot by soil microorganisms.

4.12.1. Tea

Effect of the two bacteria on development of brown root rot of tea, caused by *F. lamaoensis* was determined (Plate XX). Inoculation was done with the PGPRs and pathogen and disease assessment was done in three varieties (UP 3, HV 39 and T 17) as described under materials and methods. It was observed that *B. megaterium* reduced brown rot more significantly in comparison to *O. anthropi*, when the plants were artificially inoculated with pathogen after three days of soil drenching with bacteria (Table 23 and Fig. 19).

4.12.2. Chickpea

Since *O. anthropi* did not inhibit *S. rolfsii* or *S. sclerotiorum in vitro*, further tests were carried out with *B. megaterium* only. Seed bacterization with *B. megaterium* sown in *S. rolfsii* II infested soil showed a considerable decline in disease symptoms. After 45 days of sowing, there was 84.5 % decrease in disease symptoms by *B. megaterium* as compared to *S. rolfsii* II alone (Table 24; Plate XXI).

4.12.3. Orchids

In the present study, *B. megaterium* proved suppressive to crown rot diseases caused by *S. rolfsii* I and *S. sclerotiorum* and recorded least disease incidence. The efficiency of *B. megaterium* did not vary much with the pathogen. The percentage of disease incidence was 84.6 % in *S. sclerotiorum* inoculated untreated *Vanda* plants (Plate XXII) which reduced to 38.5 % in *B. megaterium* treated *Vanda* plants. Similarly, the percentage of disease incidence was 90 % and 75 % in untreated *Oncidium* and *Phaleonopsis* plants which reduced

Plate XX (A-B): Tea root showing symptoms of brown root rot disease caused by *F. lamaoensis*

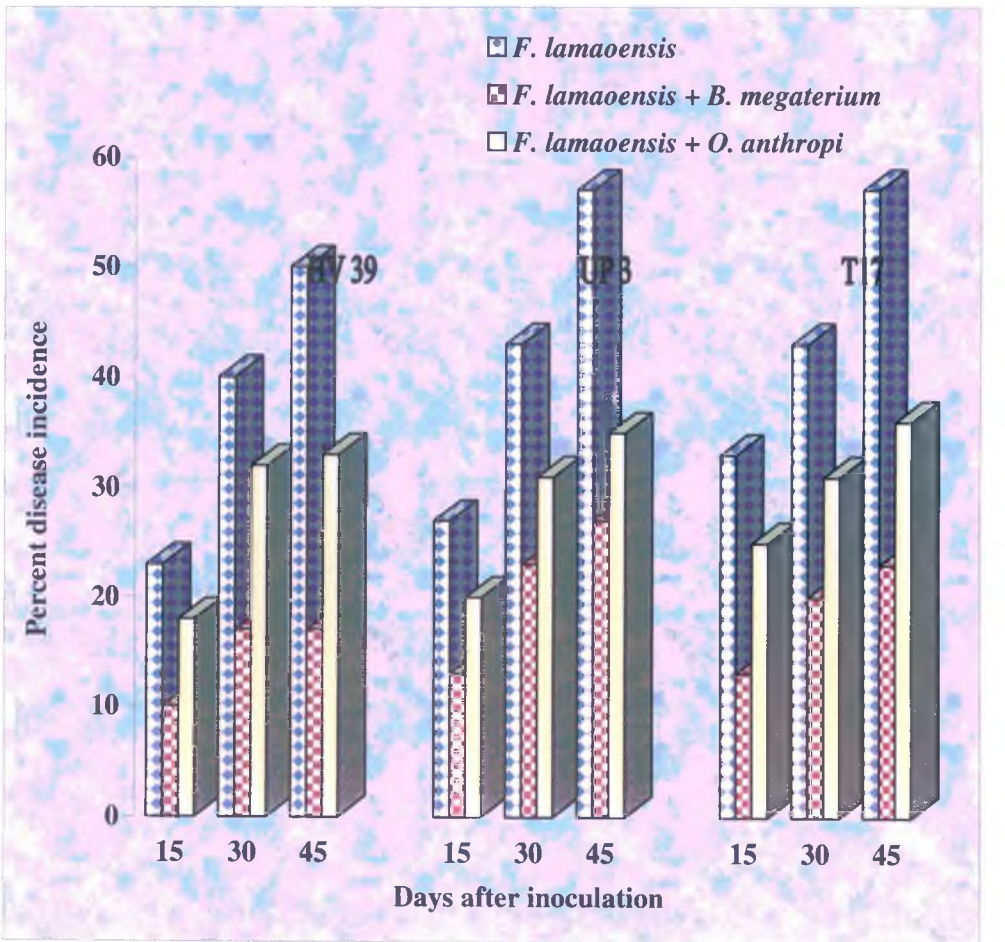


Fig. 19: Effect of *B. megaterium* and *O. anthropi* on brown root rot of tea.

Plate XXI (A-G): A: Healthy chick pea plants grown in pot; B & C: Chick pea plants artificially inoculated with *S. rolfsii* I; E: Close view of chick pea plant showing symptoms of white rot; D, F & G: Growth promotion and disease control in chickpea; [C: Control, *B.m*: *B. megaterium* treated, *B.m* + *S.r* : *B. megaterium* + *S. rolfsii* I inoculated and *S.r*: *S. rolfsii* I inoculated]

respectively to 40 % and 33.4 % in *B. megaterium* treated plants (Table 25; Plates XXIII & XXIV).

Table 23: Effect of *B. megaterium* and *O. anthropi* on the development of brown rot of tea caused by *F. lamaoensis*

Varieties	Treatment	Root rot index Days after inoculation 15	30	45
HV 39	*F. lamaoensis*	1.90	3.00	5.55
	F. lamaoensis + *B. megaterium*	0.18[a]	1.24[a]	2.35[a]
	F. lamaoensis + *O. anthropi*	0.98[b]	2.43[b]	4.3[b]
UP 3	*F. lamaoensis*	1.45	2.64	4.60
	F. lamaoensis + *B. megaterium*	0.42[a]	1.12	2.30
	F. lamaoensis + *O. anthropi*	1.02	2.23	3.78
T 17	*F. lamaoensis*	1.22	2.42	4.80
	F. lamaoensis + *B. megaterium*	0.44	0.82[a]	2.16
	F. lamaoensis + *O. anthropi*	0.87[a]	1.56[b]	3.06[a]

[a]- Difference with control significant at P= 0.05; [b]- insignificant and rest significant at P= 0.01 as done by Student's 't' test. Root index: 0- no symptoms; 1- small roots turn brownish and start rotting; 2- leaves start withering and 20-30 % of roots turn brown; 3- leaves withered and 50% of the roots affected; 4- shoot tips also starts withering; 60-70 % roots affected; 5- whole plants die, with upper withered leaves still remaining attached; roots fully rotted.

4.13. Biochemical changes during induced resistance

Application of the two PGPRs to soil was found to affect the biochemical responses of plants. Disease establishment is also known to cause biochemical changes in the host. Hence, in another series of experiments, biochemical responses of tea, chickpea and orchids following application of PGPRs and inoculation with the pathogens were determined.

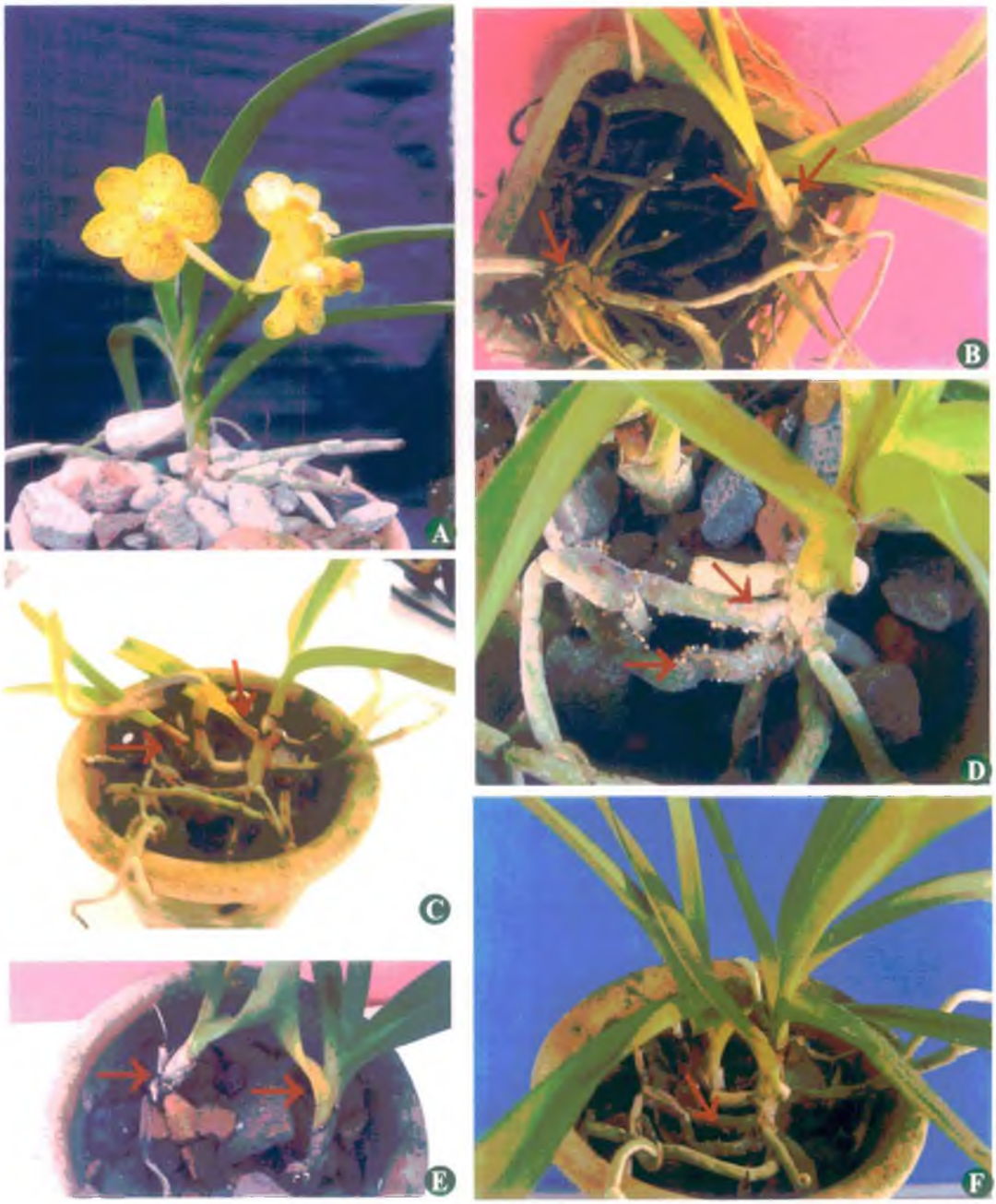


Plate XXII (A-F): A: Healthy flowering plant of *Vanda*; B-E: *S. Sclerotiorum* infected plants showing the symptoms of turning lower leaves yellow and wilting from the margin back towards the base and tiny spheres of sclerotia at the root .

Plate XXIII (A-E): A-C: A healthy plants of *Oncidium*; A: Flowering plant; D & E: *S. rolfsii* infected plants showing the leaf yellowing typical of stem rot and white mycelium of *S. rolfsii* on the base of the plant severed by the fungus

Plate XXIV (A-G): A & B:Healthy flowering plants of *Phaleonopsis*; C&D: Healthy plants of *Phaleonopsis* growing in the nursery bed; E & G: *S. rolfsii* II severed *Phaleonopsis* plants showing slerotia formed at the roots; F: Close view of sclerotia.

Table 24: Effect of *B. megaterium* on stem rot disease of chickpea in the greenhouse condition

Treatment	Percent disease incidence Days after sowing			Mean*	Percent reduction over control
	15	30	45		
S. rolfsii II	39	72	72	55.5	0.0
S. rolfsii II + *B. megaterium*	8.6	8.6	8.6	8.6	84.5

* Average of 20 plants. Difference with control significant at P= 0.01 as done by Student's 't' test.

Table 25: Efficacy of *B. megaterium* in controlling disease in orchid plants

Genus	Treatment	Percent disease incidence Days after inoculation		
		15	30	45
Oncidium	*S. rolfsii* I	36	87	92
	S. rolfsii I + *B. megaterium*	12	23	42
Phaleonopsis	*S. rolfsii* I	58	78	89
	S. rolfsii I + *B. megaterium*	13	29	34
Vanda	*S. sclerotiorum*	22	47	67
	S. sclerotiorum + *B. megaterium*	8	15	29

* Average of 20 plants.

4.13.1. Tea

Higher activities of defense enzymes like peroxidase were observed in *F. lamaoensis* andd PGPR treated tea plants. Other defense enzymes like PAL, β,1-3, glucanase and chitinase also showed enhanced activity (Table 26, Fig. 20 & 21).

Protein content was lesser in *F. lamaoensis* inoculated plants than in uninoculated plants but protein content was higher in bacteria treated plants (Fig. 22). Phenol contents were even more significantly increased when challenged with pathogen. Maximum phenol content was obtained in the

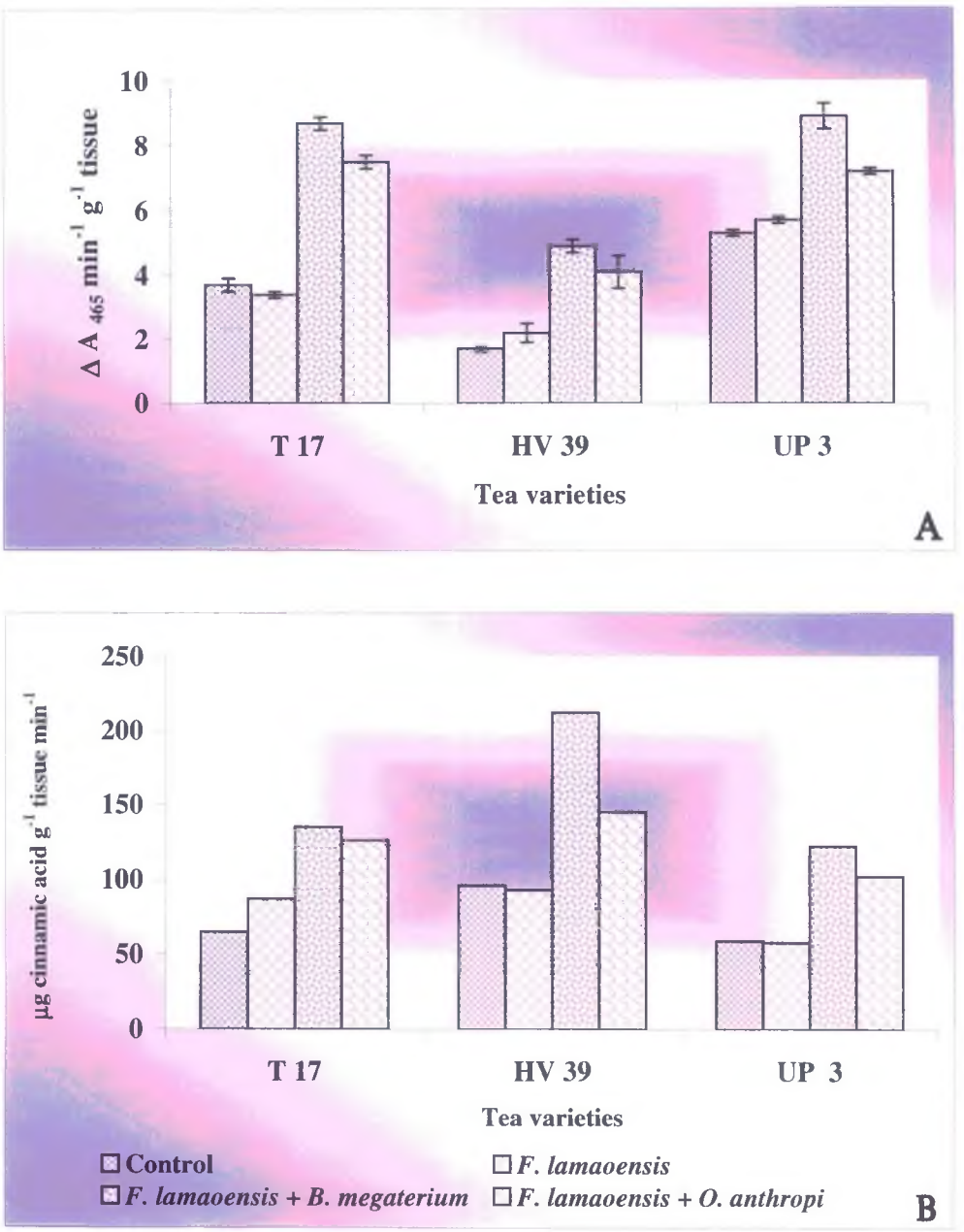
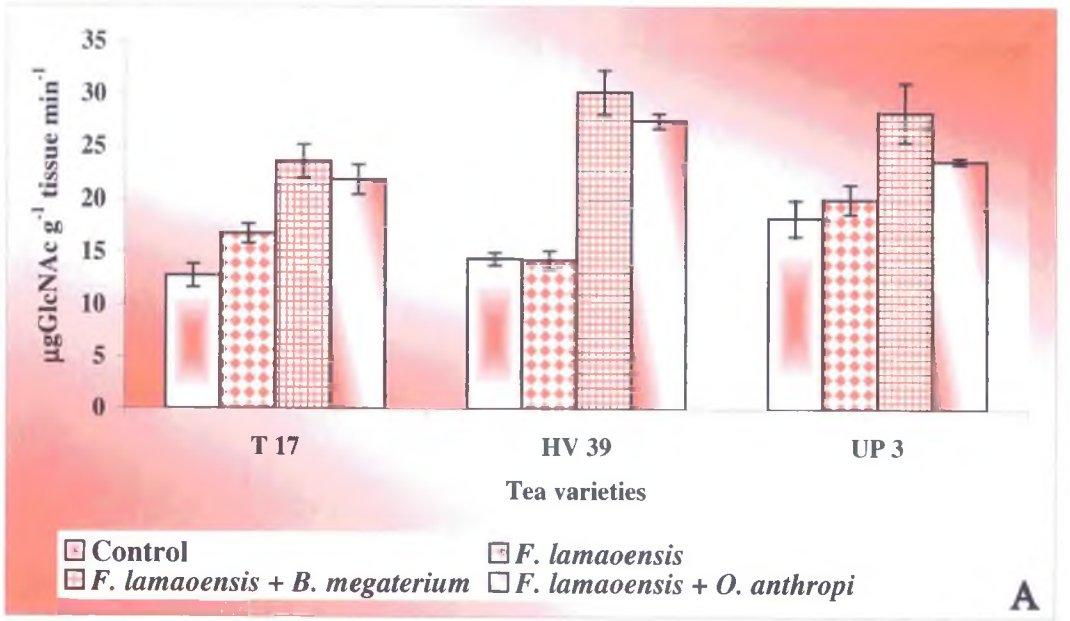


Fig. 20: Effect of challenge inoculation with *F. lamaoensis* and *B. megaterium or O. anthropi* on peroxidase (A) and phenylalanine ammonia lyase (B) activities of tea.



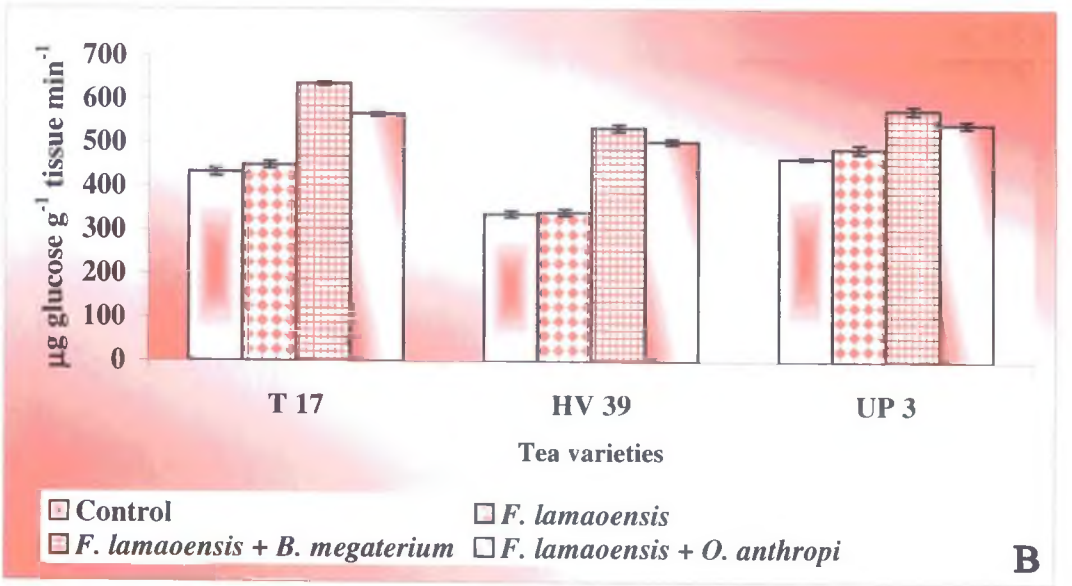


Fig. 21: Effect of *B. megaterium* or *O. anthropi* and *F. lamaoensis* on chitinase (A) and b-1,3-glucanase (B) activities of tea leaves.

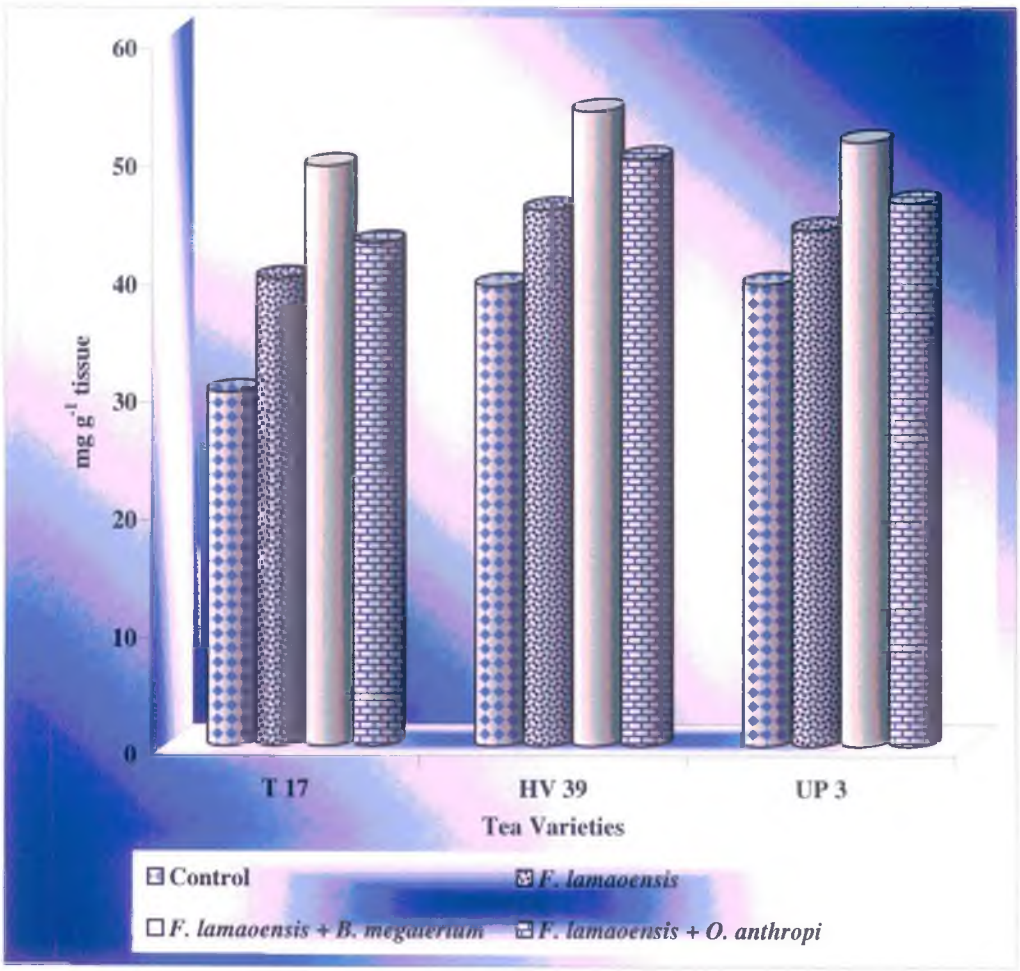


Fig. 22: Effect of challenge inoculation of *F. lamaoensis* and antagonists on protein content of tea leaves.

presence of both *B. megaterium* and *F. lamaoensis* (Fig. 23). Chlorophyll content was also found to be higher in *B. megaterium* treated plants and lower in pathogen inoculated plants than untreated control plants.

4.13.2. Chickpea

Biochemical analysis of *S. rolfsii* I inoculated plants and uninoculated plants showed significant increase in defense enzyme like peroxidase, PAL, β,1-3,glucanase and chitinase, which increased further in plants preinoculated with *B. megaterium* or *O. anthropi* (Table 27). The protein content was higher in pathogen inoculated plants than in uninoculated plants (Fig. 24). However, the protein content increased in bacteria inoculated plants challenged with pathogen *S. rolfsii* I. Again, higher phenol content was observed in all pathogen inoculated plants than in uninoculated plants (Fig. 25). The chlorophyll content was also found to be higher in *B. megaterium* treated pathogen challenged plants and lower in only pathogen inoculated plants than untreated control plants (Table 28).

4.13.3. Orchid

Biochemical changes induced by pathogen alone or in combination with *B. megaterium* were also studied. The biochemical responses tested included enzyme activities, chlorophyll, phenol and proteins. It was also observed that chitinase, β-1,3-glucanase, PAL and PO enzymes activity increased in pathogen inoculated as well as in *B. megaterium* treated plants challenge inoculated with pathogen. Relatively higher activity was shown by plants inoculated with *B. megaterium* and challenge inoculated with pathogen followed by plants inoculated with pathogen alone. Least enzyme activity was recorded in untreated control orchid plants (Fig. 26 & 27).

Appreciable increase in chlorophyll content of plants was observed in plants treated with *B. megaterium* followed by challenge inoculation with pathogen *S. rolfsii* I or *S. sclerotiorum*. The pathogen infection led to reduction in chlorophyll content of orchid plants (Table 29). The protein content of uninoculated control plant was lower than in pathogen inoculated plants (Fig. 28)

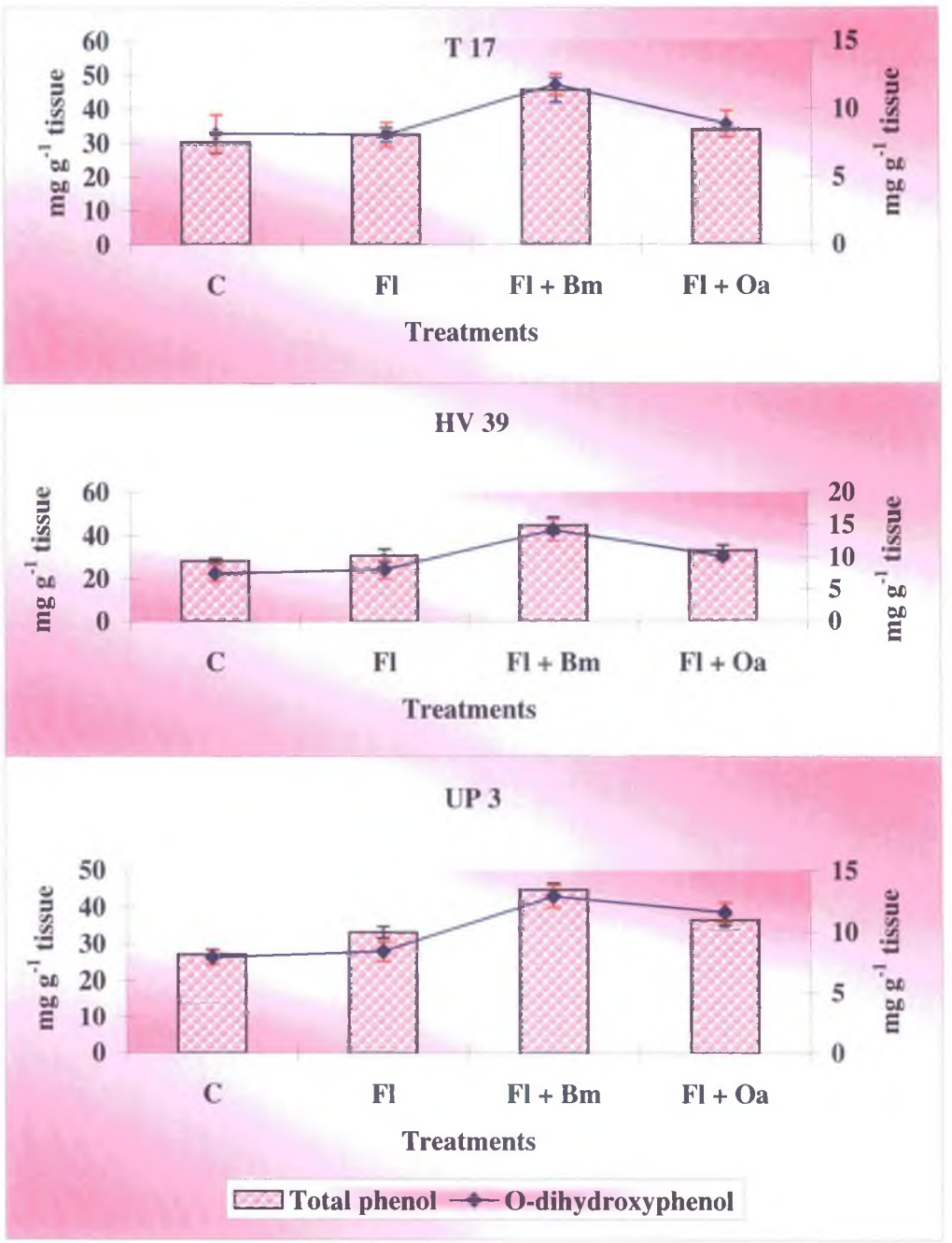


Fig. 23: Changes in phenol content of tea leaves following challenge inoculation with PGPRs and *F. lamaoensis.*

[C: =Control; Fl =*F. lamaoensis*; Fl + Bm =*F. lamaoensis* + *B. megaterium*; Fl + Oa =*F. lamaoensis* + *O. anthropi*]

while it was higher in pathogen challenged *B. megaterium* inoculated plants than in pathogen inoculated plants. The phenol content was higher in pathogen challenged *B. megaterium* inoculated plants than in pathogen inoculated and control uninoculated plants (Fig. 29).

Table 26: Enzyme activities in tea leaves of different varieties following application of PGPR and pathogen in the rhizosphere

Treatment	Enzyme activities			
	PO[a]	PAL[b]	CHT[c]	GLU[d]
T-17				
Control	3.7 ±0.2	65±0.4	12.8±1.1	432±3.9
F. lamaoensis	4.3± 0.1	87±0.6	16.8±0.9	448±5.2
F. lamaoensis+ *B. megaterium*	8.7±0.2	135±0.3	23.6±1.6	637±1.3
F. lamaoensis+ *O. anthropi*	5.1±0.2	105±1.7	19.5±1.4	525±5.2
HV 39				
Control	1.7±0.1	96±0.2	14.4±0.6	336±2.0
F. lamaoensis	2.2±0.3	93±0.5	14.3±0.9	340±1.8
F. lamaoensis+ *B. megaterium*	4.9±0.2	212±0.8	30.2±2.1	534±2.4
F. lamaoensis+ *O. anthropi*	3.1±0.5	110±0.9	19.3± 0.7	425±2.9
UP 3				
Control	5.3±0.1	59±0.4	18.3±1.7	464±4.1
F. lamaoensis	5.7±0.1	58±0.3	20.1±1.4	463±2.9
F. lamaoensis+ *B. megaterium*	8.9±0.4	122±0.7	28.3±2.8	575±5.2
F. lamaoensis+ *O. anthropi*	7.5±0.1	109±1.1	25.1±0.3	525±2.3

[a]-PO activity assayed as Δ A_{465} $min^{-1}g^{-1}$ tissue; [b]- PAL activity assayed as µg cinnamic acid produced by enzyme from 1 g tissue min^{-1}; [c]- CHT activity assayed as µg N-Acetyl glucosamine released by enzyme from 1 g tissue min^{-1} and [d]- β1,3-GLU activity assayed as µg glucose released by enzyme from 1 g tissue min^{-1}

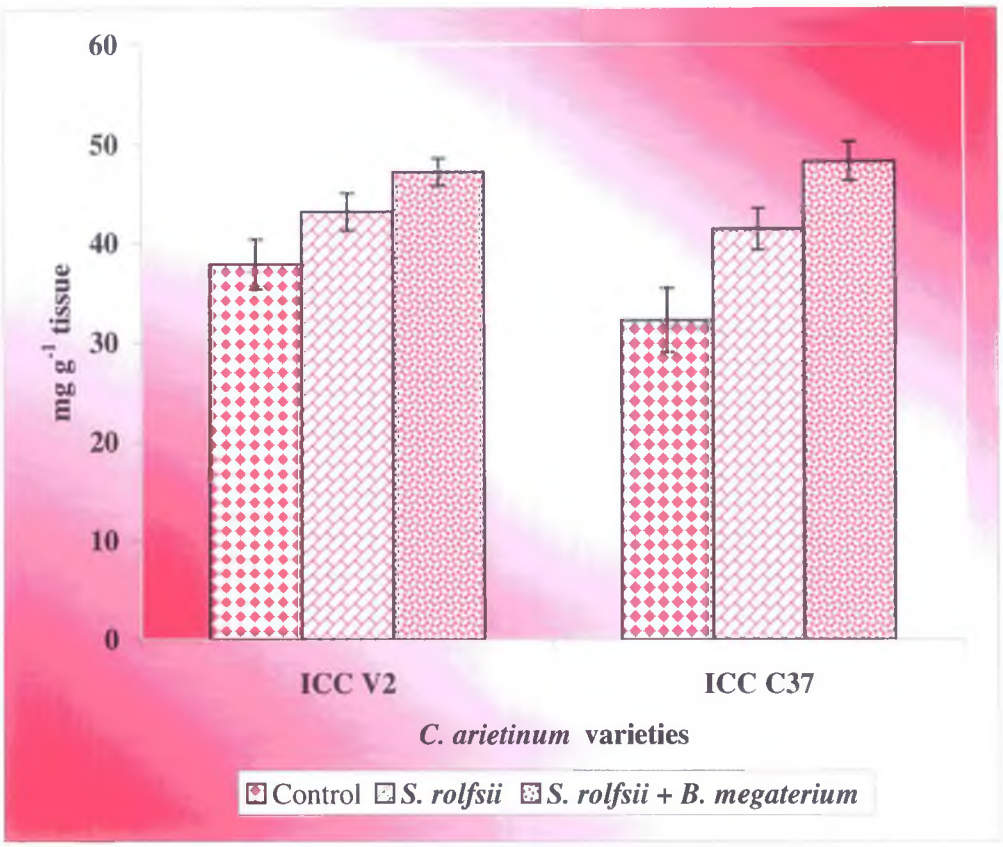


Fig. 24: Changes in protein content in *C. arietinum* following *S. rolfsii* II inoculation

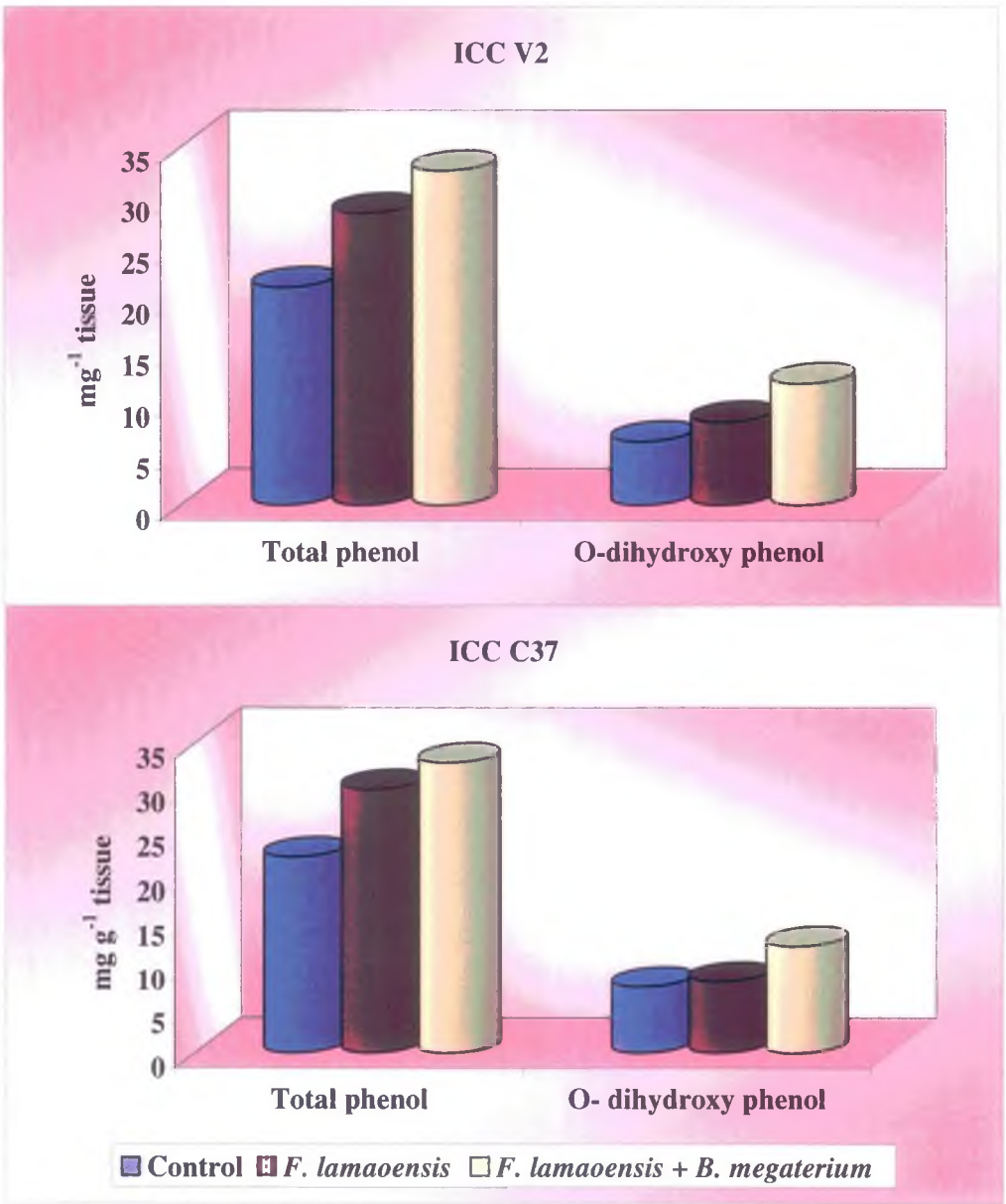


Fig. 25: Effect of *B. megaterium* and *F. lamaoensis* on the phenol content of chickpea

Table 27: Enzyme activities in *C. arietinum* plants of following application of *B. megaterium* and pathogen in the rhizosphere

Variety	Treatment	Enzyme activities			
		PO[a]	PAL[b]	CHT[c]	GLU[d]
ICC V2	Control	0.058	20.5	14.2	336.1
	F. lamaoensis	0.082	35.2	21.9	392.7
	F. lamaoensis + *B. megaterium*	0.104	45.0	25.2	413.7
ICC C37	Control	0.073	23.4	16.3	319.5
	F. lamaoensis	0.089	36.7	27.8	389.6
	F. lamaoensis + *B. megaterium*	0.106	43.3	31.1	410.4

Difference with control significant at P= 0.01 as done by Student's 't' test.

[a]- PO activity assayed as ΔA_{465} $min^{-1}g^{-1}$ tissue; [b]- PAL activity assayed as µg cinnamic acid produced by enzyme from 1 g tissue min^{-1}; [c]- CHT activity assayed as µg N-Acetyl glucosamine released by enzyme from 1 g tissue min^{-1} and [d]- β1,3-GLU activity assayed as µg glucose released by enzyme from 1 g tissue min^{-1}

Table 28: Effect on the chlorophyll content of *C. arietinum* plants following application of *B. megaterium* and pathogen in the rhizosphere

Variety	Treatment	Chlorophyll content (mg g^{-1} tissue)		
		Total Chl.	Chl a.	Chl b.
ICC V2	Control	3.89	2.03	1.86
	F. lamaoensis	5.49	2.46	3.03*
	F. lamaoensis + *B. megaterium*	8.06	4.27	3.78
ICC C37	Control	6.01	3.64	2.37
	F. lamaoensis	7.82	4.24	3.58
	F. lamaoensis + *B. megaterium*	10.88	4.90*	5.35*

* Difference with control significant at P= 0.05, rest significant at P= 0.01 as done by Student's 't' test

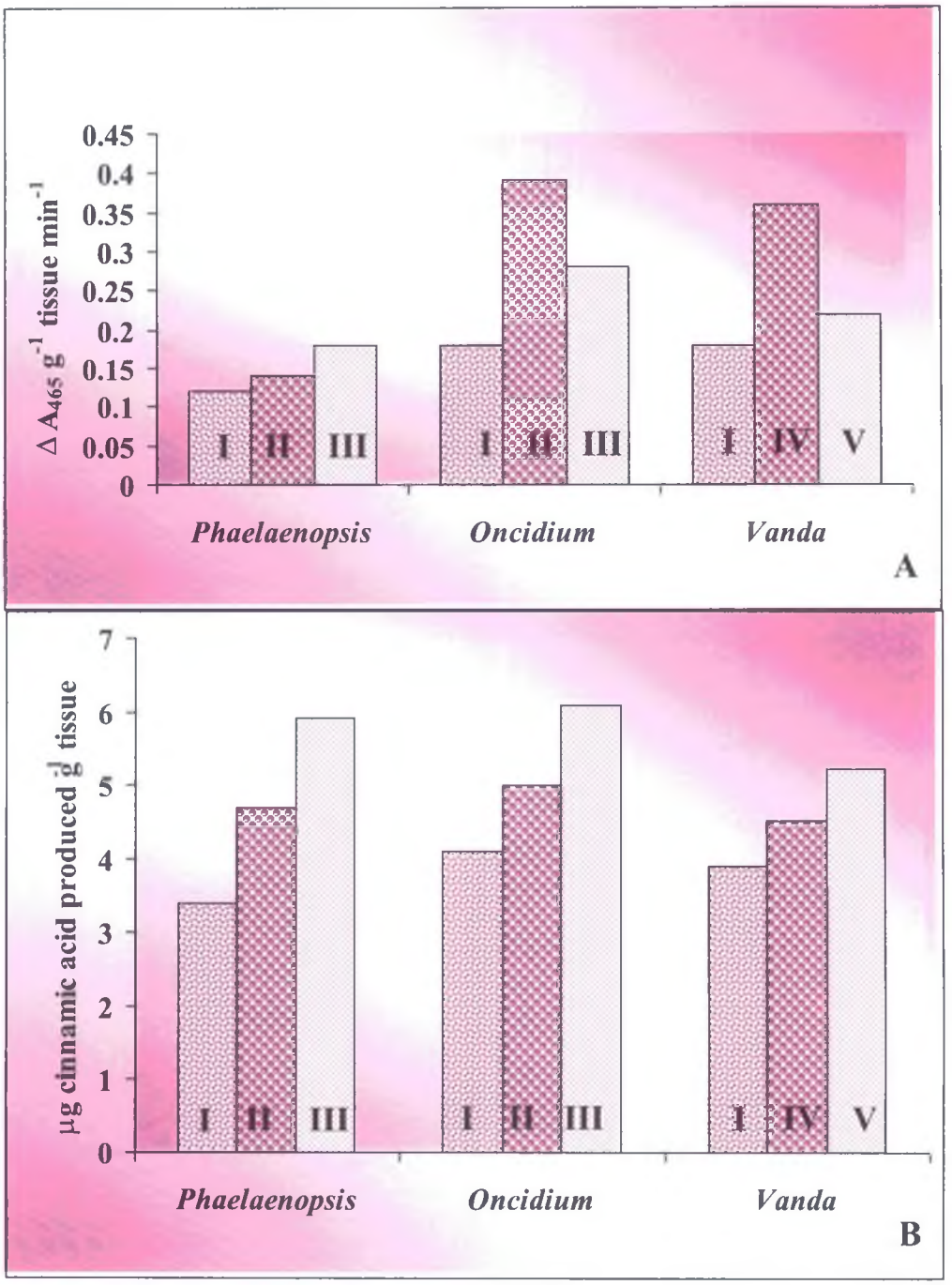


Fig. 26: Effect of inoculation of pathogens and *B. megaterium* on Peroxidas (A) and Phenylalanine ammonia lyase (B) activity in orchid plants.
[I: Control; II: *S. rolfsii* II; III: *B. megaterium* + *S. rolfsii* II; IV: *S. sclerotiorum;* V: *B. megaterium* + *S. sclrotiorum*]

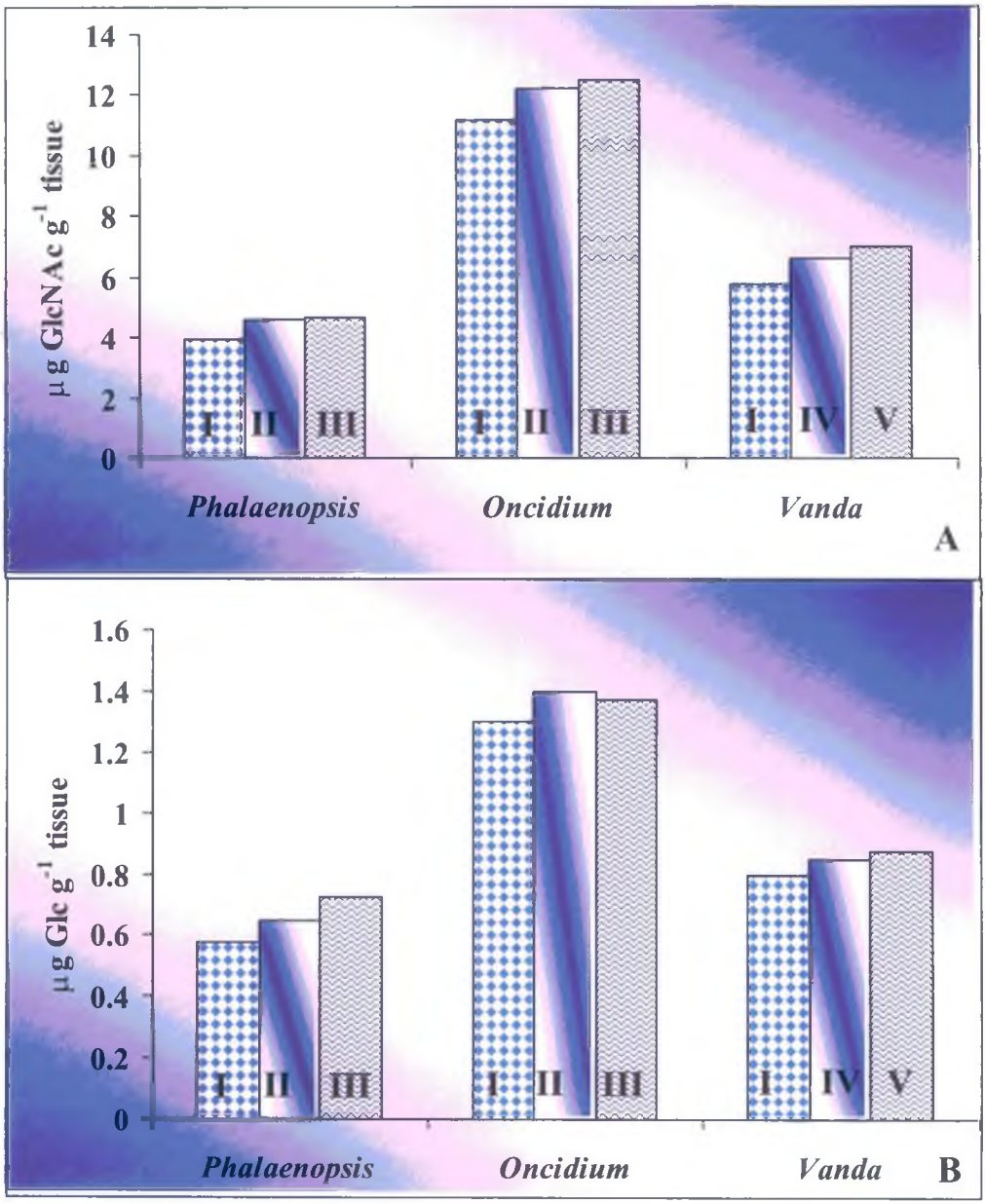


Fig. 27: Effect of *B. megaterium* and *F. lamaoensis* on enzyme activities in orchids (A: Chitinase and B: β-1,3-Glucanase).
[I: Control; II: *S. rolfsii* II; III: *B. megaterium* + *S. rolfsii* II; IV: *S. sclerotiorum;* V: *B. megaterium* + *S. sclrotiorum*]

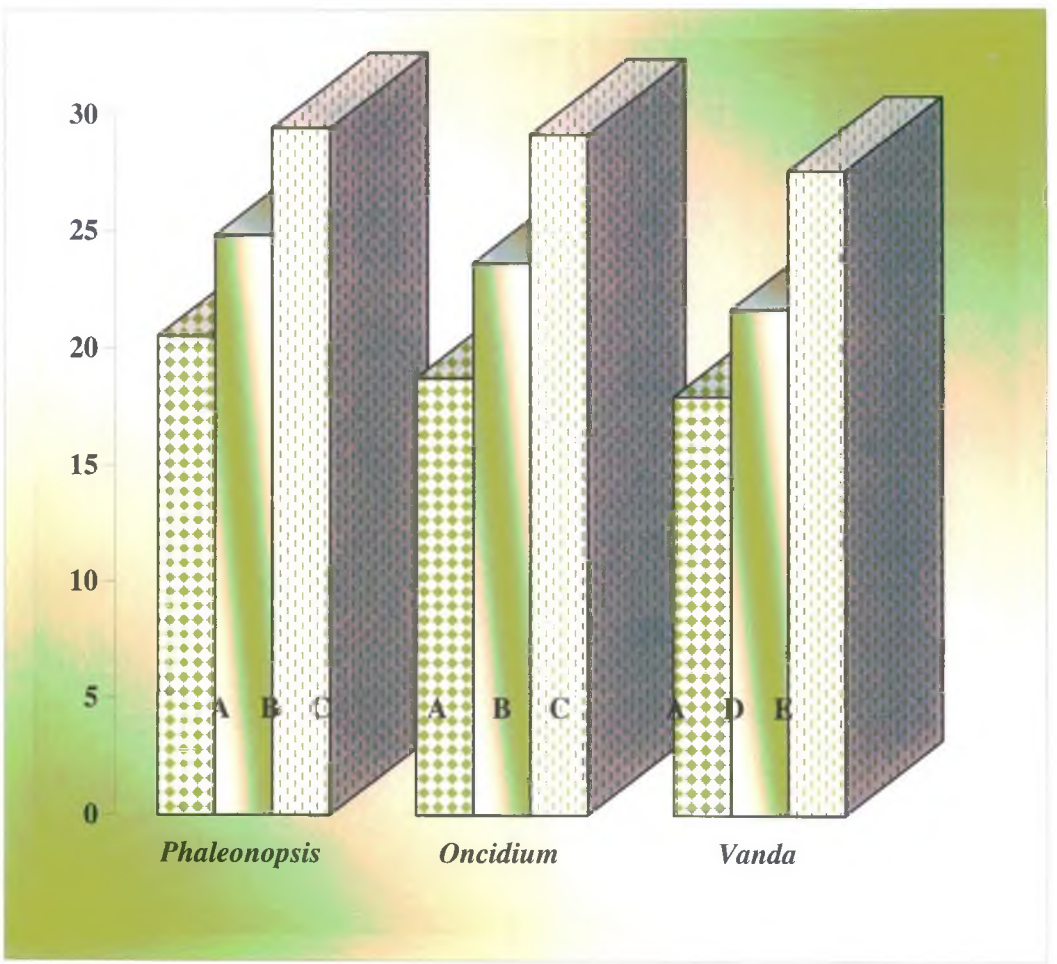
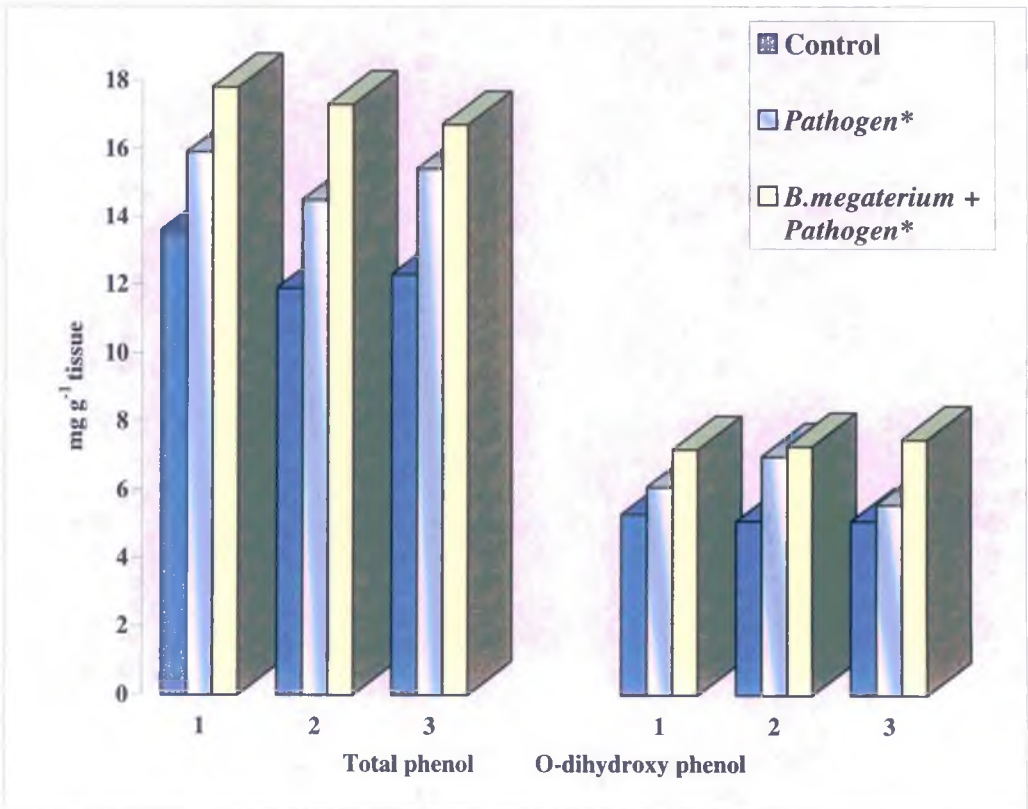


Fig. 28: Effect of challenge inoculation of *S. rolfsii* I and PGPRs on protein content of orchid

[A: Control; B: *S. rolfsii* II; C: *B. megaterium* + *S. rolfsii* II; D: *S. sclerotiorum* ; E: *B. megaterium* + *S. sclerotiorum*]



* *S. rolfsii* II in case of *Phaleonopsis* and *Oncidium* and *S. sclerotiorum* in case of *Vanda.*

Fig. 29: Changes in Phenol content in orchid infected with pathogen
(1: *Phaleonopsis* ; 2: *Oncidium* ; 3: *Vanda*)

Table 29: Effect of pathogen on the chlorophyll content of orchid plants

Variety	Treatment	Chlorophyll content (mg g^{-1} tissue)		
		Total Chl.	Chl. a	Chl. b
Vanda	Control	223.91±	132.03	91.88
	B. megaterium	244.67	146.07	98.77
	B. megaterium+ *S. sclerotiorum*	201.87	117.31*	84.56**
	S. sclerotiorum	198.36	113.17	83.19
Phalaenopsis	Control	506.17	291.37	214.80
	B. megaterium	567.34	308.58	258.93
	B. megaterium + *S. rolfsii* I	487.36	253.68	233.68
	S. rolfsii I	468.39	241.72	226.67*
Cattleya	Control	218.97	126.03	92.94
	B. megaterium	242.01	140.63	101.45*
	B. megaterium + *S. rolfsii* I	206.13*	103.19	102.94
	S. rolfsii I	196.28	98.37	97.91

* Difference with control significant at P= 0.05; ** insignificant and rest significant at P= 0.01 as done by Student's 't' test.

4.14. Studies on talc-based formulation of PGPRs

B. megaterium and *O. anthropi* as soil drench, foliar spray or seed bacterization promoted growth significantly it was further decided to prepare formulations of these bacteria in a suitable carrier and determine the efficiency of the formulations. For this, formulations of PGPR strains prepared using carboxy methyl cellulose with talcum powder as carrier material were tested under greenhouse conditions for their effect on growth promotion of tea. Seedlings of three tea varieties (UP 3, TV 18 and BSS 2) were selected. The percentage of increase in plant height and number of leaves were significantly enhanced on

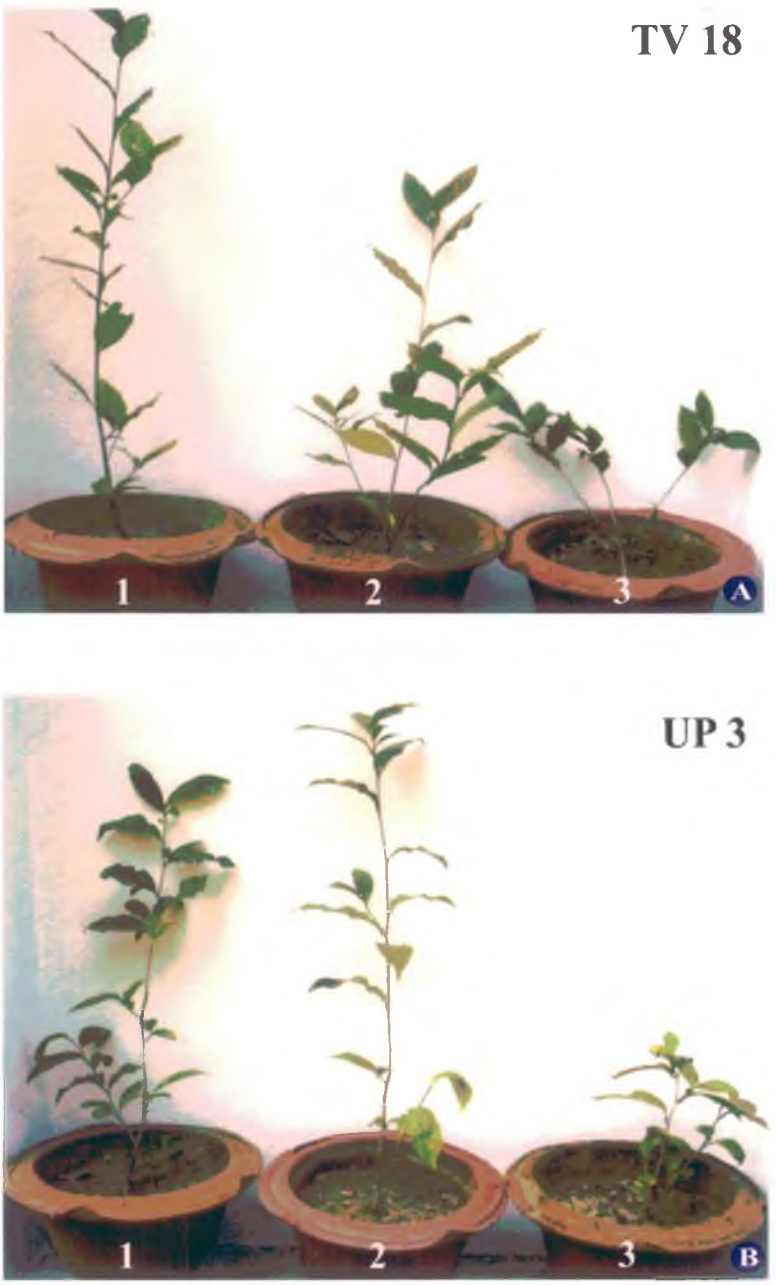


Plate XXV (A-B): Growth promotion in tea by application of PGPR based talcum formulation
[1: *O. anthropi,* 2: *B. Megaterium*; 3: Control;]

application of the talc formulation in comparison to control plants (Table 30; Plate XXV).

Table 30: Growth promotion in tea plants by talcum based formulation

Variety	Treatment	After 2 months		After 4 months	
		Increase in height (cm)	Increase in No. of leaves	Increase in height (cm)	Increase in No. of leaves
UP 3	*Control*	0.2±0.02	0.00	0.7±0.03	1±0.00
	O. anthropi	3.5±0.46[a]	3±0.33	5.0±0.29	10±0.58
	B. megaterium	5.0±0.06	3±0.58	13.0 ±0.5	7±0.58
TV 18	*Control*	0.5±0	1±0.00	1.5±0.11	2±0.00
	O. anthropi	2.5±0.11	4±0.58[a]	5.0±0.17	3±1.16[a]
	B. megaterium	4.0±0.11	2±0.00[b]	10.0±1.16	5±1.16
BSS 2	*Control*	0.5±0	1±0.00	1.5±0.23	1±0.00
	O. anthropi	2.0±1.33	2±0.58[b]	4.5±0.17	4±0.58[a]
	B. megaterium	4.0±0.11	3±0.58[a]	9.0±0.06	15±1.16

Average of 10 replicate plants per treatment; [a]- difference with control significant at P= 0.05; [b]- insignificant and rest significant at P= 0.01 as done by Student's 't' test

4.15. Immunodetection of PGPRs and pathogens

4.15.1. Detection of PGPRs in soil

4.15.1.1. Optimization

Prior to using purified polyclonal antibodies (IgG) in different tests, optimization was done.

4.15.1.1.1. Immunodiffusion

The effectiveness of antigen preparations from bacterial cell for raising PAbs against each preparation was checked by homologous cross reaction following agar gel double diffusion tests. PAbs raised to bacterial cells, which were purified were used for immunodiffusion tests. Results have been presented in Plate XXVI C & D). Strong precipitin reactions were observed in both 1st and 2nd bleedings.

4.15.1.1.2. ELISA

Polyclonal antibodies (PAbs) were raised against antigens from *B. megaterium* and *O. anthropi* and optimization tests initially were done with two PAbs separately. PAbs in each case were collected by different bleeding at definite time intervals and experiments were done with the purified IgG fraction of the PAbs.

4.15.1.1.2.1. Antigen concentration

Doubling dilution of *B. megaterium* and *O. anthropi* antigen ranging from 40 to 0.312 μg ml^{-1} tested against IgG at a concentration of 40 μg ml^{-1} (Fig. 30A). ELISA values decreased concomitantly with the antigen concentration but the values were still quite high indicating that the range of detection could be much lower.

4.15.1.1.2.2. IgG concentration

Doubling dilution of IgG purified from PAbs obtained were tested against homologous antigens at a concentration of 10 μg ml^{-1}. Absorbance values decreased from 40 to 0.312 μg ml^{-1}. A_{405} values on the other hand increased with the different bleeding (Fig. 30B).

4.15.1.1.3. Test with soil antigens

The sustainability of applied bacteria in the tea rhizosphere was determined initially by ELISA using PAbs raised against *B. megaterium* and *O. anthropi*. The result showed that the bacteria could successfully survive in the rhizosphere even after 6 months of inoculation (Table 31 & 32). The bacteria survived equally well in rhizosphere when applied individually or in combination.

4.15.1.2. Dot-Blot

In the present study, dot blot was also used to detect the antigen-antibody

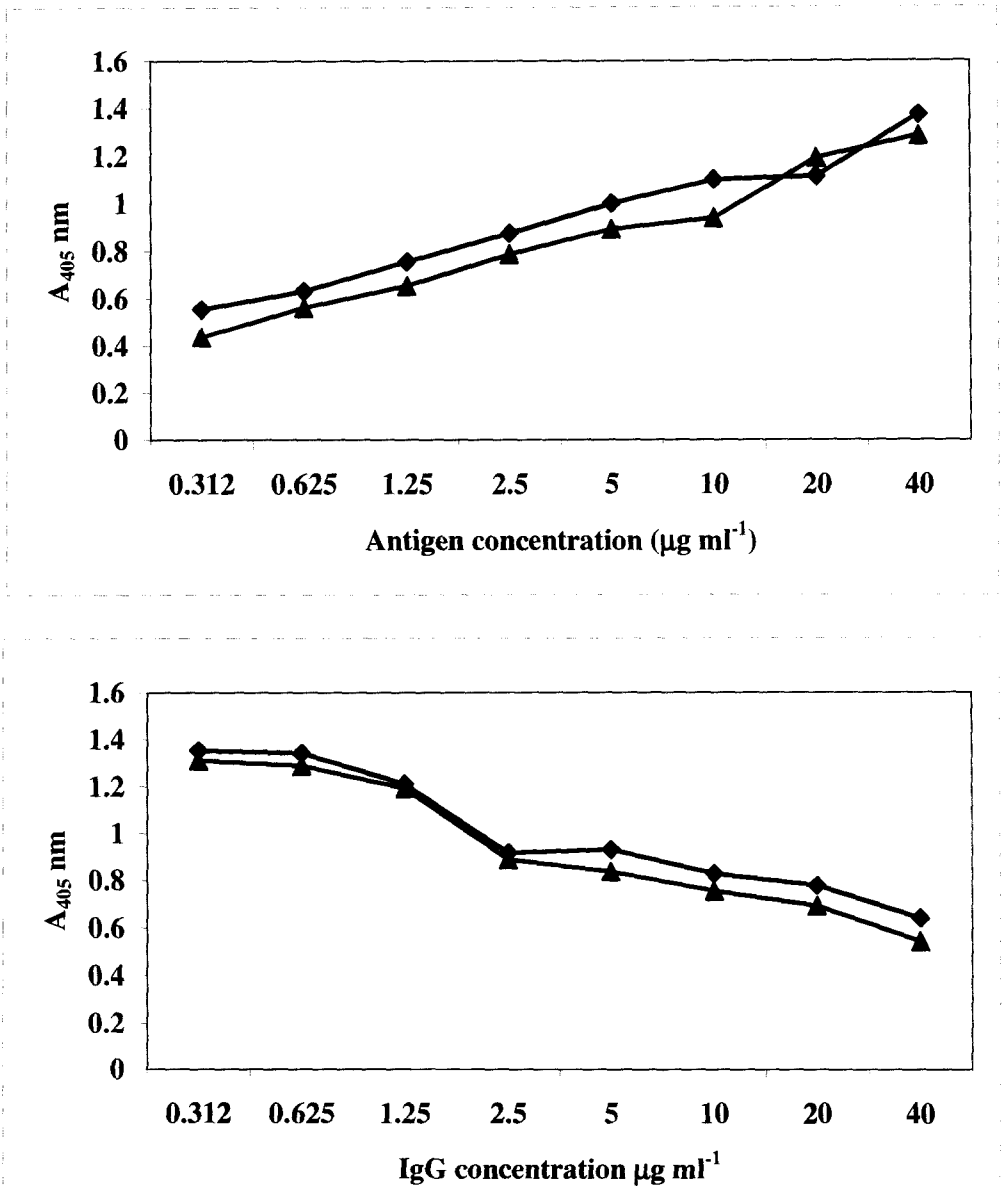


Fig. 30: Optimisation of PAbs of *B. megaterium* and *O. anthropi* and their respective antigens

reactions in different combinations. Homologous reactions of bacterial antigen showed positive reaction i.e. violet color developed when NBT/BCIP was used as the substrate PAbs used in the experiment were raised against the respective bacteria. When the antigen prepared from bacteria amended soil of rhizosphere region was probed with PAbs of bacteria, violet color developed indicating the survival of bacteria in the rhizosphere of plants (Plate XXVI E&F).

Table 31: Detection of survival of *B. megaterium* in soil after direct application

Antigen source: Rhizosphere soil from	Absorbance at 405 nm			
	Uninoculated	Time after bacterial application		
		Immediate	15 days	6 months
TV 18[1]	0.347±0.04	1.058±0.07	1.052±0.09	1.031±0.02
S 449[1]	0.243±.017	1.086±0.09	1.025±0.04	0.989±0.06
T 78[1]	0.542±0.19	1.400±0.12	1.365±0.09	1.071±0.02
T 78[2]	0.221±0.03	1.345±0.06	1.244±0.21	0.990±0.05
TV 26[1]	0.459±0.11	1.308± 0.11	1.132±0.11	1.041±0.03
TV 26[2]	0.440±0.09	1.234±0.04	1.230±0.11	1.068±0.05

Average of 3 replicates; Pab dilution : 1:1000; ±: Standard error

[1]- *B. megaterium* applied alone; [2]- *B. megaterium* applied in combination with *O. anthropi.*

Table 32: Detection of survival of *O. anthropi* in soil after direct application

Antigen source: Rhizosphere soil from	Absorbance at 405 nm			
	Uninoculated	Time after bacterial application		
		Immediate	15 days	6 months
TV 18[1]	0.347±0.04	1.157±0.07	1.150±0.08	1.050±0.02
S 449[1]	0.243±.017	1.096±0.08	1.051±0.04	0.889±0.07
T 78[1]	0.542±0.19	1.320±0.12	1.310±0.09	1.111±0.03
T 78[2]	0.221±0.03	1.354±0.05	1.204±0.12	0.999±0.07
TV 26[1]	0.459±0.11	1.343± 0.13	1.121±0.10	1.011±0.04
TV 26[2]	0.440±0.09	1.254±0.04	1.211±0.13	1.118±0.06

Average of 3 replicates; Pab dilution: 1:1000; ±: Standard error

[1]-*O. anthropi* applied alone; [2]- *O. anthropi* applied in combination with *B. megaterium*

4.15.1.3. Bacterial colony transfer

By this method, specific bacteria could be directly detected on nitrocellulose membrane. Soil suspensions were prepared by using bacteria inoculated rhizosphere soil and applied on the surface of 20 ml of solidified NA medium in sterile petriplates. The colonies that appeared after 24 h incubation were transferred to NCM and probed with respective PAb. Violet dots were observed in NCM which showed the survival of antagonistic bacteria in the soil (Plate XXVI).

4.15.2. Detection of PGPRs in formulation

The viability of formulation was tested during storage period of 60 days and 120 days by ELISA. The absorbance result showed that bacterial population declined gradually during the storage but when applied in the field the bacterial could successfully establish themselves in the rhizosphere (Table 33).

4.15.3. Detection of pathogens in soil

4.15.3.1. ELISA

The presence of *F. lamaoensis* in the soil was detected by ELISA using specific PAb of *F. lamaoensis*. Population of *F. lamaoensis* in soil was relatively much lower on treatment of the soil with *B. megaterium* and *O. anthropi* (Table 34). In ELISA, the antigens from the soil inoculated with bacteria and pathogen showed significantly lower A_{405} value than the antigens from soil treated with *F. lamaoensis* alone. This showed that population of *F. lamaoensis* in soil was reduced by the application of PGPRs.

4.15.3.2. Dot –Blot

Dot-immunobinding technique was used for detection of pathogen in the rhizosphere. When the antigen prepared from the rhizosphere soil amended with pathogen (*F. lamaoensis*) alone and from soil treated with PGPR (*B. megaterium* and *O. anthropi*) followed by pathogen inoculation was probed with PAbs of

F. lamaoensis. Violet colored permanent dots were visible in pathogen alone treated samples indicating the survival of the *F. lamaoensis* in the rhizosphere of the plants whereas the samples from both PGPR and pathogen treated showed very faint color indicating the inability of pathogen to survive in presence of PGPR (Table 34 & Plate XXVI G & H).

Table 33: Detection of survival of PGPR in rhizosphere after application as talcum based formulation

Antigen source	Probed with PAb of	Absorbance at 405 nm	
		60 days	120 days
Talcum	*B. megaterium*	1.560±0.03	0.919±0.04
Rhizosphere soil of UP 26		1.086±0.02	1.021±0.09
UP 3		1.051±0.02	1.030±0.03
CP 1		1.005±0.01	0.954±0.04
Talcum	*O. anthropi*	1.760±0.03	1.321±0.04
Rhizosphere soil of UP 26		1.039±0.04	1.010±0.05
UP 3		1.071±0.02	0.961±0.07
CP 1		1.021±0.05	0.983±0.01

Average of 3 replicates plants per treatment

Table 34: ELISA and dot blot values of soil antigens from different treatments after reaction with PAb of *F. lamaoensis*

Soil antigen*	ELISA A_{405} values	Dot Blot Color intensity**
Uninfested soil	0.208 ± 0.006	–
Treatment		
F. lamaoensis	1.086 ± 0.03	+++
B. megaterium	0.006 ± 0.00	–
F. lamaoensis + *B. megaterium*	0.554 ± 0.02	+
O. anthropi	0.009 ± 0.00	–
F. lamaoensis + *O. anthropi*	0.621 ± 0.02	+

Average of 3 replicates; Pab dilution: 1:500; *Sample collected 30 days after inoculation with pathogen; ** Color intensity of dots – Deep violet: +++ ; insignificant + ; No color :

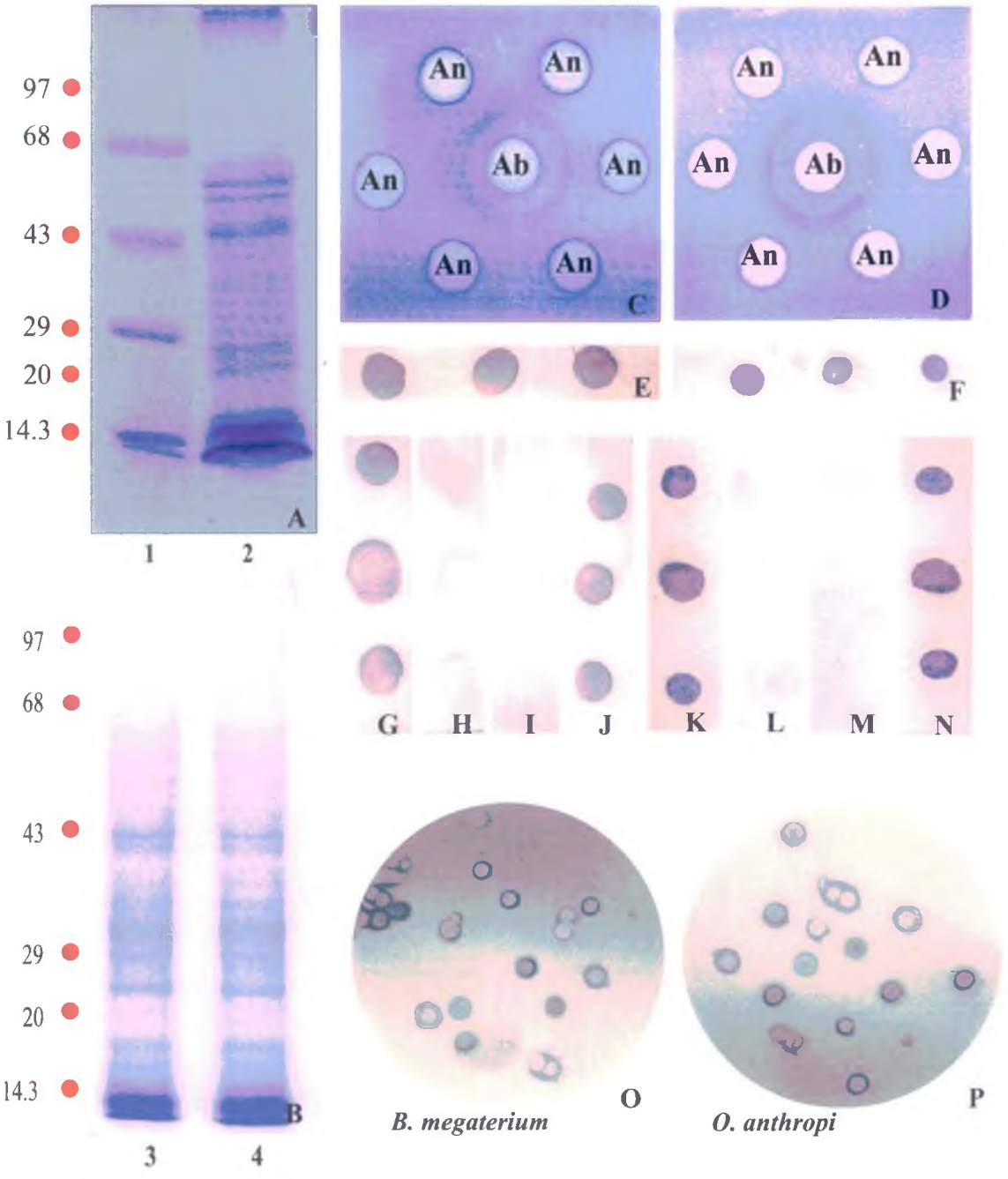


Plate XXVI (A-P) A: SDS-PAGE analysis of proteins extracted from *B. megaterium* and [Lane 1- Marker and Lane 2- Protein of *B. megaterium*] B: *O. anthropi* [Lanes 1& 2- Proteins of *O. anthropi*] C: Agar gel double diffusion tests performed with PAbs (Ab) raised against *B. megaterium* (C) and *O. anthropi* (D) with homologous antigens (An); E-N: Dot immunobinding assays. E & F: Homologous reactions of PAbs of *B. megaterium* (E) and *O. anthropi* (F); G-I & K-M: PAbs of *F. lamoensis* treated with different antigens. Antigens from soil inoculated with *F. lamaoensis* (G & K); with *B. megaterium* & *F. lamaoensis* (H); *B. megaterium* (I) *O. anthropi* & *F. lamaoensis* (L) and *O. anthropi* (M). Soil inoculated with *B. megaterium* (J) and *O. anthropi* (N) probed with homologous PAbs. O & P: Bacterial colony transferred in NCM probed with homologous Pab.

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.

SUMMARY

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

2. Materials used in this investigation and experimental procedures followed have been discussed in detail.

3. Microorganisms were isolated from soil samples collected from rhizosphere of healthy tea plants growing in different regions at foothill of Darjeeling Himalayas. Screening of bacteria was carried out against root pathogens like *Fomes lamaoensis, Sphaerostilbe repens, Poria hypobrumea, Sclerotium rolfsii* I, and orchid pathogens like *Sclerotium rolfsii* II and *Sclerotinia sclerotiorum* by dual pairing tests.

4. Four antagonistic bacteria were isolated out of which two were selected for further experimental purposes due to their better efficiency in antagonizing the test pathogens. These were identified as *B. megaterium* and *O. anthropi.*

5. The two selected bacteria were characterized *in vitro* for their cultural characteristic as well as for their plant growth promoting activities. Both the bacteria had the property of P-solubilisation, production of IAA, siderophores and volatiles.

6. Active principles were isolated both from the cell free culture filtrates and whole cells of the two bacteria which showed antifungal activity in spore germination and radial growth bioassays. Culture filtrates were heat stable.

7. Solvents extracts of culture filtrates also showed inhibitory activities in bioassays. Diethyl ether fraction was most effective.

8. The plant growth promoting ability of the antagonistic bacterial isolates were evaluated in greenhouse using different bacterization methods such as soil drenching or foliar application in tea and seed bacterization in *Cicer arietinum.* All the methods were equally efficient. The bacteria were applied singly or in dual combination.

9. The growth promotion of different varieties of tea seedlings was observed in terms of growth parameters such as increase in shoot length, number of leaves

and shoots and Leaf Area Index. It was observed that in relation to control, tea plants subjected to single or dual application showed increased growth. Both *B. megaterium* and *O. anthropi* significantly promoted the growth of the plants. Similarly, on bacterization of *C. arietinum* seeds, the germination percentage as well as vigour index of the plants were greatly increased. Foliar spraying with the suspension also led to significantly better growth of the shoots. More luxuriant growths were obtained.

10. In addition to growth promotion, biochemical changes such as enzyme activities (Chitinase, β-1,3-Glucanase, Phenylalanine ammonia lyase, Peroxidase and Polyphenol oxidase), protein, phenol and chlorophyll content associated with PGPR application were also studied. Higher activities of PO, PAL, CHT, β GLU and PPO and accumulation of higher phenolic compound in PGPR treated plants were observed.

11. On Native PAGE analysis of PO and PPO it was observed that all the isoforms were constitutively present in untreated control plants and in treated plants. However, maximum intensities of bands were noticed in the treated plants. Soluble proteins extracted from control and PGPR treated tea plants were analyzed by SDS-PAGE. Though a large number of bands appeared, no new proteins could be discerned.

12. HPLC analysis was performed with the catechins extracted from tea leaves treated with PGPRs either by soil drenching or by foliar application. Appearances of new isoforms of catechins were observed in different treatments.

13. Higher enzyme activities were recorded in plants sprayed with the PGPRs in combination than in individually sprayed plants. Total protein content was significantly higher in plants when *B. megaterium* and *O. anthropi* were sprayed in combination than in individually sprayed plants.

14. Greenhouse experiments were conducted to study the effect of *B. megaterium* and *O. anthropi* on brown rot caused by *F. lamaoensis* in tea and effect of *B. megaterium* on crown rot caused by *S. rolfsii* II in orchids (*Phaleonopsis, Oncidium*), collar and root rot caused by *S. rolfsii* I in *C. arietinum* and white rot

caused by *S. sclerotiorum* in *Vanda.* Disease development was significantly reduced in PGPR treated plants compared to control plants that were not treated with PGPR strain.

15. The biochemical response of plants such as defense related enzyme activities *viz.* (CHT, βGLU, PO and PAL), proteins, phenols and chlorophyll content were determined where PGPRs (*B. megaterium* or *O. anthropi*) as well as pathogens were inoculated in the soil. Increase in the activity of various defense related enzymes indicated the induction of systemic resistance against pathogen infection.

16. PGPR formulation was developed in talcum powder. The viability of talcum formulation was tested during 60 days and 120 days of storage by ELISA. Evaluations were conducted on tea for growth promotion. All treatments with formulation showed enhancement in growth over untreated controls.

17. The present study suggests that the tested PGPRs could survive in talc based formulations and could be used as fresh suspensions and powdered formulations.

18. Polyclonal antibodies (PAbs) were raised against antigens prepared from *B. megaterium* and *O. anthropi.* These were purified by ammonium sulphate precipitation followed by DEAE-Cellulose chromatography. The IgG obtained in each case was used for immunodiffusion and ELISA.

19. Agar gel double diffusion tests were performed using crude antibody. Strong precipitins were obtained in both the bacteria.

20. The bacterial sustainability in the soil was evaluated by ELISA and Dot-blot. This was confirmed by plating the soil suspension prepared by using the bacteria inoculated soil. The colonies thus formed were transferred to the nitrocellulose membrane and probed with respective antibodies.

21. The presence of pathogen in the soil treated with PGPRs prior to pathogen inoculation as well as in untreated plants was detected by immunological techniques such as ELISA and Dot-blot. The result revealed that the pathogen could not survive much in presence of PGPR.

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