# EXPLOITATION OF RHIZOSPHERE MICROORGANISMS OF TEA FOR PROTECTION AGAINST ROOT ROT PATHOGENS

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


PRABIR ROY CHOWDHURY, M.Sc

IMMUNO-PHYTOPATHOLOGY LABORATORY
DEPARTMENT OF BOTANY
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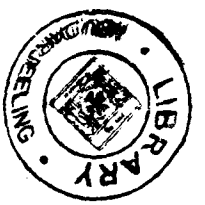


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# UNIVERSITY OF NORTH BENGAL
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September 17, 2008

TO WHOM IT MAY CONCERN

This to certify that Mr. Prabir Roy Chowdhury has carried out his research work under our supervision. His thesis entitled "***Exploitation of rhizosphere microorganisms of tea for protection against root rot pathogens***" is based on his original work and is being submitted to the University of North Bengal for the degree of Doctor of Philosophy (Science) in Botany, in accordance with rules and regulations of the University of North Bengal.

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# Acknowledgement

*Words are chameleons, which reflect the color of their environment. Hence for revealing ones emotions and feeling though words may seem to be an exercise in deception there is hardly any proxy to voice the same in such a sublime event as this. Knowing fully well that acknowledgement of any magnitude, however colossal is too small a gesture, I can't help doing that.*

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Prabir Roy chowdhury
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# Contents

Tea [*Camellia sinensis* (L.) O. Kuntze] is an important plantation crop of India and the most common non-alcoholic beverages of the world used since ancient times. India is the largest producer and exporter of processed tea with over 30% share in the global tea production. Thus, tea holds a key position in Indian economy generating over 660 million US dollars of foreign exchange annually. Tea is grown in more than 30 countries around the world from Georgia (CIS) 43° N latitude to Nelson (South Island) in New Zealand 42° S latitude, and altitudes varying from sea level to 2300 m above mean sea level. In India, it is cultivated over an area of 4, 28,000 ha, most of it in the states of Assam, West Bengal, Tamil Nadu and Kerala.The other states having fairly large area (>2000 ha) under tea cultivation area Tripura, Himachal Pradesh, Uttar Pradesh and Karnataka. There are three species namely *C.sinensis*, *C.assamica* and *C.assamica spp. laxicolyx* which have been cultivated in India.

Tea plants, as all other plants, are subjected to various environmental stresses. Being a perennial, the tea plant has to withstand such adverse conditions year after year and successful growth and production of the plants are influenced to a great degree by the response of the plant to the environment. The root environment is of great significance as the plants, growing in the same soil year after year, have to depend on the particular soil for all their nutrients and water requirements.

For excessive production and keeping away disease causing organisms, huge amount of chemical fertilizers and pesticides are used in tea gardens. The excessive application of chemical fertilizers causes soil sealing, fertility diminishing, and residual problems. The leftover of chemical fertilizers and pesticides have seriously affected the quality of products, people's health and caused environmental pollution. The over use of fertilizers has also damaged the soil's original micro-ecological balance and deteriorated the diseases spread by soil. These ecological, environmental and human health problems have increased public awareness and attempts are being made to search for effective alternative approaches which have minimal deleterious effects, more environmentally friendly, and will contribute to the goal of sustainability in agriculture. In this line plant growth promoting rhizobacteria (PGPR) present immense potential and promise as effective substitutes for agro-chemicals. Exploitation of soil microorganisms offers an attractive alternative to use of agro-chemicals.

The term 'Rhizosphere' was introduced by Hiltner in 1904, and refers to the area surrounding the root whose physiological and physical features are affected by the root. The rhizosphere has attracted much interest since it is a habitat in which several biologically important processes and interactions take place.Rhizosphere constitutes: internal, surface and external. Internal rhizosphere refers to the cell of the root components. Surface refers to the root surface; the external rhizosphere refers to the area around the root. Microbial population is of greater densities in rhizosphere than the non-rhizosphere zone. It is caused by secretion of root exudates and casting of sloughed off root tissue by plant in soil during growth phases(Baker and Snyder,1965).Rhizosphere micriflora,may vary greatly with the age of the plant and different localities of growth. Microorganisms always interact among themselves and with the plant. Some of these interactions are beneficial to the plant, while others are deleterious, and the growth of the plant is, in turn affected by these interactions. These microorganisms grow in close association with the plant roots and forming a cover around the root are referred to as Rhizobacteria (Bashan,1998).They select and regulate the root's function in secreting and absorbing nutrients.PGPR was first defined by Kloepper and Schroth (1978). PGPR mainly constitute species of *Azotobacter*, *Azospirillum*, *Pseudomonads*, *Acetobacter*, *Burkholderia*, *Enterobacter*, *Streptomyces* and bacilli. PGPRs have been applied in a wide range to the agricultural field for the purpose of growth enhancement, including increased seed emergence, weight, crop yield and disease control (Glick, 1995; Aino *et al.* 1997; Ait Barka *et al.* 2000; Dobbelaere *et al.* 2003)

The mechanism by which PGPR increases plant growth is not well understood. There are several PGPR inoculants currently commercialized that seem to promote growth through at least one mechanism; suppression of plant disease (termed Bioprotectants), improved nutrient acquisition (termed Biofertilizers), or phytohormone production (termed Biostimulants).They suppress plant disease through at least one mechanism; induction of systemic resistance, and production of siderophores or antibiotics. Exposure to the PGPR triggers a defense response by the crop as if attacked by pathogenic organisms. The crop is thus armed and prepared to mount a successful defense against eventual challenge by a pathogenic organism.Siderophores produced by some PGPRs scavenge heavy metal micronutrients in the rhizosphere (e.g. iron), starving pathogenic organisms of proper

nutrition to mount an attack of the crop.Interestingky, plants seem capable to still acquire adequate micro-nutrient nutrition in presence of these PGPR.Antibiotic producing PGPRs release compounds that prevent the growth of pathogens.

Biofertilizers are also available for increasing crop nutrient uptake of nitrogen from nitrogen fixing bacteria associated with roots (*Azospirillum*),iron uptake from siderophore producing bacteria(*Pseudomonas*), sulfur uptake from sulfur-oxidizing bacteria(*Thiobacillus*),and phosphorus uptake from phosphate-mineral solubilization bacteria (*Bacillus,Pseudomonas*). Species of *Pseudomonas* and *Bacillus* can produce as yet not well characterized phytohormones or growth regulators that cause crops to have greater amounts of fine roots which have the effect of increasing the absorptive surface of the plant roots of uptake of water and nutrients. These PGPRs are referred to as Biostimulants and the phytohormones they produce include indole-acetic acid, cytokinins, gibberellins and inhibitors of ethylene production.

There are a large number of pathogens in the soil causing a number of root diseases. Manipulation of soil environment has become a tool for biological control of such soil borne plat pathogens. Manipulation can be done by artificial introduction of antagonists in the soil or spraying these antagonists on the aerial parts of plants.

The study of rhizosphere microflora and the exploitation of potentially antagonistic microorganisms for suppression of root diseases have been carried out by several workers. Among the most commonly used microorganisms are the fluorescent Pseudomonads, *Bacilli, Serratia* and *Trichoderma* and *Gliocladium*. It has been. reported that the saprophytic microflora of the rhizosphere includes doth deleterious and beneficial organisms that have potential to influence plant growth and crop yield significantly (Lynch,1982; Chen *et al.* 1995; Bloemberg *et al.* 2001; Compant *et al.* 2005; Gnanamanickam *et al.* 2007; Chakraborty *et al.* 2007; Christopher and West, 2007).The beneficial microorganisms affect plant growth positively by mechanisms which include promotion of the availability and uptake of mineral nutrients (Loper *et al.* 1999; Kuiper *et al.* 2001), provision of plant growth substances (Gaskins *et al.* 1984; Ping and Boland, 2004), and importantly, suppression of deleterious microorganisms (Schipper *at al.* 1997; Dekkers *et al.* 1998; Ait Barka *et al.* 2000) and modify cropping environment to enhance productivity (Lynch,1990; De Weger *et al.* 1995). The deleterious microorganisms, on the other hand, affect plant growth


**Plate I:** Tea garden in Hill (A) and Plain (B).

negatively. Reports on the role of fluorescent Pseudomonad in suppression of diseases in experimental conditions or in suppressive soils are well documented. According to Cook and Baker (1983), soil microflora determines two types of mechanisms of suppression: one is called general, other is specific. General suppression is achieved by the whole microflora; the higher microflora biomass and activity, higher the suppressiveness.In contrast with the biocontrol achieved by non-pathogenic *Fusarium oxysporum* or by fluorescent Pseudomonads, suppression of *Fusarium* wilts in naturally suppressive soils operates very effectively under various experimental conditions. The consistency of suppression might be related to the diversity of the microbial population present in the soils. With a view to stimulating the complex microbial interaction responsible for soil suppressiveness, several workers have used a combination of both antagonistic microorganisms in biocontrol experiments (Fuchs and Defago, 1991; Leeman *et al.* 1996; Schisler *et al.* 1997; Park and Kloepper, 2000; Quazi and Vyas, 2007).

It has also been suggested that induction of resistance in the host may also play an important role in the observed disease suppression. Van Peer *et al.* (1991) also reported experimental evidence for the inclination of bacterial induction of host resistance against *Fusarium* wilt.Siderophore production was also postulated to be an important mechanism for the biocontrol activity of PGPR (Castognetti *et al.* 1986; Bakker *et al.* 1993). Ongena *et al.* (2000) reported that protection of cucumber against *Pythium* rot by some strains of *Pseudomonas* was neither due to the production of siderophores, nor to antibiosis, but by induction of antifungal phenolics in the host root.

The use of biocontrol agents for the protection of plants against pathogens both *in vivo* and *in vitro* have been reported by a number of authors.*T.harzianum* and *T.viride* have been widely used for biocontrol purposes especially those of root disease. Tea phyllosphere fungus *Sporothrix* sp. antagonistic to tea anthracnose fungus *Colletotricum theae-sinensis* was reported by Yamada (2004), Sain and Gour (2005) reported that *Bacillus* and *Pseudomonas sp.* could be used to control *Xanthomonas axonopodia pv. cyamopsida, X.campestris* and *X. campestris pv. glycine* causing blight in cluster bean, black rot of cabbage and leaf pustules in soybean, respectively. Meshram *et al.*(2007) were evaluated fluorescent Pseudomonas and

*Bacillus subtilis* isolated from cotton rhizosphere against *Fusarium* wilt (*Fusarium oxysporum* f. sp. vasinfectum) and *Macrophomina* stem break/dry root rot (*Macrophomina phaseolina/ Rhizoctonia bataticola*) disease in cotton. Singh *et al.* (2007) were also reported that *Trichoderma* strains protect tomato plants against *Fusarium* wilt .There are also reports of use of *Trichoderma* and *Gliocladium* as plant protecting agents (Sharma, 2000; Wahad, 2000). In tea, Chakraborty *et al.* (1995, 1997 and 1998) reported the use of antifungal strains of *Micrococcus luteus*, *Bacillus pumilus* and few other antagonistic bacteria isolated from the phyllosphere for control of foliar fungal diseases. Chakraborty *et al.* (2003, 2004, 2006 and 2007) were also reported tea rhizosphere microorganisms, *Bacillus pumilus, B. megaterium, Serratia marcescens* and *Ochrobactrum anthropi* not only control root rot pathogens but also promote plant growth.

In recent years, Indian tea is facing the prospect of losing out on tea export to traditional countries because of two major facts – the first one being that other countries like Kenya,Sri Lanka etc are increasing production rapidly and have become competitors of India; secondly countries to which orthodox Darjeeling tea were being exported are now insisting on reduced use of pesticides/fungicides for health purposes.Hence,the time has now come to look for means of reducing chemical outputs in tea, which may be achieved by increasing use of plant growth promoting or antagonistic microbes in tea phyllosphere/rhizosphere.

*Poria hypobrunnea* Petch. causes a widely prevalent stem-cum-root disease of tea in all the tea growing areas of Terai and Dooars. It is a wound parasite which gains its entrance into the frame of the bush through wound, especially on thick branches, caused by various agencies and gradually extends down to the roots when the affected bushes are completely killed. Thin films and small cushions of yellow-brown mycelia are produced on the root surface as well as on the wound, beneath the bark. Wound is yellowish, soft and decayed, marked with thin, irregular, light-brown lines and permeated with thin sheets of yellow brown mycelium.

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 special objectives:

(a) Isolation and identification of rhizosphere microorganisms from different varieties of tea plants of various ages growing in different localities and determination of their microbial population.

(b) *In vitro* testing of the isolated microorganisms against the root rot pathogens causing brown root rot, violet root rot and branch canker disease of tea.

(c) Selection of microorganisms showing antagonistic activity towards the root rots pathogens.

(d) *In vivo* testing of the isolated antagonists for disease suppression.

(e) Determination of plant growth promoting activity if any in these antagonistic microorganisms

(f) Selection of such microorganism(s) showing both disease reduction and plant growth promotion.

(g) Extraction of active principles from the selected microorganisms.

(h) Bioassay of the active principles and optimization of their production in their culture

(i) Determination of the population of the antagonists as well as pathogen in the soil after definite period.

Rhizosphere is the harbor for a large number of micro-biota, some are beneficial by their positive effect on the plant and others are parasites or pathogens of the plant host. The group of beneficial microbes is designated as the plant growth promoting rhizobacteria (PGPR) due to their growth promoting influence on the phyto-biota.The PGPR activities must be preceded by rhizospheric establishment, regulated strongly by the rhizospheric competence by the microbes.

Several possible mechanisms for plant growth promotion by the rhizobacteria have been proposed, including production of phytohormones (auxin, gibberellin, ethylene), siderophores, solubilising phosphates, fixation of atmospheric nitrogen or indirect mechanisms either by suppression of diseases caused by pathogens or by reducing deleterious rhizobacteria through forming antibiotics PGPR mediated induced systemic resistance (ISR) is an important mechanism of biological disease control. PGPR can also act as bioremediation agents. They can hold soil aggregates, creating channels through which roots grow, soil fauna move and water percolates. 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; Kloepper *et al.* 2004). 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 pathenogenesis of soil borne microorganisms. It can also be crucial for the action of microbial inoculants used as biofertilizer, biopesticides, and phytostimulators and bioremediators. *Pseudomonas* one of the best root colonizers is therefore used as a model root colonizer. A review by Lugtenberg *et al.* (2001) focused on (a) the temporal spatial description of root colonizing bacteria as visualized by confocal laser scanning microscopically 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.

Elicitation of induced systematic resistance (ISR) by plant-associated bacteria was initially demonstrated using *Pseudomonas sp* and other gram-negative bacteria. Several reviews have summarized various aspects of the large volume of literature on *Pseudomonas spp.* as elicitors of ISR. Fewer published accounts of ISR by *Bacillus sp*

are available, and Kloepper et al., (2004) revieweed this literature for the first time. Published results were summarized showing that specific strains of the species *B. amyloliquefaciens, B. subtilis, B. pasteurii, B. cereus, B. pumilus, B. mycoides* and *B. sphaericus* elicitor significant reductions in the incidence or severity of various diseases on a diversity of hosts. Elicitation of ISR by these strains has been demonstrated in green house or field triala on tomato, bell pepper, muskmelon, watermelon, sugar beet, tobacco, *Arabidopsis* sp., cucumber, loblolly pine, and two tropical crops (long cayenne pepper and green kuang futsoi). Protection resulting from ISR elicited by *Bacillus* sp has been reported against leaf-spotting fungal and bacterial pathogens, systemic viruses, a crown-rooting fungal pathogen, root-knot nematodes, and a stem-blight fungal pathogen as well as damping-off, blue mold, and late blight disease. Reductions in populations of three insect vectors have also been noted in the field: striped and spotted cucumber beetles that transmit cucurbit wilt disease and the silver leaf whitefly that transmits Tomato mottle virus. In most cases, *Bacillus* sp that elicits ISR also elicits plant growth promotion. Studies on mechanisms indicate that elicitation of ISR by *Bacillus* sp is associated with ultra structural changes in plants during pathogen attack and with cytochemical alternations. Investigations into the signal transduction pathways of elicited plants suggest that *Bacillus* sp activate some of the same pathways as *Pseudomonas sp* and some additional pathways. For example, ISR elicited by several strains of *Bacillus* sp is independent of salicylic acid but dependent on jasmonic acid, ethylene, and the regulatory gene NPR1-results that are in agreement with the model for ISR elicited by *Pseudomonas sp.* However, in other cases, ISR elicited by is dependent on salicylic acid and independent of jasmonic acid and NPR1.In addition, while ISR by *Pseudomonas sp* does not lead to accumulation of the defense gene PR1 in plants, in some cases, ISR by *Bacillus* sp does. Based on the strains and results summarized in this review, two products for commercial agriculture have been developed, one aimed mainly at plant growth promotion for transplanted vegetables and one, while has received registration from the U.S. Environmental protection Agency, for disease protection on soybean.

## 2.1. Plant growth promotion

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$^0$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$^0$C, *Serratia proteamaculans* 1-102; 17.5$^0$C, *S. proteamaculans*1-102 and *Aeromonas hydrophila* P73; 25$^0$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 the immediate protection of germinating seeds. Their strains were from the rhizosphere of wheat growing in the soil from fields where wheat had been grown continuously for many years, to help ensure that the strains were 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-diacetyphloroglucinol (PHL). The evidence is strong that PHL producing starins 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 in1997. They now have cultures of Q8R 1-96 trasformed to produce PHZ in addition to PHL.

Cattelan *et al.* (1999) selected 116 isolates from bulk soil and the rhizospere 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 sub sample 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 β-1,3-glucanase production, and two of five isolates 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 rhizobacterial able to produce ACC deaminase and, to a lesser extent, β-1, 3-glucanase or siderophores or those able to solubilze 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 filtrates of this strain exhibited three prominent bands in UV-VIS spectra between 300 and 400nm. The growth promotion assay of the extracted compound against different indicator organisms indicated the production of a compound related to a 2-0xoacid type siderophore. The HPLC of the purified ethyl acetate extract of the strains and standard 4- methyl-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).

Experiments were conducted by Lee (2000) 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 $28^0$C for certain period of time then dried in shade and stored. Seed priming with *Bacillus* strains showed even higher germination rate than SMP of chemical osmotic controllers. In pot 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 noted when compared to other treatments. Bio-primed seedling revealed twofold more root biomass than untreated control.

According to Bai (2002) *Serratia proteamaculans* 1-102 (1-102) promotes soybean-bradyrhizobia nodulation and growth, but the mechanism is unkown. 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 stimulation of 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 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 Bacterization by root dipping was chosen due to its practicability. The most efficient bacterial strains were C210, ENF 16, 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 ENF 10 plus RAB9, ENF 16 plus C2 10 and RAB9 promoted root dry weight increases of 100.3%, 88.1%, 80.1 %, respectively. Production of either IAA or HCN, and solubilisation 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.* (2002) 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 ACC deaminase. In contrast, an ACC deaminase specific product was amplified from *Pseudomonas sp.* 4MKS8 and *Klebsiella oxytoca* 10MKR7. This was the first report of ACC deaminase in *K.oxytoca*

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 (Baruah *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 increases 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.

Experiments were conducted during 2000 and 2001 to determine the effects of floral and foliar application of the bacterial 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 60 days after full bloom. 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 uncontrolled. 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 onder 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* (Be) 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 seedling. Four (Aur6, Aur9 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 the entire variable 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.

A bacterial screening was carried out by Mancro *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 characterized at genus level, *Bacillus* being the dominant genera in all cases. Fifty percent of the *Bacillus* strains isolated from each species were analyzed by PCR-RAPDs. At 85% similarity, 12 groups were selected for *D. thapsi* and 18 for *D. parviflora*. One strain of each group was selected for biological assay on *D.lanata*, growth promotion and cardenolide content in leaves after inoculation performed in the root system were noted. 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.

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 decrease of 1- aminocyclopropane-1-carboxylic acid (ACC) the immediate precursor of ethylene in plants. The enzyme

catalyzing 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 on plants, thereby lowering the concentration of ethylene.

Ramos *et al.* (2003) either inoculated alder seedling 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 inoculums levels were determined using the phospholipids 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. Effect was 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.

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 yield 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 (auxin, cytokinins, abscicic acids,lumichrome, rhiboflavin, lipo-chito-oligasaccharides and vitamins) that promote plant growth, their colonization and infection of cereal roots would be expected to increase plant development, and grain

yield. Matiru and Dakora (2003) have 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.

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 enable visualization of the bacteria omitting the fixation process during the FISH protocol. The microscopic techniques showed that the potential of strain Sp 245 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 inocula.

Activities associated with *Paenibacillus polymyxa* treatment of plants in some experiments include nitrogen fixation, soil phosphorous solubilisation, and production of antibiotics, auxin, chitinase, and hydrolytic enzymes, as well as promotion of increased soil porosity. Timmusk (2003) showed that, in stationary phase, *P.polymyxa* released the plant hormone cytokinin isopentenyladenine, in concentrations of about 1.5 nM. In a gnotobiotic system with *Arabidopsis thaliana* as a model plant, it was shown that *P.polymyxa* inoculation protects plants; challenge by either the pathogen *Erwinia carotovora* (biotic stress) or induction of drought (abiotic stress) showed that that pre-inoculated plants were significantly more resistant than control plants. By RNA differential display on RNA from *P.polymyxa* treated or control plants, changes in gene expression were tested. One mRNA, encoding ERD15 (drought stress-

responsive gene) showed a strong inoculation-dependent increase in abundance. In addition, several biotic stress genes were also activated by *P.polymyxa*.Antagonism towards the fungal pathogens *Phytophthora palmivora* and *Pythium aphanidermatum* was studied. *P.polymyxa* counteracted the colonization of zoospores of both oomycetes on *A.thaliana* roots, and survival rates of plants treated with *P.polymyxa* were much higher when challenged *P. aphanidermatum*. Using a green fluorescent protein-tagged isolate of *P.polymyxa*, colonization of *A.thaliana* roots was investigated. Authors drew two main conclusions. Firstly, the bacterium enters the root tissue (but not leaves) and is abundantly present in intercellular spaces. Secondly, the root becomes severely damaged, indicating that- under some conditions- *P.polymyxa* is a "deleterious bacterium", and in others it promotes growth. Based on his work, Timmusk proved that a balance between the activities of a PGPR, the genetic background and physiological state of a plant, and the environmental conditions employed in test system, ultimately determines the resulting effect. (Timmusk, 2003)

Timmusk *et al.* (2005) also showed that this bacterium protected Arabidopsis thaliana against pathogens and abiotic stress. They studied colonization of plant root by a natural isolate of *P.polymyxa* which had been tagged with a plasmid-borne *gfp* gene. Fluorescence microscopy and electro 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 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.

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-formimg 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 population 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 PGPRs can be successfully incorporated into soilless media in vegetable transplant production systems.

According to Jaizme-Vega *et al.* (2004) soil microbiota communities have demonstrated their crucial role in maintaining the soil ecological balance and therefore the sustainability of either natural ecosystems or agro ecosystems. 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. In a micropropagated plant system, bacterial inoculation at the beginning of the acclimatization phase must also be observed from the perspective of the establishment of the soil microbiota rhizosphere. The authors evaluated 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 froml.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 pots (up to 14.7% increase over control) and field experiments (up to 27.5% increase over control); however, the response varied with cultivars 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 cultivars. Their study suggested that potential for auxin biosynthesis by rhizobacteria could be used as a tool for the screening of effective PGPR strains.

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 population of Q8r1-96 than Q2-87 within 14 days post-inoculation (dpi), two cultivars supported relatively high population densities of the strains. Population densities normalized to root weight

were 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 the field conditions by Donate-Correa *et al.* (2005). They collected samples in two localities of the Tenerife Island: La Laguna and EI 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-positive 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 riches was found in La Laguna. The species *Pseudomonas fluorescens* showed the highest number of properties related to plant growth promotion (PGP): 1-aminocyclopropane-1carboxylate (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.

In search of efficient PGPR strains with multiple activities, a total of 72 bacterial isolates to *Azotobacter*, fluorescent *Pseudomonas*, *Mesorhizobium* and *Bacillus* were isolated from diffent 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 (50-500µg/ml), IAA production was highest in the *Pseudomonas* followed by *Azotobacter* and *Bacillus* isolates. *Azotobacter* isolates ($AZT_3$, $AZT_{13}$, and $AZT_{23}$), *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 plant system is needed to uncover their efficacy as effective PGPR.

A study was conducted by Cakmakei *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, reffered 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 solubilzing 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 green house, 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 inocula 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 depending on the inoculant strains, 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* 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 the genus *Bacillus*, nine to genus *Rhodococcus*, seven to genus *Arthrobacter*, six to genus *Serratia* and one each to genera *Chryseobacterium*, *Delftia*, *Gordonia* and *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 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.

Hafeez *et al.* (2006) isolated seventeen rhizobacteria from different ecological regions, i.e. Brazil, Indonesia, Mongolia and Pakistan 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, irrespective of the region. Fifteen isolates fixed N at rates ranging from 20.3-556.8 nmole $C_2H_2$ reduced/h/vial. Isolate 8N-4 from Mongolia produced the highest amount of indole-3-acetic acid (42.1µg/mL), produced siderophores (0.3 mg/mL) and was the only isolate that solubilized phosphate (188.7 µg P/mL). 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

Hameeda *et al.* (2007) carried out studies to re-cycle crop residues and prepare composts. Their work included isolation and characterization of bacteria for different plant growth promoting traits and antagonistic activity. Two hundred and seven bacteria were isolated from farm waste compost (FWC), rice straw compost (RSC), *Gliricidia* vermicompost (GVC) and macrofauna present in FRC. Percentage of isolates having plant growth promoting traits were 54% from FWC, 56% from RSC, 64% from GVC and 41% from macrofauna. Antagonistic bacteria were 19% from FWC, 38%fromRSC, 39%from GVC and 23% from macrofauna. Twenty-three of 2007 isolates showed plant growth- promoting traits and antagonistic activity against test fungi. These were tested for their plant growth promoting ability on sorghum and pearl millet. Twelve strains significantly increased the plant growth of sorghum and pearl millet. Five of the twelve strains were phosphate- solubilizing bacteria(PSB) and two strains were *Serratia marcescens* EB 67 and *Pseudomonas sp.* CDB 35 showed highest gluconic acid production (67 and 27 mM) and P-solubilization (1036 and 560 uM). Evalution of these potential bacteria in glasshouse condition revealed that there was significant increase in plant growth parameters of sorghum and pearl millet.

Das *et al.* (2007) reported the potentiality of native bacteria of sorghum rhizosphere for early growth stimulation in rabi sorghum. Around 10-15 per cent isolates of the native bacteria in rabi sorghum rhizosphere showed positive effect on early growth in sorghum seedlings. Selected isolates increased seed broad spectrum inhibitory effect on the growth of charcoal rot pathogen (*M. phaseolina*). Two isolates SRB26 and SRB28 had advanced the flowering time of the rabi cultivar M25-1 by 3-5 days under due to production of plant growth hormone, indole acetic acid (IAA), and pathogen suppressing siderophore by rhizobacteria. SRB28 colonized on sorghum

root and developed micro – colonies on the root epidermis which might have improved its survival and bio-efficacy in the rhizosphere. It was concluded that sorghum rhizosphere, harbored plant growth promoting as well plant growth deleterious bacteria and seed treatment with selected strains of native rhizobacteria could enhance seedling growth in sorghum, a property which might be advantageous to rainfed sorghum.

## 2.2. Biological Control

### 2.2.1 Rhizobacteria

*Pseudomonas fluorescens* strains which effectively inhibited mycelial growth of *Fusarium udum*, the pigeon pea (*Cajanus cajan)* pathogen, were isolated from the rhizoplane of different crops (Vidhyasekaran, 1997). Various powder formulations of two efficient *P. fluorescens* strains 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 pigeon pea rhizophere throughout the crop-growth period. The talc-based powder formulations effectively controlled pigeon pea will and increased yield in two field trials. According to the authors, development of powder formulation of P. *fluorescens* will aid large-scale application of biological control in farmers' fields.

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 by Raupach and Kloepper (1998). Investigations under greenhouse conditions were conducted with three cucumber pathogens-*Colletotrichum orbiculare* (causing antracnose), *Pseudomonas syringae pv. Lachrymans* (causing angular leaf spot), and *Erwinia tracheiphils* (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 inoculums. 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 seed treatment showed that intensive plant growth promotion and disease reduction and disease reduction to a level statistically equivalent to the synthetic elicitor Actigard applied as a spray.

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 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 coinnoculated. A single *P. agglomerans* application 3 days prior to the pathogen inoculation was sufficient to provide control since population of about $10^7$ CFU per kernel were established consistency, while *Pseudomonas syringae pv. Syringae.* Population dropped 100-fold to $2.0 \times 10^4$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 concentration $10^3$ 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 antagonist 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 formulation 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 bio control efficacy.

Antibiotic resistant mutants of strains of fluorescent pseudodomonads were isolated by Yeole and Dube (2001) from the rhizosphere of chili, cotton, groundnut and soybean. Isolates produced siderophores and showed plant growth promoting activity with parent crops and showed varied response in their root colonizing capability. The groundnut isolates had highest rhizosphere competence followed by soybean, cotton and chili.

The efficacy of various *P. fluorescens* isolated was tested for the management of fruit rot of chilli caused by *Colletotrichum capsici.* Among the various isolates tested *P. fluorescens isolates viz. Pfl and ATR* increased the plant growth and produced the maximum amount of indole acetic acid. P. *fluorescens* Pfl 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 (Pfs17),* 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 *Pfs3* 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 resistence, 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 *Pfs3* 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. It also caused maximum accumulation of total phenolics and Gallic acid in

all chickpea plant parts as compared to other treatment and untreated control. A direct relationship between the level of total phenolics and seedling survivability was observed. PGPR-mediated induction of phenolics compounds as a biochemical barrier in *C. arietinum* against *S. rolfsii* infection was envisaged by the authors.

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 net house under artificial inoculum conditions. Tomato seedlings var. local treated with *A. chroococcum* before transplanting along with soil application of nitrogen @ 60, 80 and 100kg $ha^{-1}$ showed complete inhibition of plant mortality (7.36%) was also observed when seedlings were treated with *A. chroococcum* only as compared to the seedling 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 incubation. Light and scanning electron microscope examination showed hypal 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 1and PS 2 also inhibited the colony growth of *M. phaseolina* by 25 and 32% respectively. Inhibitory effect of volatile substances, however, decreased with advancing 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 deficent condition. PS 2 showed higher antagogonistic activity than PS1, as evidenced by pronounced colony growth inhibition.

Fourteen plant growth promoting rhiobacteria (PGPR) isolated from rhizoticzones of field-grown green gream (*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 rhizophere bacteria was found to repress or simulate the growth of any of the *Bradyrhizobium* strains tested. However, they produced antibiotic and siderophores

and plant growth promoting substances. Nitrogen fixation and phosphate solubilization was not detected by any of the isolates. Under *in-vitro* condition, 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.

A series of laboratory, greenhouse and field experiments were conducted by Niranjan *et al.* (2003) on strains of plant growth promoting rhizobacteria (PGPR). The PGPR were tested as suspensions of fresh culture and talc-based powder formulation. 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 formulation 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 *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 62%, respectively, over the untreated control. Under experimental plot condition, prominent enhancement in growth also was observed in the disease tests. Yield was enhanced 40 and 375 over the untreated control by seed treatment with powdered formulation 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 (57%), followed by the PGPR both under greenhouse and field condition. With fresh suspensions, treatment with INR7 resulted in the highest protection (57%), followed by *B. pumilus*

strain SE34 and B. subtilis GB03, which resulted in 50 and 43% protection, respectively, compared with the untreated control. With powdered formulation, PGPR strain INR7 suppresed downy mildew effectively, resulting in 67% protection, while SE34 resulted in 58%, followed by GB03 with 56% protection. Treatment with Apron (Metalaxy) resulted in the highest protection against downy mildew under both greenhouse and field condition. Thus, the present study suggest that the tested PGPR, both as powdered formulation 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 chitson 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 application were tested: seed treatment, soil amendment, and seed treatment+soil amendment. In general, irrespective of application method, most of formulations, in comparison with the control, increased growth and vigor as measured by seed germination, seedling vigor, plant height, fresh and dry weight, leaf area, tillering capacity, number of ear heads, length and girth of ear head, 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 formulation. Formulation LS 256 and LS 257 besides being the best growth promoters were also the most efficient resistance inducers. None of the formulation 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 formulation. 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 formulation 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 PGPRs would lead to broad-spectrum protection against several pathogens under field condition 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 disease and hosts tested were southern blight of tomato (*Lycopersicon esculentum)* caused by *Sclerotium rolsii,* anthracnose of long cayenne pepper (*capsicum annuum* var. *acuminatum)* caused by *colletotrichum gloeosporisdies,* and mosaic disease of cucumber (*cucumis sativus)* caused by cucumber mosaic virus (CMV). Results showed that some PGPR strain IN937a+*B. pumilus* strain IN937b, significantly protected (p=0.05) plants against all tested disease in both seasons. Further, cumulative marketable yields were positively correlated with some treatments.

Root colonization by certain non-pathogenic bacteria can induce systemic resistance to pathogen infection 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+1.1μg $ml^{-1}$salicylic acid (SA) in a liquid casamino acid medium under laboratory condition. The bacterial inoculants induced resistantce 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 $FeCl_3, 6H_2O$) 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.

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 seedling with varied amount. The maximum amount of total phenolic 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 growth of chickpea. In the presence of culture filtrate of *S. rolfsii*, the two *Pseudomonas* strain 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 phenolic 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 trough 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 antagonist to AF 11-4 (highly toxigenic strain) *in vitro*, in field to determine their biocontrol potential. The antagonists were applied 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 reduce seed contamination occurred due to significant reduction in AF population in the rhizosphere of groundnut.

*Sclerotinia* stem rot of mustard incited by *Sclerotinia sclerotiorum* Lib. De Bary is one of the most important seed as well as soil borne diseases of mustard. Native rhizobacterial isolates of maize, wheat and chili were evaluated by Samanta and Dutta (2004) *in vitro* through dual culture method for their antagonistic activities against four impotant soil borne plant pathogens viz., *Rhizoctonia solani, Macrophomina phaselina, Sclerotium rolfsi* and *Sclerotinia sclerotiorum.* MPf-1 was found to be most effective isolate and suppressed the mycelial growth of all the four soil borne plant pathogenic fungi. Based on the culture, morphological and biochemical characteristics, five rhizobacterial isolates, MPf-1, MPf-2, ChP-1, ChP-2 and PfW1 were tentatively identified as *Pseudomonas sp.* belonging to fluorescent group and another P-2 was tentatively identified as Bacillus sp. Maximum seed germination was observed in MPf-1 and PfW1 treated seeds. Isolates from maize were found to be superior in respect to vigour index of mustard plant. Maximum phosphate solubilization and IAA production (10µg/ml) were observed in MPf-1 isolate. Four metabolites produced by MPf-1 isolate were identified and purified using TLC and HPLC. One metabolite Mp-III was identified as phenolic compound. Crude extract of MPf-1 inhibited the growth *Sclerotinia sclerotiorum* (84% at 2000 ppm). MPf-1 isolate as seed treatment and foliar spray showed better bio-protectant by causing a reduction in incidence of *sclerotinia* stem rot of mustard.

Greenhouse experiments were conducted by Anith *et al.* (2004) to the study the effect of plant growth promoting rhizobacteria (PGPR; *Bacillus pumilus* SE 34, *Pseudomonas putida* 89B61, Bio Yield, and Equity), acibenzolar-S-methyl (Actigard), and a soil amedement with S-H mixture (contains agriculture 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 transplant at the time of seeding and 1 week prior to inoculation with *Ralstonia solanacearum.* Bio Yield, formulated PGPRs that contained two *Bacillus* strains, decreased disease

significantly in three experiments. Equity, a formulation containing more than 40 different microbial strains, did not reduce wilt incidence compared with the untreated control. With inoculums at low pathogen densities of 1 x $10^5$ and 1 x $10^6$ CFU $m^{-1}$, disease incidence of Actigard-treated plants was significantly less than with nontreated plants. This is the first report of actiguard-mediated reduction of bacterial wilt incidence in a susceptible tomato cultivar. When PGPR and Actigard application were combined, Actigard plus *P. putida* 89B61 or Bio Yield 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 Actigurd with the S-H mixture significantly reduced bacterial wilt incidence in tomato in two experiments.

A pool of 11 randomly selected, uncharacterized *Bacillus pumilus* isolates from sugar beet were evaluated by Bargabus *et al.* (2004) using a high-throughput screened that utilized labotary-based tests for 2 pathogenesis-related proteins, chitinase and β-1,3glucanse, 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 laborty-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-1,3glucanse, and was preceded by biphasic hydrogen peroxide production, also found in incompatible plant-pathogen interaction in which systemic resistance is induced. A combination of glycol chitin and aniline blue plate assays correctly identified all in plant inducers of systemic acquired resistance without the inclusion of false positive identification, reducing the workload in subsequent disease challenge assays by nearly 70%.

Jeun et *al.* (2004) 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 orbicutaure.* 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-3-aminobutric 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 autoflourescent 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 autoflourescent epidermal cells at the sites of fungal penetration. Their findings suggest that PGPR and chemical inducers cause different plant response during induced resistance.

Talc based bioformulation containing cells of *Pseudomonas fluorescens, Bacillus subtilis* and *Saccharomyces cerevisiae* were evaluated for their potential to attack the mango (*Mangifera indica* L.) anthracnose pathogen C*olletotrichum gloeosporioides* Penz. under endemic conditions by Vivekananthan *et al.* (2004). The pre harvest 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 minimum induction of flowering, a yield attribute in the pre harvest stage, consequently reduced latent symptoms were recorded at the post harvest stage. An enormous induction of the defense-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 defense-mediating enzymes may collectively contribute to suppress the anthracnose pathogen, leading to improved yield attributes.

Khabbaz *et al.* (2005) isolated five bacteria from different ecosystems viz., phylloplane of paddy leaves, water pond and industry effluent were screened against the major fungal diseases of rice viz., blast, sheath blight, brown spot and foot rot. Among the collected isolate, isolate no. S12 from phylloplane of rice leaves showed promising results against *Pyricularia oryzae, Rhizoctonia solani, Helminthosporium oryzae* and *Fusarium moniliforme* by producing strong inhibition in dual culture technique. Percent inhibition of mycelial growth was recorded based on the inhibition zone. The isolate S12 was identified as *Bacillus* sp. based on the biochemical tests. The same isolate can be formulated and used effectively under field conditions.

Pure culture of *P. aeruginosa* isolated from soil and charecterised according to Bergey's manual of determinative bacteriology were studied by Sharma *et al.* (2005) for siderophore production as well as antifungal activity. Siderophore production was determined by CAS reagent using top layer method. Antifungal activity of this strain against *Fusarium moniliformae*, *Alternaria* and *Helminthosporium halbdes* was assayed by seeding the bacterial lawn with fungal dics and incubating the plates at $37^0$ C. Antifungal activity of cell free filtrate of *P. aeruginosa* was also studied. Eight-millimeter diameter wells were made in an agar plate seeded with fungal discs and filled with cell free filtrate of 5 days old culture of *P. aeruginosa*. The plates were inoculated at $37^0$ C Inhibition of growth of all three fungi by as well as cell free filtrate was observed. Inhibition of fungal pathogens is due to production of antifungal secondary metabolites by *P. aeruginosa*. Use of cell free filtrate of as a bio control agent can thus provide an eco-friendly option and hence suggested.

Bhatia *et al.* (2005) studied ten isolates of fluorescent pseudomonads from rhizosphera of sunflower, potato, maize and groundnut. All the isolates produced fluorescent pigment in succinate broth displayed siderophore production. Production of hydrocyanic acid (HCN) and indole acetic (IAA) by all the isolates was reduced besides phosphate solubilisation. Out of the ten strains, *Pseudomonas* PS I and PS II was found most potential. Bacterisation of sunflower seeds with fluorescent *Pseudomonas* PS I & PS II resulted in increased seed germination, root length, shoot length, fresh and dry weight of roots ands shoots, and yield of sunflower. Seed bacterisation with strains of fluorescent *Pseudomonas* PS I & PS II reduced incidence

of collar rot by 69.8% and 56.9% respectively, in *Sclerotium rolfsii*-infested soil, making the organism a potential bio control against collar rot of the sunflower.

A number bacterial strains isolated by from rhizophere of different crop plants including tea (*Camellia sinensia* L. (O) kuntze) were screened for their ability to suppress two root disease-brown rot and charcoal rot of tea under gnotobiotic and nurseray condition. The strains were initially selected based on their *in vitro* antibiotic against *Fomes lamaoensis* and *Ustulina zonata*, the causative organism of brown and charcoal rot disease of tea. Three fluorescent *Pseudomonas* strain designated as RRLJ, B4, RRLJ 04, RRLJ 706 and a non fluorescent *Pseudomonas* strain AMJ showed significant suppression of both the disease under nursery condition. Seed dressing of the stem cutting with these strain also enhanced the percentage of survival of cutting in the nursery condition besides enhanced the number of leaves with high chlorophyll content. The application of these strain reduced the disease incidence of charcoal rot in field condition. The bio active metabolites isolated from these strain also showed plant growth protein and disease suppression properties. RRLJ 134 produced six different bioactive metabolites of which three have been identified as phenazine analogues. (Dileep Kumar *et al.*2005)

The *Pseudomonas flouresens* 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 poly phenol oxidase (PPO) by the Pfl isolate was studied against *P. grisea* by Radjacommare (2005). Analysis of 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 Pfl 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 cinematic surface of open blue berry flowers suppresses floral infection by the mummy berry fungus *Monolinia vacciniicorymbosi.*

Out of 500 rhizobacteria isolated from soil, rhizosphere and rhizoplane of healthy tomato plants one isolate was previously selected by Romeiro *et al.* (2005) in laboratory, green house 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 gives 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) to determine the Avocado flower inoculated with a liquid commercial formulation of *Bacillus subtilis* B246 were observed at different time intervals under the scanning electron microscope (SEM). Population dynamic of the antagonistic on the flower were determined by means of total viable counts using reference culture and background counts from the control. Flowers were also inoculated with antagonistic-pathogen (*Dothiorella aromatica* and *Phomopsis perseae*) in combination to determine *in vivo* interactions. The SEM observation and population dynamics study confirmed that the antagonistic 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 *Bacillus subtilis.*

Different formulations of *Bacillus licheniformis* were evaluated on their own and in combination with prochloraz and stroburilin for their ability to reduced 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 antharacnose and SR as effectively as the chemical control of post-harvest disease when fruit were kept in cold storage to simulate export conditions. In two of 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 which can be successfully incorporated into the existing pack line.

Chakraborty *et al.* (2006) isolated *Bacillus megaterium* De Bary TRS- 4 from tea rhizosphere and tested for its ability to promote growth and cause reduction in tea plants. *In vivo* studies revealed the ability of this bacterium to promote growth of tea plants very significantly. Brown root rot disease, caused by *Fomes lamaoensis* was markedly reduced by application of the bacterium to the soil. Population of *F. lamaoensis* in soil before and after supplication of *B. megaterium*,as determined by ELISA and dot-blot using PAb raised against the pathogen, was shown to be greatly reduced in presence of the bacterium. Biochemical changes induced in tea plants were also examined. Root colonization by *B. megaterium* and subsequent inoculation with *F. lamaoensis* also led to an increase in polyphenolics, as well as in defense related enzyme- peroxidase, chitinase, □-1,3-glucanase and phenyl alanine ammonia lyase. Determination of mechanism of action of this bacterium revealed it to be able to solubilize phosphate, produce IAA, siderophore and antifungal metabolite. The plant growth promotion and reduction of disease intensity have been shown to be due to a combination of several mechanisms.

In greenhouse experiments, plant growth promoting rhizobacteria (PGPR) *Serratia marcescens* NBR11213 was evaluated for plant growth promotion and biological control of foot and root rot of betelvine caused by *Phytophthora nicotiane* (Lavania *et al.*,2006) . Bacterization of betelvine (*Piper betle* L.) cuttings with *S. marcescens* NBR11213 induced phenylalanine ammonia-lyase, perxodase, and polyphenoloxidase activities in leaf and root. Qualitative and quantitave estimation of phenolic compounds was done through high-performance liquid chromatography (HPLC) in leaf and root of betelvine after treatment with *S. marcescens* NBR1213 and infection by *P. nicitianae.* Major phenolics detected were gallic, protocatechuic, chlorogenic, caffeic, ferulic, and ellgic 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 phenolic was increased in *S. marcescens* NBR11213-treated plants infected with *P. nicotianae.* In a greenhouse test, bacrerization using *S. marcescens* NBR11213 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 biological control and their probable role in protecting betelvine against *P. nicotianae, an important soil-borne phytopathogens*fungus.

Saika *et al.* (2006) attempted to control an algal pathogen of tea, *Cephaleuros parasitica* by biological means. The pathogenic alga was isolated from the leaves and twigs of red rust effected tea plant from Gotonga Tea Estate, Jorhat, Assam during July, 2002. Algal colony on the culture medium was detected after 48 hours of inoculation. The organisms (RR3) used for preparation of algaecide is a heterotrophic bacterium isolated from tea garden soil of Assam which grows well in a specific medium at pH 6.9. *In vitro* algaecides activity of the bacterium was studied by using live culture, culture filtrate and bacterial metabolite. Results showed complete inhibition of growth of the algal pathogen at a concentration of 20:100 when live culture and culture filtrate was used. Bacterial metabolite at a concentration 0.6 g/ 100ml was effective against the algal pathogen. Besides the algaecidal activity, the bacteria (RR3) and its metabolite enhance plant growth. The growth was determined by measuring the biomass and number of new off-shoot emergence in the treated tea plants.

Pieterse *et al.* (2006) developed an *Arabidopsis*-based model system using *Fusarium oxysporum f sp raphani* and *Pseudomonas syrigae pv tomato* as challenging pathogens, in order to study the molecular basics underlying the systemic resistance. Colonization of the rhizosphere by the biological control strain WCS417r of *P. flourescens* resulted in a plant-mediated resistance response that significantly reduced symptoms elicited by both challenging pathogens. Moreover, growth of *P. syringae* infected leaves was strongly inhibited in *P. flourescens* WCS417 treated plants. Transgenic *Arabidopsis* NahG plants, unable to accumulate SA, and wild-type plants were equally responsive to *P. flourescens* WCS417r-mediated induction of resistance. Furthermore, *P. flourescens* WCS417r-mediated systemic resistance did not coincide with the accumulation of PR mRNAs before challenge inoculation. The result indicated that *P. flourescens* WCS417r induces a pathway different from the

one that controls slassic systemic acquired resistance and 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 *P. flourescens* Pfl at 7-d intervals consistently reduced the disease incidence of blister 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, $\beta$-1, 3-glucanase and phenolics were studied. The enzyme accumulation was greater in *P. flourescens* Pfl-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 bio formulation (Saravanakumar *et al.* 2006).

*Pseudomonas corrugate,* 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. oxyporum,* 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. oxyporum* (76.9) in Kings BSNL 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 siderphore, ammonia, lipase and chitase in growth medium by *P. corrugata* were considered contributing to the antagonistic activites of the bacterium.

Two tea rhizosphere microorganisms, *Bacillus megaterium* and *Ochrobactrum anthropi* inhibited the growth of four tea pathogens, *Fomes lamaoensis, Sphaerostilbe repens, Poria hypobrunnea* and *Sclerotium rolfsii,* to a

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

Peanut are frequently invaded by toxigenic *Aspergillus fiavus* prior to harvest, especially in hot, dry condition. Strategies for reducing aflatoxin contamination of peanut have concentrated on minimizing postharvest infestation of seed with toxigenic strains of *A.flavus* and *A. parasiticus.* Historically, these strategies have emphasized cultural practices, since chemical control and prevention of preharvest fungal infestation have generally been ineffective.Biological control offers one potential strategy for preventing seed invasion by *Aspergillus flavus* or preventing production of aflatoxin *in situ.* One approach for biocontrol which is currently being investigated is the incorporation of nontoxigenic strains of *A. parasiticus* into peanut field soil which results in displacement of toxgenic *Aspergillus* spp. The date, there has been no extensive evaluations of bacteria as potential biological control agents against aflatoxigenic fungi on peanuts. In assay optimization experiment, two *in vivo* assays (seed and root radical) were developed and optimized for screening 119 strains of geocarposphere bacteria as candidate biological control against *A. flavus*,. Seven bacterial strains viz. *Bacillus megaterium, B. laterosporus, Cellulomonas cartae, Flavobacterium odoratum, Phyllobacterium rubiacearum, Pseudomonas aurofaciens* and *Xanthomonas* maltophila prevented colonization of seeds and root radicales by the fungus. Root growth promotion, noted with some strains, may indicate that bacteria alter the host physiology, which in turn, could be advantageous for control of aflatoxin production. In green house experiment, inoculation of root regions of 1 to 2 – week old peanut plants with toxigenic *Aspergillus flavus* and geocarposphere bacterial resulted in lower synthesis of aflatoxin $B_1$ in the peanut kernels at maturity, than those in plants inoculated with the toxigenic strains alone. Of seven bacterial strains tested, four strains showed reduction in aflatoxin production in varying extents. Pre-inoculation of bacterial strains (1-day earlier) resulted in greater

inhibition of aflatoxin accumulation. However, toxin level was not much reduced when the bacterial strains were introduced 1-day after inoculation of toxigenic *A. flavus* strain. *Bacillus megaterium* showed maximum inhibition of aflatoxin biosynthesis as compared to remaining three potential bacterial strains. The morphological interactions among A.flavus and other bacteria were also examined on peanut extract agar medium. The results suggest the potential of bacteria as biological control agents against pre- harvest aflatoxin contamination of developing peanuts. (Chourasia, 2007)

Growth promotion and pathogen suppression are the two well known mechanisms exhibited by PGPR. Bio–innovation research with PGPR and biocontrol research has led to the development of bacterial inoculants that consistently promise higher crop productivity. Gnanamanickam *et al.* (2007) conducted research programmed in India and US during 2006 and 2007 for three of their products whose active ingredients are *Bacillus* or *Pseudomonas* strains. EcoGuard GN is a EPA-registered biofungicide whose active ingredient is *Bacillus licheniformis* 3086. In a field test conducted during 2006 at Coimbatore, weekly applications of Eco Guard GN at 260 l/ha led to yields of 30.5 t/ha while the untreated control crop produced 12.6 t/ha (58% increase). The biofungicide used at this concentration also reduced the incidence of down mildew (caused by *Plasmopara viticola*). The percent disease index (PDI) in treated plots was reduced by 38.2% compared to untreated control. TAEGRO, also an EPA- registered biofungicide formulated in cornstarch contains 24.5% of freeze-dried spores of *Bacillus subtilis* var *amyloliquefaciens* FZB24. In US trials carried out in 2007, TAEGRO afforded substantial control of *Xanthomonas campestris* pv. *vesicatoria* (bacterial speck) and Phytophthora root rot of tomato better than. Kocide 2000. In addition to its well known fungicidal properties against *Rhizoctonia*, *Fusarium*, and Phytophthora (in particular the ridomilresistant isolates of pink-rot of potato pathogen, *Phytophthora erythroseptica*), TAEGRO has also shown consistent enhancement of crop yields in crops such as tomato, potato, cucumber and several ornamental crops. In recent greenhouse tests, the incorporation of TAEGRO in potting mix at 0.613 g/gallon pot suppressed *Ralstonia solanacearum* (bacterial wilt of tomato) by 75% over that of the untreated control. The third product under investigation is stain 3621 of *Pseudomonas congelans*. It suppresses the growth of *Ralstonia solanacearum*, X.c. pv. *vesicatoria* and *Erwinia amylovora* in laboratory

and greenhouse assays. This Gram-negative strain with proven PGPR and biocontrol traits now available as a wheat bran product and also in alginate beads is yet to be field tested. At present their believe that TAEGRO has the potential of a biobactericide that can control important diseases such as bacterial with and fire blight and a mixture of TAEGRO and *P. congelans* would be a much superior microbial product for disease control and growth enhancement/crop productivity in the organic and global agricultural market segments worldwide.

Liquid formulations of PGPR (*Rhizobium, Azotobacter, Azospirillum*, PSM, etc.) have become a preferred method for inoculating plants for improved availability of nutrients in soil and thereby promote growth of plants. A seed survival study was conducted by Acharya *et al.* (2007) during May-2007 of *Azotobacter chroococcum* (ABA-1), Anand isolate in liquid and carrier formulations and for their ability to support growth and promote survival of *Azotobacter* during storage on *Pennisetum glaucum* cv. GHB-558. In a polyethylene bag, 100g seeds were treated with 0.5 ml of liquid or 2.5 g carrier based formulation and approximately 2-5 ml sterile distilled water was added for uniform coating of inoculants on seeds. *Azotobacter* count was done on Ashby's mannitol agar medium by serial dilution method, various additives like glycerol and polyvinyl phrolidone (PVP) @ 2% to liquid inoculants promote higher cell of all the formulation depended on the *Azotobacter* strain and additives, when stored at room temperature. Cell count up to $6.8 \times 10^5$ x, $8.0 \times 10^5$, $6.6 \times 10^5$ CFU/g seed respectively after 48 hours at $30^0$C, whereas Charcoal and Lignite based formulation maintained $6.4 \times 10^4$, $6.0 \times 10^4$ CFU/g seed respectively. After 48 hours survival of bacteria on seed was reduced drastically. Seed germination effect of all inoculants formulation was observed on seed agar keeping an uninoculated check. After 48 hours of incubation in dark increased root and shoot length with secondary root formulations gave better germination compared to untreated check. The present investigation indicated that liquid inoculants with additives promote survival of bacteria on seed and also increase germination.

Effect of different plant growth promoting rhizobacteria (PGPR) and the method of application of PGPR were investigated by Hariprasad and Umesha (2007) to determine whether biocotrol of bacterial spot disease of tomato caused by *Xanthomonas vesicatoria* (Doidge) Dye could be improved. The PGPR strains

(*Bacillus subtilis* strain GB03, *Bacillus amyloliquefaciens* strain IN937a, and *Brevibacillus brevis* strain IPC11) were selected based on the reported capacity to induce resistance against various bacteria pathogens of tomato. PGPR applications were made by seed, root and fohar spray treatment separately and in combinations in field. Among them, GBB03 was the most effective in providing significant suppression of bacterial spot and was well correlated with increased activity of defense related enzymes viz, peroxides and phenylalanine ammonia lyase. Combination treatments proved to be the best in reducing the bacterial spot incidence. Plant growth promoting rhizobacteria that were effective in green house were also able to induce resistance in under field conditions against bacterial spot of tomato.

Maiti *et al.* (2008) made an attempt to isolate and utilize a potential biocontrol agent against two fungal diseases of *Stevia rebaudiana* caused by *Alernaria alternata* and *A. steviae*. *Pseudomonas aeruginosa* WS-1, a rhizosphere isolate among 134 isolates, showed in vitro antagonistic activity against both the phytopathogens. Microscopic examination after antagonism showed hyphal shriveling, swelling, vaculation, short branching and granulation of cytoplasm resulting in lysis of hyphae of the pathogens. Correlation of antifungal activity activity of this isolate has been found to be linked with the production of siderophore, proteases and chitinases. Furthermore, Talc based formulation of the antagonist @ 4 gm/ 1 (containing $10^6$ cells/ ml) showed 84% and 71% protection against leaf spot disease caused by *A. alternata* and leaf blight disease caused *A. steviae* respectively when applied at an interval of 15 days in field condition.

Vermicompost based bioformulation of *Pseudomonas aeruginosa* was effective in suppression of bacterial wilt (Ralstonia solanacearum) incidence of chilli (*Capsicum annum*) in field. Quantitative assays of the population dynamics of *P.aeruginosa* revealed that the shelf-life of this bioagent was maintained upto 200 days of storage at room temperature ($26\pm2^0$C). Bioformulation of vermicompost with *P.aeruginosa* carboxy methyl cellulose and mannitol was the best to maintain shelf-life and high population recovery of *P.aeruginosa* during storage ($10^6$ x $10^7$ cfu/ml). Application of this bioformulation as seed treatment followed by root and soil application at transplanting and soil application at 30 days after transplanting of chilli, showed minimum wilt incidence( 8.7% ), maximum yield ( 87.39/ha ) and high

recovery (58.93 x$10^7$ cfu/g) of *P.aeruginosa* from the crop rhizosphere (Bora and Deka, 2008)

### 2.2.2. Fungi

Madi and Katan (1998) reported that infiltration of *Penicillium janczewskii* conidia or its culture filtrate into melon and cotton leaves induced systemic resistance and protected the lower part of the stem of melon and cotton plants against *Rhizoctonia solani*, leading to up to a 100% reduction in the incidence of damping-off. Hypersensitive reaction like responses was observed in melon and tobacco, but not in cotton. Peroxidase activity, associated with induced systemic resistance, increased in treated plants of both species compared to the control plants. Gel elctrophoresis of peroxidases from melon plants treated with culture filtrate exhibited enhanced activity of all three isozymes present in untreated plants, but mostly of the slowest migrating isozyme. Phenylalanine ammonia-lyase activity in stems of melon plants treated with *P. janczewskii* or its culture filtrate increased two-fold compared to the untreated plants. Western blot analyses revealed induction of β-1, 3-glucanase, a pathegenesis-related protein, and HSP 70, a member of the heat shock protein family in melon. Altered root development was observed in cotton plants infiltrated with *P. janczewskii* conidia or its metabolites. Our results suggest that treating leaves with *P. janczewskii* or its culture filtrate triggers the signal transduction cascade, activating different defense genes in melon and cotton, thus protecting the lower parts of the stem.

Assam and Tamil Nadu isolates of *Trichoderma harzianum*, *T. viride* and *T. virens* were tested by Hazarika and Das (1998) for their potential to suppress *Rhizoctonia solani*, the French bean root rot pathogen under *in vitro* conditions. All isolates inhibited growth of *R. solani*. Culture filtrates of *T. harzianum* and *T. viride* inhibited mycelial growth and sclerotial germination. Wheat bran substrate supported maximum growth of all isolates followed by farm yard manure and tea waste. Both *T. harzianum* and *T. viride* effectively controlled the bean root rot disease when they were applied as seed and soil treatment.

Singh *et al.* (1998) *Trichoderma viride* and *T. harzianum* when applied as soil inoculation plus seed treatment proved most effective in reducing incidence of dry root rot and increase phenolic compound and carbohydrate contents of chickpea

followed by *Pseudomonas fluorescens, Bacillus subtilis* and *Aspergillus flavus.* Soil inoculation plus seed treatment was the best method of application followed by soil inoculation and seed treatment alone.

Seven *Trichoderma* spp., seven isolates of *Pseudomonas fluorescens,* two isolates of fluorescent pseudomonad, *Bacillus subtilis* and one yeast, namely, *Saccharomyces cerevisiae* were screened against *Colletotricum capsici* (Syd) Butler and Bisby, both in *in vitro* and on the plant. Jeyalakshmi *et al.* (1998) found, among the fungal antagonists, *S. cerevisiae* exhibited maximum reduction of the mycelia growth followed by *T.viride* Among the 10 bacterial antagonists, *B. subtilis* showed the maximum growth reduction followed by *P.fluorescens* isolate 27. In pot culture experiment *S. cerevisiae* recorded the maximum reduction of fruit rot intensity and incidence and die-back incidence followed by *B. subtilis,* when sprayed on 105 and 120 days after sowing. However, it was next only to carbendazim (0.1%)

Potential of seven promising biocontrol agents (BCAs) *Chaetomium globosum, Coniothyrium minitans, Gliocladium virens, Laetisaria arvalis, Trichoderma harzianum, T.hamatum* and *T. viride* against *Rhizoctonia solani* Kuhn, causing root rot of French bean (*Phaseolus vulgaris* L.) was studied by Mathew and Gupta (1998) under *in vitro* and glasshouse conditions. *In-vitro* evaluation of BCAs by dual-culture method revealed that *T. harzianum* caused maximum inhibition, followed by *T.hamatum,T. viride* and *G. virens.* In pot experiments, *G. virens* and *T. harzianum* proved superior to other antagonists in reducing pre-emergence root rot to 6.7 and 13.3%, respectively, as compared to 36.7% in control. *T. harzianum* was also effective to reduce post-emergence root rot. Pre-inoculation of antagonists proved to be a superior method to check post-emergence root rot.

Rhizome rot of ginger is caused by either *Pythium* or *Fusarium* spp. or both (mainly *P. myriotylum* and *F. solani*). Resident biocontrol agent (BCA) *Trichoderma harzianum* isolated from rhizome rot suppressive soils reduced the disease and increased plant stand and yield. In order to further enhance the efficiency of disease suppression, Ram *et al.* (1999) used a bacterial BCA *Pseudomonas* sp individually, in combination with and also with fungicidal rhizome treatment. Combination of both BCAs resulted in better germination and plant stand, reduced disease, and increased yield. Soil application of BCA was more effective compared to their seed treatment.

Integration of soil application of BCA with fungicidal rhizome treatment (bavistin + ridomi MZ) increased the efficiency of disease control as compared to their individual treatments. Soil application of and *T. harzianum* rhizome treatment with *Pseudomonas* sp and fungicides was the most effective among all the tested treatments.

Bunker and Mathur (2001) evaluated three biocontrol agents(BCAs) individually and in combinations, and in integration with Bavistin seed treatment in pathogen infested soil in pots, for suppression of dry root rot pathogen *Rhizoctonia solani* in bell pepper (*Capsicum frutescens* cv. *california* Wonder. Seed treatment with the biocontrol agents was as effective and bavistin seed treatment. Integration of seed and soil application of individual BCA resulted in higher germination and reduced mortality due to disease. Combination of two biocontrol agents, particularly of *Trichoderma harzianum* and *T.aureoviride* was better than the individual ones. Population of BCAs in chilli rhizoshere and soil was directly related to suppression of *R.solani*. Application of mixture of *T.harzianum* and *T.aureoviride* as seed and soil treatment was the most promising in increasing the germination and suppression of chilli root rot pathogen and the disease.

Wilt caused by *Fusarium oxysporum f.sp. cumini* (Foc) is the most important and destructive disease of cumin (*Cuminum cyminum*).To reduce the population of this pathogen and the incidence of wilt on cumin in field, efforts were made to evolve environmentally sound method of management by utilizing native bio-control agents. *Aspergillus versicolor* highly antagonistic to *F .oxysporum* was isolated from arid soils. Dual culture tests were performed to confirm its antagonistic activities and were also compared with that of *Trichoderma harzianum*, a known biocontrol agent. In Foc and *A.versicolor* infested soil, an initial population of $4.2 \times 10^4$ CFU $g^{-1}$ soil of Foc drastically declined to $2.8 \times 10^3$ CFU $g^{-1}$ soil after 15 days of incubation causing 93.3% reduction compared to 73.8% reduction in Foc prop gules in the presence of *T.harzianum*. In liquid culture tests, cell-free filtrates even at 0.5 ml concentration of both the bio-control agents could inhibit mycelial growth of Foc. Reduction in mycelial growth of Foc in the the cell-free filtrates of *A. versicolor* and increase in reduction with increased concentration of cell-free filtrate is a clear evidence that *A. versicolor* has released certain antibiotics. Studies related to thermal resistance to

*A .versicolor* showed that it was able to survive and multiply even at $65^0$C. An initial count of 6.3 x $10^4$ CFU $g^{-1}$ of *A. versicolor* increased many fold with the increase in the time interval at a temperature range of 50-$55^0$C under moist conditions. Studies on integration of soil amendments and bio-control agents revealed that amending soil with *A. versicolor* alone or in combination with *T.harzianum* or residues was significantly better in reducing Foc propagules compared to non-amended control. Better survival and multiplication of *A .versicolor* at low soil moisture content and at high soil temperature are of beneficial consequences of utilizing its potentials against control of *Fusarium* in dry sandy soils, where temperatures often reaches in the ranges of 50-$60^0$C during hot summer months. (Isreal *et al.* 2002)

Fungal isolates antagonistic to *Phomopsis vexans* were isolated from plant rhizosphere and phylloplane. An isolate Bb-III of *Beauveria bassiana* was found very effective during preliminary investigations. Efficacy of this isolate was tested *in vivo* in pots using soil infested with *P.vexans* virulent isolate. This soil was amended by Chani *et al.* (2002) with different concentrations viz. $1x10^5$, $1x10^6$, $5x10^6$ and $1x10^7$ cfu/100 g and seeds of brinjal variety Pb. Barsati were sown in each pot. The biocontrol isolate reduced seed rot by 70% and seedling blight by 57%, the damping off incidence was reduced by 65.6% and final plant stand was 87.3% compared to 51.4% in control. Highest concentration $1x10^7$ cfu/100 g soils was the most effective. Biocontrol agent performed better than seed dressing with captan. Further studies on its formulation and application technology could lead to its development as a biofungicide

Collar rot disease caused by *Phytophthora cactorum* (Leb. and Cohn) Schrot results in 12-15 percent mortality of apple plants in the nurseries and also causes extensive economic losses in established orchards by out rightly killing the grown up plants. Since it is difficult to manage this disease by chemical treatments, therefore two non-chemical methods.viz.employment of biocontrol agents (BCAs) and addition of amendments (plant leaves, cakes) in the soil were evaluated in controlling this disease under pot culture conditions. Out of seventy three fungal BCAs and thirty two bacterial BCAs isolated from the soil samples collected from apple orchards and nurseries, twenty three (fungal) and twelve ( bacteria) were found effective against the target pathogen under laboratory conditions. Pot culture evaluation of *in vitro*

effective BCAs of fungal origin revealed that five namely, *Trichoderma longibrachiatum, T.harzianum,T. viride,T virens* and *Penicillium funiculosum* were found highly effective and gave 89.96 percent disease control. Similarly, out of twelve bacterial BCAs four namely, *Bacillus subtilis, Enterobacter aerogenes, Pseudomonas putida* and *Bacillus spp.* were found effective to c heck the seedling mortality up to an extend of 78.6-89.2 percent. Further, in evaluation of BCAs of mycorrhiza origin (8 No.) separate addition of two viz. *Glomus mosseae* and *G.macrocarpus* checked the disease development up to the extent of 71.3 and 68.7 percent, respectively under pot conditions. In another experiment, screening of fifteen different soil amendments (plant leaves, cakes) against the target pathogen indicated that addition of dried leaves of *Lantana camara, Vitex negunda, Melia azadirachta, Murraya koeningii* and Mustard cake were found effective in controlling the disease up to 66.3-78.4 percent. (Sharma *et al.* 2002)

Indiscriminate use of synthetic pesticide for controlling plant diseases not only causes environmental pollution, species disappearance, pressure on natural resources but their toxic residues enter into animals and humans and causes several health hazards. Alternatively biological management of plant diseases using soil borne antagonistic fungi are ecofriendly and reduces use of pesticides and may be employed as a component of sustainable agriculture. The chickpea wilt complex is caused by pathogens viz., *Fusarium oxysporum f. sp. Ciceri* (Padwik), *Sclerotium rolfsii* Sacc. and *Rhizoctonia solani* Kuhn. Use of various antagonistic fungi such as *Gliocladium virens* /in combination with fungicide (vitavax. 0.1%) not only controlled chickpea wilt reduce environment pollution. Preparation of powdered base *G. virens* / *T. harzianum* using different carriers were made which costs about Rs. 25 per kg, other preparations that are available in the market costs about Rs. 125 per kg hence, it may be suggested that use of along with (vitavax. 0.1%) in the form of powdered preparation not only controls chickpea wilt complex effectively but also shows growth promoting activities. (Singh *et al.* 2004)

Yamada (2004) isolated *Sporothrix* sp. from tea phylloplane and screened for their antagonistic activity against tea anthracnose fungus *Colletotricum theae-sinensis.* Screening was done by pathogen- antagonist dual culture test. Culture filtrate of *Sporothrix* sp. inhibited elongation of germ tube of conidia of *C. theae-sinensis.*

Lesion size of anthracnose was decreased when conidia suspension of *C. theae-sinensis* was inoculated together with *Sporothrix* sp. by wound inoculation method using detached mature leaves

Singh and Singh (2005) reported the case of a formulation of *Trichoderma harzianum* as vine treatment to control collar rot of betel vine (*Piper betle* L.) caused by *Sclerotium rolfsii*. The strains were isolated from disease suppressive soil in the conservatories located at Mahoba, UP, India.The per cent mortality in the vines treated with *T. harzianum* formulation was 9.39% as compared to 76.91% in inoculated control. In addition to the disease control, the yield of betel vine in terms of number of leaves was also increased in *Trichoderma* treated vines in comparison to both inoculated and uninoculated control.

Chili (*Capsicum frutescens* L.) is an important spice cum vegetable crop. Leaf spot and fruit rot caused by *Alternaria alternata* (Fr.) Keissler is severe and common disease in entire chilli growing area in the country. It infects all the aerial parts of the plant causing severe losses. Eight known antagonists viz., *Trichoderma viride*, *T. harzianum*, *T. longibrachiatum*, *Aspergillus flavus*, *A. niger*, *Chaetomium globosum*, *Gliocladium virens* and *Bacillus subtilis* were evaluated *in vitro* by dual culture, pathogen at periphery and pathogen at centre method. Gohel *et.al* (2005) reported that all the antagonists were significantly superior in checking the growth of the pathogen. In case of dual culture method, *T. longibrachiatum* produced maximum inhibition (73.21 %) of the pathogen.Whereas *T. harzianum* (49.46%) proved best in pathogen at periphery method. While in case of pathogen at centre method.*A.flavus* (77.00%) produced maximum inhibition. Overall *Trichoderma* spp., A.*flavus* and *B.subtilis* were found better against *A.alternata.*

In a study by Jha and Jalali (2005) the culture filtrates of different fungal antagonists' viz., *Trichoderma viride, Aspergillus niger, A. flavus, A. terreus, A. sydowi* and *Spicaria sylvatica* were tested at 5%, 10% and 20% concentration in relation to their effect on radial growth of *Fusarium solani f sp.pisi* by dual culture method. They found that the culture filtrates of all the tested antagonists showed significant inhibition of radial growth of the pathogen *F. solani f.sp.pisi* and an increase in the concentration of culture filtrates resulted in greater inhibition of the growth of pathogen.The culture filtrate of *T. viride* recorded the maximum inhibition

of radial growth of pathogen by 38.8,54.12 and 60.11% at 5,10 and 20% concentration, respectively, followed by that of *A. niger,* where the radial growth of the pathogen was inhibited by 21.32,42.27 and 52.38% at 5,10, and 20% concentration of culture filtrate,respectively.Like-wise the culture filtrates of *S. sylvatica and A. terreus* also showed marked inhibition of radial growth of the pathogen which was 24.40 and 19.30%,respectively, at 10% concentration and 29.41 and 25.73% at 20% concentration of filtrates. On the other hand the culture filtrate of *A.flavus* recorded relatively lesser inhibition while that of *A. sydowi* exhibited the least inhibition of radial growth of the pathogen.The inhibition of radial growth of *F. solani f.sp.pisi* by culture filtrates of various antagonists might be due to production of antifungal metabolite by the respective antagonists.

Collar rot of brinjal (*Solanum melongena* L.) caused by *Sclerotium rolfsii* Sacc, was also reduced by different antagonists viz., *Trichoderma viride, T. harzianum, T. longibrachiatum, Aspergillus flavus, A. niger, Gliocladium virens* and *Bacillus subtilis* which were evaluated by Patel *et al.* (2005). The antagonists were applied @ 50/ Kg soil in previously inoculated (60g inoculun/Kg soil) pots. *T.harzianum* and *T. viride* were found superior in reducing pre and post-emergence seedling mortality, enhanced germination and resulted in better final plant stand.The next best in order of efficacy were *A. niger* and *T. longibrachiatum.*

Multilocational field studies were conducted (1999-2000 to 2002-2003) by Gaur *et al.* (2005) to evolve the best application technique of *Trichoderma harzianum* for controlling dry root rot (*Rhizoctonia solani*) of chickpea. The studies (rain fed and irrigated) conducted on the basis of preliminary green house results showed that dry root rot of chickpea can be effectively and economically managed either by the soil application of 10-15 days pre-inoculate . *T.harzianum* (TG-1) @ 10 Kg ha-$^{1}$ in 200 kg 0f FYM or by the practice of seed treatment with talc-based formulation of *T.harzianum* containing 2x10$^{6}$ c.f.u./g @ 10 g Kg-$^{1}$ seed in combination with soil application of $ZnSO_4$ @ 25 Kg ha$^{-1}$.Under rainfed situation, seed treatment with talc based formulation of *T.harzianum* @10 g Kg-$^{1}$ seed proved economically better. Therefore, it may serve as an ecofriendly substitute to carbendazim.Wheat bran-based formulation of *Trichoderma* gave better control of disease than talc-based formulation for soil application. Similarly soil application of *Trichoderma* was found significantly

superior over seed treatment. *Trichoderma* @ 10g $Kg^{-1}$ seed gave better control than the lower dose of 4 g $Kg^{-1}$ seed. Technique of seed dip and solid matrix priming did not prove effective.

Chandel *et al.* (2005) isolated different microorganisms from the rhizosphere of carnation plants which were tested under *in vitro* in pot and field conditions to ascertain their antagonistic property. Different microorganisms namely, *Aspergillus sp., Fusarium sp., Penicillium sp., Rhizopus* sp., *Trichoderma, T. viride, T. hamatum,* bacteria *Bacillus* sp. *Pseudomonas* sp. and actinomycetes were isolated from the carnation rhizosphere. Maximum inhibition of the mycelial growth of the fungus (*Fusarium oxysporum f. sp. Dianthi)* was shown by *T. harzianum* (76.54%) with minimum radial growth (9mm) that was superior over the treatment and was closely followed by *T. viride*(70.06%). However, minimum inhibition was recorded in *Rhizopus* sp. (28.70%) that was statistically on par with the In pot culture, all the antagonists were able to reduce the wilt disease incidence over control, maximum being recorded in *T. harzianum* followed by *T. viride, Aspergillus sp.,* and *Bacillus* sp. while in field conditions *T. harzianum* followed by *T. viride* gave more than 80% disease control. However, *Penicillium sp.,* were found least effective in disease control. Both the root dip and soil drench method were found equally good with no significant difference in the disease incidence. The present disease incidence was comparatively less after 35 days of planting as compared to 60 days.

*Aspergillus sp.,* followed *Trichoderma* was found to be dominant in the turmeric rhizosphere (Dutta *et al.* 2005b). On the basis of microscopic observation and growth characterisatics native Tri-Pun and Tri-Pun 2 isolates were tentatively identified as *T. virens* and *T. hamatum* respectively. Tri-Pun2 followed by Tri-p-Pun inhibited growth of soil-borne plant pathogens during dual culture method. Potato dextrose agar and Oat meal agar media were found to be most suitable for growth and sporulation of native *Trichoderma* isolates. Though the competitive saprophytic ability (CSA) of Thdl (*T. hamatum* Delhi isolate) was best as compared to native isolates, Tri-Pun and Tri-Pun2 also belong to higher group. Paddy grain and paddy husk were found to be the best locally available substrates for mass multiplication of native Tri-Pun 2 isolate.Eighty one percent spore viability was observed after 137

days at normal temperature in paddy grain substrate. Tri-Pun 2 was found to be most effective after Bavistin for management of web blight disease of black gram.

Betel Vine (*Piper betle* L.) is an important commercial crop of Karnataka, mainly cultivated for its leaves, known as 'Pan'. Raghavebdra *et al.* (2005) isolated *Pythium vexans*, a soil-borne fungal pathogen of betel vine in the Tarikere taluk of Southern transition agroclimatic zone of Karnataka, from diseased plant parts by baiting technique. The pathogencity tests revealed that the isolated fungus *P. vexans* was pathogenic. The *in vitro* control of *P. vexans* was carried out by following 'Poison food technique'. The cold-water extracts of *Allium sativum* (Garlic) and *Azadirachta indica* (Neem) showed significant inhibition of colony growth. *A. sativum* showed maximum inhibition of *P. vexans* colony at 80% and 100% concentrations by 49.57% and 52.59% respectively. *A. indica* at 80% and 100% concentration showed maximum inhibition of P. vexans colony by 18.53% and 48.71% respectively. The dual culture studies revealed that the native isolates of *Trichoderma harzianum* was effective against *P. vaxans*. The average inhibition percentage of *P. vexans* by *T. harzianum* was 22.29%. Experiment on *in vivo* control of the disease was carried out under green house conditions. The test plants (*P. betle*) were raised in the pots, for the treated plants, the soil was amended with 100g of *T. harzianum* that was mass multiplied in the substrate 'paddy straw-sorghum' mixture, for the untreated plants (control) only substrate was added to the soil. For both treated and control plants four mycelial discs were placed around the root zone. After ten days the roots of the treated host plants were observed for the disease symptoms. The treated host plants containing the inoculums of *T. harzianum*, did not develop any disease symptoms and remained healthy and the disease was expressed in untreated host plants

Devi and Paul (2005) collected soil and wilt/root rot infected plant samples from pea growing areas. Five pathogens viz., *Fusarium oxysporum f. sp. pisi, F. solani f. sp. pisi, Rhizoctonia solani, Phoma medicaginis var. pinodella* and *Sclerotionia sclerotiorum* were found to be associated with the disease. Thirteen antagonists were isolated from the soil and screened against the pathogens. Out of these, JMA-4( *Trichoderma harzianum*), SMA-5 (*T. harzianum)*, DMA-8 (*T. koningi)* and JMA-11 ( *T. koningi*) were found to be more promising on the basis of their broad

spectrum activity and growth characteristics. Hyphal interaction studies revealed several hyphal abnormalities in the pathogens such as swollen hyphae, excessive branching, hyphal sliming, hyphal apex deformation, excessive vacuolation and lysis. Soil factors like 30% soil moisture, $25^0$C soil temperature and 6.6 pH were found to be most suitable for the growth of JMA-4. Among ten plant extracts evaluated, *Ranunculus muricatus* showed strong antifungal activity against pathogens and biomass. *Eupatorium adenophorum* did not affect the growth of bioagents but was inhibitory to pathogens. Spictaf (0.1%) was ineffective to biomass whereas, it caused more than 50% inhibition of pathogens. Out of three fungicides tested, Bavistin was found to be highly toxic to pathogens as well as the bioagents. Different delivery systems evaluated against test pathogens revealed that wheat bran based formulation was most effective against *R. solani* and *F. solani*. Application of bioagent in the form of sodium alginate pellets resulted in lowest disease caused by *F. oxysporum* and *P. medicaginis*. Seed treatment was found to be effective against wilt/root rot complex disease in pot experiments. Under field conditions use of sodium alginate pellets was found to be highly effective delivery system. Integration of seed treatment with bioagent + soil application with wheat bran based formulation plus mulch followed by seed treatment with Spictaf (0.5%) + soil application with wheat bran plus mulch were found to be most effective in managing the disease.

Thakur *et al.* (2006) used different bioagents against *Rhizoctonia bataticola in vitro* and *in vivo*. *In vivo* studies revealed that seed treated with spore suspension of *Trichoderma harzianum* increases significantly. *Trichoderma harzianum* was found superior over *T. viride*, EM solution of *Bacillus subtilis*. Seed treatment with *T. harzianum* influenced the seed germination and there was considerable increase in plant height also. *In vitro* experiment revealed that *T. harzianum* was superior in arresting the growth of *Rhizoctonia bataticola* followed by *T. viride* over control. EM solution was found to be effective even at lower concentration of 2 percent and *Bacillus subtilis* was also found effective in restricting *Rhizoctonia bataticola*. *T. harzianum T. viride* significantly reduced the root rot incidence to 23.18% and 28.35% respectively compared to 83.6% incidence in control. Thus, it may be inferred that seed treatment with *T. harzianum* could bring down the menace of root rot caused *Rhizoctonia bataticola* (Taub.) Butler in Sesamum.

Chakraborty *et al.* (2006) reported that two tea rhizospheric bacteria- *Bacillus pumilus* TRS3 and *Bacillus megaterium* TR S4, as well as native strains *Trichoderma harzianum* and *T. viride* were found to be highly antagonistic *in vitro* against *Poria hypobrunnea*, *Fomes lamoensis* and *Spherostilbe repens*, causing root rot, brown root rot and violet root rot, respectively. *In vivo* applications of the bacteria and fungi led to enhancement in growth of tea plants over respective untreated control. Significant control of root rot diseases was also achieved due to treatment with the bacteria and *Trichoderma sp.* Species of *Glomus*, along with other mycorrhizal fungi were isolated from tea rhizosphere. *In vivo* application of *Glomus* to the rhizosphere also significantly increased plant growth and reduced root rot intensity. Dual inoculation of VAM and PGPR markedly enhanced the growth of the tea seedlings in terms of plant height, number of leaves, number of branches and dry weight of root and shoot. Phosphorous uptake was also enhanced by tea seedlings by combined inoculation with VAM and PGPR. Significant increases were obtained in mojor biochemical components- proteins, total phenols and O-dihydroxy phenols following various treatments. Defense related enzymes- phenyl alanine ammonia lyase, peroxidase, β-1, 3 glucanse and chitinase also showed increased activity when treated with VAM and PGPR, either singly or in combination. Catechins, which are major flavonoid flavour components of tea leaves, were analyzed by HPLC to determine changes in the different isoforms. Several new peaks were observed following bacterial treatments. Antifungal phenolics were extracted from treated tea roots, analyzed by HPLC and bioassay was performed using spore germination of *S. repens* and radial growth assay of *F. lamoensis*. Treatment with *Trichoderma* did not enhance activity of defense enzymes or phenolics. Population of pathogens in soil and root tissues, as determined by immunodiagnostic tests, were greatly reduced in soil after application of *Trichoderma* sp. Results of present study indicate while *Bacillus megaterium* and *Bacillus pumilus* act by inducing systemic resistance in tea plants, *Trichoderma sp.* act by their direct antibiosis against the pathogens.

"Som" (*Persea bombycina* Kost) the primary food plant of muga silk worm is prone to various diseases that affect the quality and quantity of leaves and cocoon production. Among them Grey blight is one of of the major disease of *P.bombycina* caused by *Pestalotiopsis disseminate* (Thum) Stey. The leaf loss due to grey blight disease is estimated at 1273 kg per hectare per annum. Indiscriminate use of

chemicals to control plant diseases is a threat to environment; therefore the alternative approach is necessary to adopt integrated disease management using eco-friendly methods by biological controlling agents.With this view, an *in vitro* experiment was carried out to study the effect of three fungal antagonists of *Trichoderma spp viz. T.harzianum, T. viride,T.hamatum* and four bacterial antagonists isolates of *Pseudomonas fluorescens* against mycelial growth and conidial development of *Pestalotiopsis disseminate* the causal organism of grey blight disease of "Som" by dual culture technique.The antagonists' treatment on the mycelial growth showed that the maximum growth inhibition of the pathogen was exerted by *T. viride*( 51.7%) followed by *T.hamatum*(45.9%)The plates amended with *Trichoderma spp* and isolates *Pseudomonas fluorescens* did not show any reduction in sporulation of the pathogen and was almost at par with check.( Das *et al.* 2006)

Potentiality of *Trichoderma harzianum* Rifai as seed treatment agent on reduction of white mold incidence of French bean caused by *Sclerotinia sclerotiorum* (Lib.) de Bary was assessed in FYM amended soil under field condition. Further, the effect of FYM amended soil on the distribution of *Trichoderma spp* and associated microflora in root rhizosphere was also studied by Gohain and Das (2006). In the present investigation, efficacy of four different concentrations of water extract of FYM amended soil viz., 0.1%.0.5%, 1.0% and 1.5% at three different period of incubation. Viz., 1, 2 and 3 weeks were tested for stimulation of growth of *T.harzianum* and inhibition of *S sclerotiorum.* The highest radial growth of *T.harzianum* was recorded in 1.5 percent water extract of FYM after two weeks of incubation *in vitro*, which was statistically at par with 1.0 percent after same period of incubation. On the other hand, minimum radial growth of was observed in 1.5 per cent of water extract of FYM amended soil after 2 weeks of incubation which was statistically at par with 1.0 per cent after same period of incubation. In field condition, seed treatment with *T.harzianum* along with 0.05% carbendazim in 1.0% FYM amended soil was found effective in reducing the white mold incidence of French bean. Increased dry weight of roots shoots and yield of crop was also observed in the field due to the same treatment as compared to inoculated control. The population density of *Trichoderma spp* in root rhizosphere of French bean was found highest in amended soil irrespective of different concentrations than unamended soil in field condition. Maximum population of *Trichoderma spp* in rhizosphere soil (both surface

and soil of 6 cm depth) was found when seeds were treated with along with 0.5% of carbendazim. At the end of the experiment, microflora associated with rhizosphere of French bean were identified as *Aspergullus sp.*-1, *Aspergullus sp-2, Rhizopus sp., Penicillium sp., Bacillus sp-1, Bacillus sp-2* and *Bacillus sp-3.* The sclerotial productions of *S sclerotiorum* were arrested significantly when seeds were treated along with 0.05% carbendazim in 1.0% FYM amended soil under field condition. From the analysis of soil before and after amendment with FYM, decreased soil pH with increased organic carbon was observed in amended soil as compared to unamended soil.

Anwer and Khan (2006) carried out an investigation to examine effectiveness of selected soil isolates of *Aspergillus niger* against *Rhizoctonia solani in vitro* and *in vivo.* Numerous isolates of *A. niger* were collected from different crop fields and pure culture on potato dextrose agar. The isolates were characterized for production of ammonia, hydrogen cyanide, hydrogen sulphide, indole acetic acid, siderophores, phosphate solubilization and antagonism against *R. solani in vitro.* Four soil isolates of *R. solani* viz. $Anc_2$, $AnR_3$, $AnPP_2$ and $AnS_2$ were selected on the basis of above characters to evaluate their effectiveness against root rot caused by *R. solani* on egg plant, *Solanum melongena* cv. Pusa Kranti in 25 cm clay pots filled with 2 kg sterilized soil ( field soil+ compost, 3:1). The isolates were applied in soil (1 g/kg soil) and on roots (bare root dip treatment).Before seedling planting the soil in pots was inoculated with 2 g sorghum seed colonized by *R. solani* (2g/kg soil). Inoculation with *R. solani* resulted to 75% decrease in the seed germination. The cultivar Pusa Kranti was found highly susceptible to the infection by *R. solani* and exhibited significantly decrease in the plant growth (16%) and flowering (21%). Application of *A. niger* isolates increased the seed germination, decreased the root rot and improved the growth variables ($P< 0.05$). Soil application with the isolate $AnC_2$ checked the root rot (21%) and improved the dry matter production (11%) and flowering (17%).

Reddy and Raghavender (2006) used arbuscular mycorrhizal fungus, *Glomus fasciculatum* in biological suppression of charcoal rot fungus *Macrophomina phaselina.* It is clear from their study that charcoal rot disease was controlled (physical and physiological process) to a large extend when the sorghum plants are inoculated with the mycorrhzal fungi. No disease was observed in the varities of RS-29 and E-36-1 when inoculated with mycorrhizae and pathogen. The disease

incidence was reduced upto 80% even in the variety CSV-8R which was considered as highly susceptible to charcoal rot. The reduction in disease severity may be due to competition of sites, through the main reason is by strengthening the host plant so that damage caused by the pathogen was offset by improved plant growth.

Fungal biocontrol agents (BCA) viz, *Trichoderma viride, T.harzianum, T.hamatum, T.virens* and bacterial isolates B1 and B2 of *Bacillus* sp. were tested by Verma and Sharma (2007) *in vitro* and *in vivo* against *Fusarium solani* causing mango seedling wilt. Fungal BCA were more effective than bacterial BCA under *in vitro* as well as in pot studies. *T.harzianum* caused a maximum (72%) *in vitro* inhibition, followed by *T. virens*(71%), *T. viride* (67%), *T.harzianum* (58%), *Bacillus* isolates B1 and B2 were the least effective. In pre-as well as simultaneous applications, *T.harzianum* recorded the least population of *F.solani* in pot soil and *T. virens, T. viride* and *T.hamatum* were the next best treatments. *Bacillus* isolates were the least effective. The pre-applications of biocontrol agents *T. virens* and *T. viride* were more effective. Maximum disease control was obtained when the pathogen was inoculated 10 days after the application of biocontrol agents. The least level of disease control was in post-application of biocontrol agents *T.harzianum* and *T. virens* recorded the highest disease control and *T. viride* and *T.hamatum* were the next best in pre- application treatments. Mango seedlings were healthier and recorded maximum increases in shoot and root lengths as well as weights.

*Trichoderma harzianum* and *Pseudomonas fluorescens* the compatible antagonists to the pathogen *Phytophthora capsici* were used in combination to control root rot of chillies by More and Baig (2007). *T.harzianum* inhibited mycelial growth of *P. capsici in vitro* after 48 h by dual culture method with a drastic growth reduction by 89%. *T.harzianum* arrested the spread of the pathogen by covering the complete surface of the pathogen colony and sporulated as evident of microscopic studies where as *P.fluorescens* produced a zone of inhibition. The formulation prepared on a mixture of soil and vermiculite was effective against the pathogen up to 2 years when stored at room temperature in the laboratory. The optimal dose of the antagonists in the compound formulation was $3.5x10^{-8}$ spores/ml of *T.harzianum* $1.0x10^{-9}$ cfu/ml of *P.fluorescens.* The two antagonists in the formulation were effective at pH from 3.5 to 5.6 at 20-40$^0$C.

### 3.1 Soil samples

In order to isolate rhizosphere microorganisms, soil samples were collected from both the hills and plains. In hills, Margaret's Hope Tea Estate, Darjeeling (2000 meters above sea level) was selected and in the plains 6 sites of Dooars and Terai were selected as follows:

**Table 1:** Sources of rhizosphere soil for isolation of microorganisms

| Name of estate | Age of bushes(years) |
|---|---|
| Hansqua Tea Estate, Terai | 90 |
| Kadommini Tea Estate, Dooars | 50 |
| CoochBehar Tea Estate, Dooars | 70 |
| Chandmoni Tea Estate, Terai | 80 |
| Tea Research station, Nagrakata, Dooars | 40 |
| Tea Experimental Garden, NBU | 10 |

Collected soil samples were used for determining microbial population and isolation of microorganisms.

### 3.2. Isolation of microorganisms from soil

Rhizosphere soils of healthy tea plants were chosen for the determining microbial population and isolation of microorganisms. In each case, 5gm of soil particles loosely adhering to the roots were collected. The soil suspension was made 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 pippetted out and serial dilutions were prepared. 1ml of each of $10^{-3}$ and $10^{-5}$ 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, $K_2HPO4$, anhydrous 1.5g, $MgSO_4.7H_2O$ 1.5 g, Glycerol 15 ml in 1 L distilled water, pH 7.4± 0.2) and Potato Dextrose Agar (PDA-potato 400g, dextrose 20 g, agar 20 g, distilled water 1 L; pH 7.3± 0.2) as the growth media. The petriplates were then incubated for 1-5days at 28±2$^0$C for the observation of microbial growth.

### 3.3. *In vitro* testing for antagonism to pathogens

#### 3.3.1. Solid medium

To know the antagonistic activity of individual bacterial strain against root fungal pathogens, *in vitro* tests were done in dual culture using PDA or NA. Agar blocks (6mm) containing 7 day old mycelia of fungi were placed 3.5cm apart on the medium in a petridish. Each bacterial isolate was streaked at one side of the agar plate about 1 cm away from the edge or by placing the fungal inocula in the centre and streaking the bacteria around the fungal inocula. For each test three replicate plates were used. The plates were inoculated for 5-7 days at 28±2$^0$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

To assess the possible mutualistic antagonisms between the fungus and bacteria in mixed culture method outlined by Chakraborty and Chakraborty (1989) was followed. PDB and NB were selected because it allows vigorous growth of both the fungus and bacteria. One agar block (6mm dia.) containing 7 day old mycelia of the fungus and 0.5 ml of bacterial suspension (1x10$^6$ cfu/ml) were used as inocula for each flask (50 ml NB/250 ml flask). The mycelium grown without the bacterial strain in the similar medium was taken as the control sample. The cultures were inoculated at 28$^0$±2$^0$C and after 15 days of incubation mycelia were washed thoroughly with sterile distilled water to remove bacteria as far as possible and harvested by staining through muslin cloth and mycelial dry weights were determined

### 3.4. Selection and identification of antagonistic microorganisms

#### 3.4.1. Microscopic observation

##### 3.4.1.1. Fungi

The isolated fungi were allowed to grow in Petridishes (7cm) containing sterile PDA medium for 7 days, then nature of mycelia growth, rate of growth and time of sporulation were observed. For identification, spore suspensions were prepared from individual culture. Drops of spore suspensions were placed on clean, grease free glass slides, mounted with lactophenol-cotton blue, covered with cover

slip and sealed with wax. The slides were then observed under the microscope following which spore characteristics were determined and size of spores measured.

### 3.4.1.2. Bacteria

Gram staining technique was followed for microscopic identification of rhizobacterial cultures. 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 tap water for 5 seconds. Next, the slide was flooded with the iodine solution and allowed it to stands for a minute as well. After a minute the slide was rinsed with tap water for 5 seconds, after which ethanol was added. The ethanol was added drop wise until no blue-violet color was emitted from the slide. Finally the slide was rinsed with water for 5 s, dried and observed under microscope.

### 3.4.2. Biochemical tests

#### i. Endospore stain

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

#### ii. Fermentation of Carbohydrates

Three test tubes containing 0.5% solution of dextrose, sucrose or lactose, nutrient broth, and a Durham tube were inoculated with the unknown isolate and incubated at room temperature for 24 h. Yellow color indicates acid production and air in the Durham indicates gas production.

#### iii. Voges Proskuser reaction

Culture tubes containing 10 ml sterile VP broth were inoculated with the bacterial isolates and incubated at $37^0$C for 3days.To the cultures 0.6 ml of 5% w/v α-ethanol-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.

**iv. Catalase**

Bacterial culture (24 h) was flooded with 0.5 ml of 10 % $H_2O_2$ solution and gas bubble production indicated the positive reaction.

**v. Urea digestion**

A test tube containing nutrient broth, urea, and phenol red was inoculated with the unknown isolate and incubated at room temperature for 24 h. A fuchsia color indicates the digestion of urea.

**vi. Esculine hydrolysis**

The slants containing esculine hydrolysis test medium were inoculated with 24 h old bacterial culture and incubated at $30^0$C for 7 days. Blackening of the medium indicates the hydrolysis of esculine.

**vii. Casein hydrolysis**

The milk agar was streaked with the bacteria and was incubated at $37^0$C and observed for the clear zone around the streaks.

**viii. Starch hydrolysis**

The bacteria were streaked on sterilized starch agar plates (NA + 0.1 % soluble starch) and inoculated for 5 days at $37^0$C. The plates were flooded with Lugol's Iodine solution. The clear zone underneath and around the growth indicates starch hydrolysis.

**ix. Indole test**

10 ml of Davis Mingoli's broth supplemented with 0.1 % tryptophan was inoculated with the isolates and incubated an aerobically at $37^0$C for 7 days. The culture were layered carefully with 2 ml of Ehrlich-Bohme (p-dimethyaminobenzaldhyde 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.

**x. Reduction of nitrate to nitrite**

Sterile nitrate broth was inoculated with test isolates and incubated at $37^0$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 napthylamine and 100 ml acetic acid. The solutions A and B were mixed in equal volume just before use) was mixed

in 1 ml of the cultures and observed for the development of red or yellow color indicating the presence of nitrite.

**xi. Gelatin liquefaction**

The organism was inoculated (stabbed) into a solid medium containing gelatin; liquefaction at room temperature or inability to resolidify at refrigerator temperature indicates the presence of photolytic enzymes.

**xii. $H_2S$ production**

Organism was inoculated into peptone iron medium; formation of black sulfides indicates that organism produces hydrogen sulfide.

**xiii. Citrate utilization**

Organism was inoculated into citrate agar medium in which citrate is the sole carbon source; an indicator in the medium changes color if citrate is metabolized; use of citrate indicates the presence of the permease that transports citrate into the cell

### 3.5. Test organisms

#### 3.5.1. Fungal Culture

For *in vitro* evaluation of antagonistic activity of rhizobacterial isolates, following root fungal pathogens viz., *Poria hypobrunnea, Fomes lamaoensis, Sphaerostilbe repens, Ustulina zonata, Sclerotinia sclerotiorum* and *Sclerotium rolfsii* were used.

#### 3.5.2. Source of culture

Tea roots pathogen *Poria hypobrunnea* Petch. was obtained from UPASI Tea Research station, Valparai, Tamil Nadu. The cultures are being maintained in the laboratory with regular sub culturing in PDA for subsequent test. Other pathogens used for the experimental purposes are mentioned in Table 2. *Sclerotium rolfsii* I Sacc. causing seeding blight in tea, *Sclerotium rolfsii* 11 Sacc. causing crown rot in orchid and collar and root rot disease in *Cicer arietinum* and *Sclerotinia sclerotiorum* (lib.) de Bary causing white rot, isolated from *Vanda roxburgii* were obtained from Immuno-Phytopathological laboratory, Department of Botany, North Bengal University .

**Table 2:** List of fungal culture used in investigations.

| Species | Host | Source |
|---|---|---|
| *Poria hypobrunnea* | Tea | UPASI Tea Research station, Valparai |
| *Fomes lamaoensis* | Tea | Tea Experimental Station, Tocklai, Assam |
| *Armillaria mellea* | Tea | UPASI Tea Research station, Valparai |
| *Rosellinia arcuata* | Tea | UPASI Tea Research station, Valparai |
| *Sphaerostilbe repens* | Tea | Tea Experimental Station, Tocklai, Assam |
| *Ustulina zonata* | Tea | Tea Experimental Station, Tocklai, Assam |
| *Sclerotium rolfsii*- i | Tea<br>Soybean | Immuno-Phytopathologicallaboratory,<br>Department of Botany, N.B.U |
| *Sclerotium rolfsii*- ii | Pea<br>Vanda | Immuno-Phytopathologicallaboratory<br>Department of Botany, N.B.U |
| *Sclerotinia sclerotiorum* | Vanda | Immuno-Phytopathologicallaboratory<br>Department of Botany, N.B.U |
| *Alternaria alternata* | Tea<br>Soil | Immuno-Phytopathologicallaboratory<br>Department of Botany, N.B.U |
| *Fusarium oxysporum* | Soil | Immuno-Phytopathologicallaboratory, NBU. |

### 3.6. Assessment of mycelial growth

Mycelial growth of the fungal cultures was assessed in both on solid media and liquid media to know their culture characteristic.

#### 3.6.1. Solid media

To assess the mycelial growth of *Poria hypobrunnea* in solid media, the fungus was first grown on petridishes, each containing 20 ml of PDA and incubated for 5-8 days at 30±2$^0$C. Agar block (6 mm diameter) containing the mycelia was cut with sterile cork borer from the advancing zone of mycelial mat and transferred to each petridish containing 20 ml of sterilized solid media. Finally diameter of mycelia was measured at regular interval of time. The media were as follows: Potato Dextrose Agar, PDA (Peeled potato-40.00g, Dextrose-2.00g, Agar- 2.00g, Distilled water-100ml); Richard's Agar, RA ($KNO_3$- 1.00g, $KH_2PO_4$- 0.50g, $MgSO_4$, $7H_20$ 0.25g Sucrose- 3.00g, Agar-1.50g, Distilled Water-100ml); Elliot's Agar, EA ($KH_2PO_4$-0.136g, $Na_2CO_3$-0.106g, $MgSO_4$, $7H_2O$-0.050g, Dextrose-0.500g, Asparagine-0.100g, Agar-1.50g), Carrot Juice Agar, CJA (Grated carrot-20.00g, Agar-2.00g, Water-100ml), Potato Sucrose Agar, PSA (Peeled potato-40.00g, Sucrose 2.00g, Agar-2.00g,

Water-100ml); Czapek-Dox Agar, CDA ($NaNO_3$ .0.20g, $K_2HPO_4$ .0.10g, KCl-0.05g , $FeSO_4$, $7H_20$-0.05g, Sucrose-3.00g, Agar-2.00g, Water-100ml); Yeast Dextrose Agar, YDA (Yeast extract-0.75g, Dextrose-2.00g, Agar-1.50g, Agar-1.50g)

### 3.6.2. Liquid media

To assess the mycelial growth of *P. hypobrunnea* in liquid media, the fungus was first grown on petridishes, each containing 20 ml of PDA and incubated for 5-8 days at $30^0 \pm 2^0$C. From the advancing zone, mycelial block (6mm) was cut with sterilized cork borer and transferred to Erlenmeyer flask (250ml) 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 aluminum foil cup of known weight and dried at $60^0$C for 96h. cooled in desiccators and weighed.

## 3.7. Assessment of bacterial growth

For assessment of bacterial growth in liquid medium, 1 ml of bacterial suspension was inoculated into the medium and allowed to grow for desired period. Following growth, absorbance was noted in a colorimeter at 600 nm. Absorbance was counted into cfu/ml from a standard where known concentration of bacterial suspension was used. The cfu values were counted to log whenever needed. For assessment of bacterial growth, different media were used. These were Potato Dextrose Broth, PDBA (Potato 400g, Dextrose 20g, Distilled $H_20$ 1L, pH 7.3 ± 0.2); Nutrient Broth, NB (Peptone 5.0g, NaCl 5.0g, Beef extract 1.5g, Yeast extract 1.5g, Distilled $H_20$ 1L, pH 7.4 ± 0.2); King's B, KB (Peptone 20.0g, $K_2HPO_4$ 1.5g, $MgSO_4.7H_20$ 1.5g Glycerol 15 ml, Distilled $H_20$ 1L, pH 7.4 ± 0.2); Luria Broth, LB (Peptone 10.0g, NaCl 5.0g, Yeast extract 1.5g, Distilled $H_20$ 1L, pH 7.2 ± 0.2); Nutrient Sucrose Broth, NSB (Sucrose 1.5 g, Yeast extract 1.2 g, Peptone 1.2 g, Beef extract 0.6g, Distilled $H_20$ 300ml, pH 7.2 ± 0.2); Glucose Yeast Peptone, GYP (Glucose 6.0 g, Peptone 3.0g, Yeast extract 1.5g, Distilled $H_20$ 300 ml, pH 7.2 ± 0.2)

## 3.8 Plant materials

### 3.8.1. Tea

#### 3.8.1.1. Selection

Tea clones were originally collected from different geographical locations of India: (a) Darjeeling Tea Research Centre, Darjeeling, (b) Tea Experimental Station,

Tocklai, Assam and (c) Tea Research station, United Planters Association of South India (UPASI), Tamil Nadu and maintained in the Germ Plasam Bank at Department of Botany, North Bengal University. For present investigation following tea varieties were used for the experimental purposes. Darjeeling varieties (K1/1, CP1, S449 and HV 39), Tocklai varieties (TV 20, T 78, TV 18 and T 17) and UPASI varieties (UP 26, UP 3 and BSS 2)

**3.8.1.2. Propagation of Tea**

Tea plants were propagated by cutting method or by seeds. In case of propagation by cuttings, plants were raised by planting cuttings made from shoots of the mother bushes. The standard form of a tea cutting is a piece of stem 2.5cm to 3.5 cm long with a good mature leaf 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 % aluminum sulphate.

Polyethylene sleeves (8" x 6") were filled up with the prepared soil and stacked 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. (Plate 2)

In case of young seedling, manuring (aluminum phosphate-8 parts by weight, ammonium phosphate- sulphate 16:20-35 parts by weight) was done after rooting following 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.

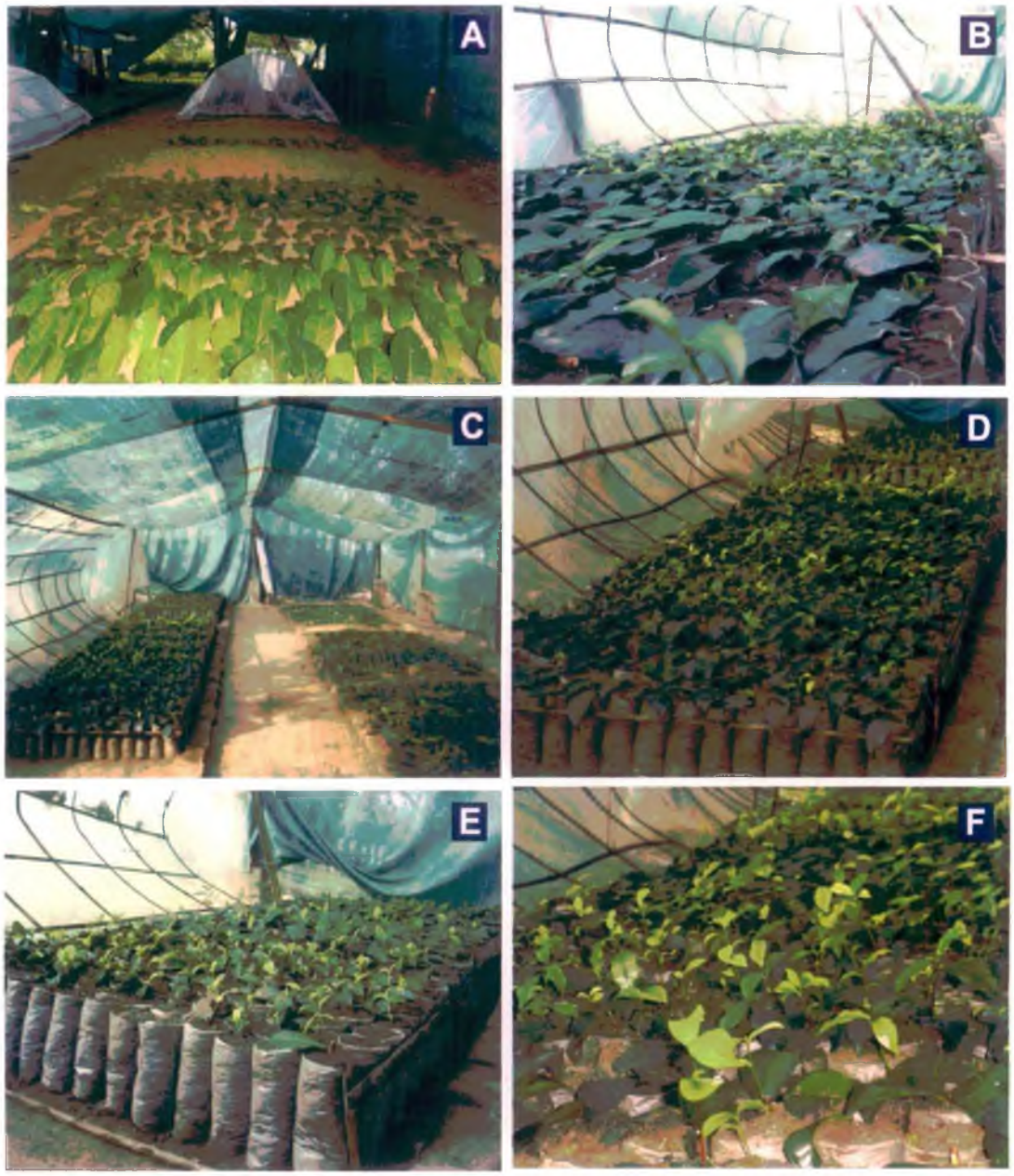


**Plate II (A-F):** Stages of Tea propagation by cutting in Nursery.

### 3.8.1.3. Plantation

For the plantation of tea varieties in the experimental plots pits were prepared for each plant. Before that, simazine @ 75 g / 20 liter water and glyphosphate @ 1:200 were used in the experimental plots for the suppression of weeds. Then pits (1 ½' X 1 ½' X 1 ½') were dug at the intervals of 2' between plant and 3.5' between rows to row. Planting 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 ) phosphate dithioate ).At the bottom of the pit, little bit of rock phosphate was placed following which half portion was covered with cattle manure soil mixture. Phorate was mixed with a portion of excavated soil and applied approximately 5cm below the ground level.

Following soil conditioning, plants were inspected, selected and brought to the experimental garden and planted in the prepared soil and pits were refilled upto ground level with conditioned soil. For experimental purposes tea plants of all the varieties were also grown in earthen pots (one plant/pot, 30cm dia.) each containing 5kg soil mixture 1:1). Ten month seedling 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.

### 3.8.2. Chickpea

The seeds of chickpea (*Cicer arietinum*) viz. ICCV2 (Swetha) 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 were then grown in the Experimental garden of Botany Department, North Bengal University.

### 3.8.3. Mungbean

The seeds of mungbean (*Vigna radiata* L.) viz. Pusa Baishakhi was collected from Pulses and Oils Seed Research Station, Berhampore, West Bengal.

### 3.8.4. Marigold

Seedlings of Marigold (*Tagetes erecta* L.) were collected from the local market and maintained in the Experimental garden of Immuno-PhytoPathology Lab. Department of Botany, North Bengal University.

## 3.9. *In vitro* characterization of PGPR activity of selected bacteria

### 3.9.1. IAA production

To determine the production of IAA, the selected bacterial cells were grown for 24h to 48h C/N ratio medium. Tryptophane (0.1mM) was added in order to enhance Indole acetic acid (IAA) production by the specific bacteria (Prinsen *et al.*, 1993). Production of IAA in culture supernatant was assayed by Pillet-Chollet method as described by Dobbelaere *et al.*, 1999. For the reaction, 1 ml of reagent, consisting of 12 g $FeCl_3$ per liter in 7.9M $H_2SO_4$ 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

### 3.9.2. Phosphate solubilisation

To assess the phosphate solubilization potential of selected bacterial Pikovskaya's agar medium (Pikovskaya, 1984) was used. Bacterial strains were individually spot inoculated at center of the agar plates followed by incubation at $30\pm2^0$C for 5 – 6 days. Formation of clear zone around the bacteria is an indication of the phosphate solubilising capacity.

### 3.9.3 Siderophore production

Production of siderophore was detected following standard method (Schwyn and Neiland, 1987) using blue indicator dye chrome azurol S (CAS). For production CAS agar, 1 L, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1mM $FeCL_3$. $6H_20$ 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 (20 ml per plate). The bacteria were spot inoculated at the center of the plates and incubated for 12-15 days. The change in the colour of the medium around the bacterial spot is an indication of siderophore production.

The bacterial strains were streaked separately into the Petriplates containing PDA amended with $FeCl_3$ at concentration of 150µg $ml^{-1}$, 300µg $ml^{-1}$ and 600µg $ml^{-1}$.The actively growing discs of fungal test pathogen *P .hyprobrunnea* was placed

opposite the bacterial streak simultaneously and plates were incubated at 30±2$^0$C for 7 days. Then zone of inhibition around the bacteria was measured. The plate containing the same medium but without $FeCl_3$ was treated as control. The experiment was done in replicates of three.

### 3.9.4 HCN production

Method as described by Wei *et al.* (1991) was followed with slight modification for determination of HCN production by selected bacterias. Bacteria were grown on NA medium supplemented with glycine (4.4 g $L^{-1}$) in a Petri plate. A strip of sterilized filter paper saturated with a solution containing picric acid 0.5% and sodium carbonate 2% placed on upper lid of petriplate. The plates were sealed with paraflim and incubated for 2-4 days. HCN production was indicated by the changes in color of the filter paper strip from yellow to brown to red.

### 3.9.5. Chitinase production

Secretion of chitinase by the selected bacteria was determined following conventional plate method using 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 30±2$^0$C for 7-8 days. Formation of clear zone around the bacteria was considered positive reaction (West and Colwell, 1994). The colloidal chitin was prepared by following the method described by Roberts and Selitrennkoff (1988), 5 g of chitin powder was slowly added to 60 ml of concentrated HCl and left at 4$^0$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$^0$C.The precipitation formed was collected by centrifugation at 7,000 rpm for 20 min at 4$^0$C and washed with sterile distilled water until the colloidal solution became neutral (pH 7.0). The prepared colloidal chitin solution (5 %) was stored at 4$^0$C until further use.

### 3.9.6. Volatile production

The antagonist and pathogen were inoculated in different Petri plates on PDA. Petri plate containing pathogen was inverted over the Petri plate-containing antagonist and sealed with adhesive tape (paraflim) keeping antagonist in lower and pathogen in

upper Petri plate (Dennis and Webster, 1971). Four sets of experiments were conducted. In one ste, the antagonists and pathogens of the same age were taken. In the second set, the antagonist culture was one day older than pathogen; in the third, the antagonists were 2 days old and in the fourth, the antagonists were 3 days old than pathogen. Each plate was incubated at 28±2$^0$C for 4 days. Radial growth of pathogen was measured after definite time interval. The plate without antagonist served as control. Each experiment considering a single bacterial isolate was performed in triplicate.

### 3.10. Application of bacteria

#### 3.10.1. Soil drench

The bacteria were grown in Nutrient Broth for 3 days at 30$^0$±2$^0$C with constant shaking. Then the bacteria culture centrifuged at 12,000 rpm for 15 min. and the pellet were collected and suspended 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 rhizosphere of potted plants either singly or in combination during transplantation of seedling from sleeves. Application was 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.

#### 3.10.2. Foliar spray

The bacterial suspension was also applied to the foliar parts of tea bushes. After addition of few drops of Tween-20, bacterial suspension was sprayed to the pruned bushes of tea to observe the shooting patterns. The spraying was done every fortnight till the new shoots started appearing. The growth parameters such as number of leaves, branches and height were observed.

#### 3.10.3. Seed bacterization

Seeds were surface sterilized with sodium hypochlorite and rinsed in distilled water after which seeds were dried under sterile air stream. 10ml of bacterial suspension containing 3 x $10^6$ cfu $ml^{-1}$ was taken in Petri plates. To this, 100mg of carboxymethyl cellulose was added as adhesive material. 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.

### 3.11. Inoculation techniques

#### 3.11.1 Preparation of pathogen inocula

The inocula of *P. hypobrunnea* were prepared in media of sand maize meal, tea root pieces and tea waste. Following preparations were used for experimental purpose.

(i) Sand maize meal media (SMM): The medium was prepared following the method described by Biswas and Sen (2000). SMM medium (maize meal: washed sterilized sand: water = 1:9:1.5, w: w: v) in autoclavable plastic bag (150g) was sterilized at 20 lb. for 20 min.

(ii) Tea root pieces: Inocula in tea root pieces was prepared following the method of Hazarika *et al.* (2000). First root and root lets were cut into small pieces (3cm x1.5cm x 1.5cm). Root pieces (22-25) were taken in 250ml flask and pieces were dipped in water for 2h and autoclaved as described above. On the next day water was decanted in a sterile chamber and root pieces were inoculated with *P. hypobrunnea*

In another method (Dhingra and Sinclair, 1985) the root pieces were boiled for 30min and sterilized in same way.12-15 sterilized root pieces were then transferred in Erlenmeyer flask (250ml) containing 20 days old *P. hypobrunnea* culture on PDB.

(iii) SMM + root pieces: In this medium SMM along with 6-8 root pieces were sterilized.

(iv) Tea waste: In this medium tea waste and sand were mixed in ratio 1:1, wetted with water and sterilized in polythene packet.

In all cases media were inoculated with *P. hypobrunnea* and incubated for 15-20 days $30^0 \pm 2^0$C.

#### 3.11.2 Inoculation of healthy tea plants

##### 3.11.2.1. Pot grown plants

In case of potted plant, 2-3 year old potted plants were used. Then 100g of *P. hypobrunnea* inoculums were added carefully in the rhizosphere and ensured that

inocula were attached to healthy tea roots. Disease assessment was done after 15, 30and 45 days of inoculation.

**3.11.2.2. Field grown plants**

Tea plants of desired ages from different variety to be tested were selected from plots in the Experimental Garden. The inoculation technique was same as described for potted plants except that 200g inocula were added in each pit. Different combinations were set with PGPRs and after inoculation maintenance was done with regularly watering.

**3.12. Disease assessment**

In order to determine the effect of bacterial isolates on disease reduction, four treatments were taken in each case

i) Untreated control.

ii) Inoculation with pathogen

iii) Inoculation with bacterial isolates.

iv) Inoculation with both bacteria and fungal pathogen.

Initially the rhizosphere was drenched with bacterial suspension prepared in sterilized water two days prior to pathogen application. *P. hypobrunnea* grown in sand maize meal medium were used as fungal inocula. Disease assessment was done after definite time intervals. 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 star 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.

### 3.13. Determination of plant growth promoting activity

#### 3.13.1. Tea

Determination of plant growth promoting activity by the bacteria was assessed under green house condition. The growth parameters such as increase in height, number of leaves and shoot were observed in treated plants in relation to the untreated control plants under the same environmental and physical condition (temperature 30-34$^0$C; R.H. 60-80 %; 16 h photoperiod). The experiment consisted of tea replicates in each treatment in completely randomized design. Observations were recorded after 2 and 4 months of final application of bacterium. 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.

#### 3.13.2. Chickpea and Mungbean

The plant growth promoting activity of bacteria in chickpea and mungbean were assessed based on the seedling vigour index by standard roll towel method. Percentage of seed germination was calculated then the root and shoot length of individual seedlings was measured to work out the vigour index. The vigour index was calculated by using the formula by Baki and Anderson (1973):

Vigour Index = (mean root length + mean shoot length) x germination percentage. The experiment was carried out in three replicates with 30 seeds in each plate

#### 3.13.3. Marigold

In case of Marigold, plant growth promotion was assessed on the basis of increase in height and no. of flowers after application of bacterial suspension as a soil drench to the rhizosphere of 2 month old plant.

### 3.14. Extraction of antifungal compounds from rhizobacteria

#### 3.14.1. Cell free culture filtrate

To prepare bacterial culture filtrate loopful of bacteria from a 24h old Nutrient Agar slant were inoculated into Nutrient Broth (50ml/250 flask ) and incubated at 35±2$^0$C with vigorous shaking on a platform shaker at 175 rpm for 96h.Centrifugation was done at 12000 rpm for 20 min. and supernatant was sterilized by cold and hot sterilization. Cold-sterilization was done by passing through sterilized micro filter (

0.22 μm pore size ).Heat-killed culture filtrate was prepared by autoclaving the supernatant at 15 lb pressure for 15 min.

### 3.14.2 Solvent extraction

In order to isolate the active principle from the culture filtrates, the bacteria were grown in NB for 96h at 35±2$^0$C in shaking condition at 175 rpm. Centrifugation was done at 12000 rpm for 20 min. and supernatant was pooled and were used as the culture filtrates. Cell-free sterilized culture filtrate was extracted separately three times with equal volume of acetone, benzene, chloroform, ethylacetate and diethyl ether The organic fraction and corresponding aqueous fraction 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 and stored in a capped bottle at 4$^0$C and used for the bioassays.

### 3.14.3. Bacterial cell

Beside solvent extraction, antifungal compounds were also extracted directly from the bacterial cells. Extraction was done with slight modification of the method described by Howell and Stiponovic (1980). The bacterial lawn was prepared in NA plate for 7 days. The agar plates were cut into 1cm squares and extracted with 200ml of 80% aqueous acetone, filtered through cheese cloth and the filtrate was centrifuged at 12,000rpm for 10 min. The supernatant was evaporated to remove acetone. To residue (5g per 100ml), NaCl was added and centrifuged at 12,000rpm for 10 min. Each portion of supernatant was extracted twice in diethyl ether and dried over anhydrous calcium chloride. The compound in a crystalline form was removed from this residue. This was recrystalized by dissolving in methanol for further studies

## 3.15. Bioassay of active principle

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

### 3.15.1. Spore germination

Fungal spores of test fungi were bioassayed against active principle obtained from selected bacteria on glass slides following the method of Trivedi and Sinha (1976). A drop of the test solution was placed on a clean, grease free slide and allowed to evaporate following which a drop of fungal 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 Petri plate for 24 h at $30^0 \pm 2^0$C. Finally one drop of lacto phenol-cotton blue was added to each spot to fix the germinated spore. The slides were observed under microscope and the percentage of germination was calculated. For each case, a total of about 200 spores were counted from about 5-8 microscopic fields.

### 3.15.2. Radial growth

The method of Fiddaman and Rossall (1994) was followed with modifications. Autoclaved PDA medium (20ml) was mixed with 0.5 ml of test compound solution and plated into petriplates (70mm dia.). After solidification, an agar block (6mm dia.) containing growing mycelium of the test fungus (4 day old) was placed in the centre of each petriplate. Control plates were mixed with solvent alone. The plates were incubated at $30^0 \pm 2^0$C and radial growth of mycelia was measured after every 2, 4, 6 and 8 days interval.

### 3.15.3. Agar cup bioassay

Antifungal activity was also determined by agar cup bioassay technique. Initially, wells were made in the periphery of the plates with a sterile cork borer and test active principle (0.2 ml) was added to each well. Following this, an agar block (2mm dia.) containing growing mycelium of the test fungal inoculums (6mm) was placed at the centre. The plate was inoculated at $30^0 \pm 2^0$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 respectively. The experiment was performed in replicates of three and was repeated thrice.

### 3.15.4. Liquid medium

Cell free culture filtrate was added to the liquid medium (PDB and NB) at different percentage (20-80 %) and inoculated with test pathogen. Then whole setup was incubated at $30^0 \pm 2^0$C for 6-7 days to observe the growth of pathogen. The media without the cell free culture filtrate was treated as the control.

### 3.16. Biochemical analyses

#### 3.16.1. Chlorophyll

#### 3.16.2.1. Extraction

For the extraction of chlorophyll from leaves of plants the method of Harborne (1973) with modifications was used. 1g leaf tissues were crushed in a mortar and pestles using 80% acetone in the dark to prevent the photo oxidation of chlorophyll. The crushed samples were filtered thorough Whitman No.1 filter paper. Final volume was made up 25 ml with adding sufficient amount of acetone. A tube containing 80% acetone was used as blank.

#### 3.16.1.2. Estimation

For the estimation of chlorophyll, absorbance of the acetone extracts was 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 = $(20.2\ A_{645}+8.02A_{663})$ μg/ml.

Chlorophyll a = $(12.7\ A_{663} - 2.69\ A_{645})$ μg/ml.

Chlorophyll b = $(22.9\ A_{645} - 4.68\ A_{663})$ μg/ml.

#### 3.16.2. Phenols

#### 3.16.2.1. Extraction

Method of Mahadevan and Sridhar (1982) was followed for the extraction of phenol. 1g of fresh leaf tissues were cut into small pieces and immediately immersed in 10 ml of 80% boiling ethanol. After 15 minutes of boiling, it was cooled and crushed in mortar and pestle followed by filtration through Whatman No1 filter paper. Final volume was adjusted with 80% ethanol. The whole extraction of phenol was done in dark to prevent induced degradation of phenol. The filtrate was used for further assay.

#### 3.16.2.2. Estimation

##### 3.16.2.2.1. Total phenol

For estimation of total phenol content of leaves, to 1 ml of the alcoholic extract, I ml of I N Folin Ciocalteau's reagent and 2 ml of 20 % sodium carbonate solution was added in test tube. The reaction mixture was shaken and heated on a boiling water bath for 1 min. After cooling under running tap water, the volume of the reaction mixture was raised to 25 ml by adding distilled water. 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.

**3.16.2.2.2. O – dihydroxy phenol**

For the estimation O-dihydroxy phenol content of leaves, to 1ml of alcoholic extract, 2 ml of 0.5N HCl, 1ml of Arnow's reagent ($NaNO_2$, 10g $Na_2$ $MoO_4$ – 10g distilled water – 100ml) and 2 ml of 1N NaOH was added. The reaction mixture turned pink. The volume of the reaction mixture was raised to 10 ml by adding distilled water. 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.

**3.16.3. Extraction of Enzymes.**

**3.16.3.1. β- 1, 3- glucanase** (EC .3.2.1.39)

Enzyme was extracted following method described by Pan *et al.* (1991). 1g of tea leaves were crushed in liquid nitrogen using 5 ml of chilled 0.05 M sodium acetate buffer (pH 5.0). The extracts were collected for mortar and pestle and then centrifuged at 10,000 rpm for 15min at 4°C and the supernatant was used as crude enzyme extract.

**3.16.3.2. Chitinase** (EC. 3.2.1.39)

Chitinase was extracted following the method described by Boller and Mauch (1988) with modifications. 1g of tea leaf sample was crushed in liquid nitrogen and extracted using 5 ml of chilled 0.1 M sodium citrate buffer (pH 5.0). The extract was centrifuged at 12,000 rpm for 20 min. The supernatant was collected and used as enzyme source for further assay.

**3.16.3.3. Phenylalanine ammonia lyase** (EC.4.3.1.5)

1 gm of leaf tissues was crushed in 5 ml of 0.1M sodium borate buffer (pH 8.8) containing 2 mM β– mercaptoethanol at 4°C followed by centrifugation at 12,000 rpm for 20min at 4°C. The supernatant was collected and after recording its volume, was immediately used for assay or stored at -20°C. (Chakraborty *et al.* 1993)

**3.16.3.4. Peroxidase** (EC. 1.11.1.7)

The enzyme was extract by crushing 1 gm of leaf sample in 5ml of 0.1 M sodium borate buffer (p11 8.8) containing 2mM β-meraptoethanol at 4°C followed by centrifugation at 12,000 rpm for 20min 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)

**3.16.4. Assay of enzyme activities**

**3.16.4.1. β-1, 3-glucanase**

Estimation of the β-1, 3 glucanase activities was measured 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 $40^0$ 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 preparations mixed with laminarin with zero time incubations. The enzyme activity was expressed as µg glucose liberated from laminarin by activity of enzyme from 1g tissue/min.

**3.16.4.2. Chitinase**

For estimation of chitinase activity of plants, to 10 µl of 1 M sodium acetate buffer (pH 4.0), 0.4 ml of enzyme solutions and 0.1 ml of colloidal chitin were added. Mixtures were incubated at $37^0$C for 2h then the reaction was stopped by centrifugation at 10,000 rpm for 3 min. An aliquot of supernatant was pipetted into a glass reagent tube containing 30 µl of 1M potassium phosphate buffer, pH 7.1 and incubated with 20 µl of (3% w/v) desalted snail gut enzyme Helicase (Sigma) for 1h, after which pH of the reaction mixture was brought to 8.9 by addition of 70µl of 1M sodium borate buffer (pH 9.8).The mixture was incubated in boiling water bath for 3 min and then rapidly cooled over ice. After addition of 2ml β-dimethylamino benzaldahyde (DMAB) reagent, the mixture was incubated for 20 min at $37^0$C

Immediately thereafter the absorbance value at 585 nm was measured using a UV-VIS spectrophotometer. Activity was expressed as mg N-acetyl glucosamine (GleNAc) released from chitin by activity of enzyme from 1g tissue/min.

### 3.16.4.3. Phenylalanine ammonia lyase (PAL)

PAL activity was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. For the estimation, 300µl of L-phenylalanine, 300µl of 0.0003M sodium borate (pH 8.0), 500µl of enzyme extract and 1.9ml of water was added in test tube followed by incubation at $40^0$C for 1h in water bath. The absorbance was recorded at 290nm against a bank without the enzyme in the assay mixture in UV-VIS spectrophotometer. The enzyme activity was expressed as µg cinnamic acid produced in 1 min/g fresh weight of tissue.

### 3.16.4.4. Peroxidase

For estimation of peroxidase activity in treated and untreated control plants, 100µl of freshly prepared crude enzyme extract, 1 ml of 0.2 M Sodium phosphate buffer (pH 5.4), 100 µl of ortho-dianisidine (5mg $ml^{-1}$methanol) and 1.7 ml of distilled water were added in a spectrophotometer cuvette. To initiate the reaction 0.1ml of $H_2O_2$ was added to the cuvette and changes in absorbance were recorded at 460nm in a UV-VIS spectrophotometer per minute. Peroxidase activity was expressed on fresh weight basis as changes in absorbance $min^{-1}g^{-1}$ (Chakraborty *et al.* 1993).

## 3.17. Catechins

### 3.17.1. Extraction

Catechins were extracted from tea leaf tissues following the method of Obanda *et al.* (1994) with slight modification. At first 10g leaves samples were heated in 100ml of acetone at $45^0$C in water bath for 30 min. The 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 adding two drops of 2N 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 filtered though milipore filter (Milipore 0.4µm HA filter paper).

### 3.17.2. HPLC analysis.

The extracted catechins were analyzed by HPLC using C-18 hypersil column with linear gradient elution system as follows: mobile phase A 100% acetonitrile, 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 ul and monitored at 278 nm. Peaks were compared with the peaks obtained for standard catechins. (Sigma chemicals, USA)

### 3.18. Immunological studies

### 3.18.1. Preparation of antigens

#### 3.18.1.1. Bacterial antigens

Bacterial antigen was obtained by strong sonication of bacterial cells in 0.05 M sodium phosphate buffer (pH 7.2) under chilled condition and centrifuged at 4°C for 20 minutes at 10.000 rpm. Then supernatant was collected and used as antigen for the preparation of antibody.

Prior to injection quantity of protein was measured and these was also analyzed by SDS-PAGE.

#### 3.18.1.2. Fungal antigens.

Mycelial protein was prepared following the method as outlined by Chakraborty and Saha (1994). Mycelia mats were harvested from 7-10 days old culture and washed with 0.2% NaCl then again rewashed with sterile distilled water. Washed mycelia were crushed with sea sand using a chilled mortar and pestle and homogenized with cold 0.05M sodium phosphate buffer (pH 7.2) supplemented with 0.85% NaCl, 10mM sodium metabisulphite and 0.5mM $MgCl_2$ in ice-bath. The homogenate mixture was kept for 2h or overnight at $4^0$C and then centrifuged at 10,000 rpm for 30min. at $4^0$C to eliminate cell debris.

The supernatant was collected and stored in -$20^0$C and used as antigen for the preparation of antiserum.

#### 3.18.1.3. Soil antigens

Soil antigens were prepared following the method of Walsh *et al.* (1996). Soil samples were collected and 1gm of soil was crushed in 2ml of 0.05 M sodium carbonate–bicarbonate buffer (pH 9.6) in a mortar and pestle and kept overnight at $4^0$C. Next day centrifugation was done at 10,000 rpm for 10 min. The supernatant was collected and used as antigen for micro plate trapping and blotting purposes.

### 3.18.2. Estimation of protein content and SDS-PAGE analysis of antigenic proteins

#### 3.18.2.1. Estimation of soluble protein

Soluble proteins were estimated following the method as described by Lowry *et al.* 1951. To 1 ml of protein sample (taking $10^{-1}$ to $10^{-2}$ dilution) 5 ml of alkaline reagent (0.5ml of 1% $CuSO_4$ and 0.5ml 0f 2% sodium potassium tartarate added to 50ml of 2% $NaCO_3$ in 0.1 N NaOH) was added and incubated for 15-20 min. at room temperature. Then 0.5 ml of Folin ciocalteau reagent (diluted 1:1 with distilled water) was added and again incubated for 15 min and colour was developed following absorbance values were measured at 700 nm. Quantity of protein was measured from standard curve made with Bovine Serum Albumin (BSA)

#### 3.18.2.2. SDS-PAGE analysis of protein

Sodium dodecyl sulphate polyacrilamide gel electrophoresis was perfprmed for the detailed analysis of protein profile following the method of Laemmli (1970)

#### 3.18.2.2.1.Preparation of stock solutions

Following stock selections were prepared:

**A. Acrylamide and N'N'- methylene bis acrylamide**

A stock solution containing 29% acrylamide and 1% bis acrylamide was prepared in warm water. As both of them are slowly dominated to acrylic and bis acrylic acid by alkali and light, the pH of the solution was kept below 7.0 and the stock solution was filtered through Whatman No. 1 filter paper and was kept in brown bottle, stored at 4°C and used within one month.

**B. Sodium Dodecyle sulphate (SDS)**

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

**C. Tris buffer**

(a) 1.5 m Tris buffer i)1.5 M Tris buffer was prepared for resolving gel. (pH adjusted to 8.8 with concentrated HCl and stored at $4^0$C for use).

(b)1.0 M Tris buffer was prepared for use in the stacking and loading buffer. (pH adjusted to 6.8 with conc. HCl and stored at $4^0$C for use).

**D. Ammonium persulphate (APS)**

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

**E. E. Tris-Glycine electrophoresis buffer**

This is a running buffer and consists of 25mM Tris base, 250mM Glycine (pH-8.3) and 0.1% SDS; A 1x solution can be made by dissolving 3.02g Tris base, 18.8g glycine and 10 ml of 10% SDS in 1L of distilled water.

**F. SDS loading buffer**

This buffer consists of 5mM Tris HCl (pH 6.8), 10mM β-mercaptoethanol; 2% SDS; 0.1% bromophenol blue, 10% glycerol. A 1x solution was made by dissolving 0.5 ml of 1M Tris buffer (pH 6.8), 0.5 ml of 14.4M β-mercaptoethanol, 2ml of 10% SDS, 10mg bromophenol blue, 1ml of glycerol in 6.8ml of distilled water.

**3.18.2.2.2. Preparation of Gel**

To analyze the protein pattern through SDS-PAGE slab gels (mini) were prepared. Two glass plates (size 8cm x10cm) 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 the three sides and were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solutions during pouring. Resolving gels were prepared by mixing stock solutions in the following order and poured leaving sufficient space (comb+1cm) for the stacking gel.

**10% resolving gel**: Composition of resolving gel solution of 7.5ml was as follows

| | Selections | Amount |
|---|---|---|
| a) | Distilled water | 2.95ml |
| b) | 30% Acrylamide mixture | 2.50ml |
| c) | 1.5 m Tris (pH 8.8) | 1.0ml |
| d) | 10% SDS | 0.075ml |
| e) | 10% APS | 0.075ml |
| f) | TEMED | 0.003ml |

The gel was immediately overlayered with isobutanol so that surface of gel remains even after polymerization. The solution was kept for 1h for polymerization of resolving gel. After polymerization isobutanol was poured off and washed with distilled water to remove any unpolymerized acrylamide. Then stacking gel (5%) was prepared by mixing the stock solutions.

**5% stacking gel**: Composition of gel solution was as follows:

| | Selection | Amount |
|---|---|---|
| a) | Distilled water | 2.10ml |
| b) | 30% Acrylamide mixture | 0.50ml |
| c) | 1.0 m Tris (pH 6.8) | 0.38ml |
| d) | 10% SDS | 0.03ml |
| e) | 10% APS | 0.03ml |
| f) | TEMED | 0.003ml |

Stacking gel solution was poured over the resolving gel and comb was inserted immediately leaving a space of 1 cm between resolving gel and comb and overlayered with water. The gel kept for 30 min. After polymerization of stacking gel the comb was removed and washed thoroughly. The gel was then mounted in the electrophoresis apparatus after removing the spacer at the bottom. Tris-glycine running buffer was added sufficiently in both upper and lower reservoir. Precaution was taken to remove any bubble trapped at the bottom of gel.

**3.18.2.2.3. Sample preparation**

Sample was prepared by mixing the sample protein (34µl) with 1 x SDS gel-loading buffer (16µl) in cyclomixer. All the samples were floated in boiling water bath for 30mim to denature the protein sample. The samples were immediately loaded in a predetermined order into the bottom of the well with T-100 micropipette syringe. Along with the samples, protein marker consisting of a mixture of six proteins ranging in molecular weight from high to low (phosphorylase-b-97, 400; bovine serum albumin- 58,000; ovalbumin-43, 000; carbonic anhydrase- 29,000; soybean trypsin inhibitor-20, 000; lysozyme-14, 300 daltons) was treated as the other samples and loaded in separate well.

**3.18.2.2.4. Electrophoresis**

Electrophoresis was performed at constant 18mA current for a period about 3h until the dye front reached the lower end of gel.

**3.18.2.2.5. Fixing and Staining**

After completion of 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 250mg of coommassie brilliant blue (Sigma R250) in 45ml methanol. When the stain was completely dissolved 45ml of distilled water and 10ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No. 1 filter paper.

The gel was removed from fixer and stained in this staining solution for 4h at 370C with constant shaking at very low speed. After staining, the gel was finally destained in destaining solution containing methanol, distilled water and acetic acid (4.5: 4.5:1) at 400C with constant shaking until background became clear.

**3.18.3. Raising of polyclonal antibodies**

**3.18.3.1. Rabbits and their maintenance**

Polyclonal antibodies were prepared against fungal and bacterial antigens in New Zealand white male rabbits approx. 2kg of body weight. Before immunization, the body weights of rabbits were recorded and observed for at least one week inside the cages. Rabbits were maintained in Animal House (Antisera Reserve for Plant Pathogens), IP Lab., Dept. of Botany, NBU. They were regularly fed with green grass, soaked gram, carrot and green vegetables like cabbage, carrots etc, and twice a day. Beside this, they were given saline water after each bleeding for three consecutive days. Cages and floor were cleaned everyday in the morning for better hygienic conditions.

**3.18.3.2. Immunization**

Method of Alba and Devay (1985) and Chakraborty and Saha (1994) were followed for immunization. Before immunization, normal sera were collected from rabbit. For developing antisera, intramuscular injections of 1 ml antigen emulsified in equal volume of Freund's complete adjuvant (Genei) were given into each rabbit 7

days after pre-immunization bleeding and repeating the doses at 7days intervals for consecutive week followed by Freund's incomplete adjuvant (Genei) at 7days intervals upto 12-14 consecutive weeks as required.

### 3.18.3.3. Bleeding

The rabbits were bled by marginal ear vein puncture. First bleeding was taken 3 days after first six injections and then subsequently seven times more every fortnight. During bleeding the rabbit was placed in their backs on a wooden board, fixed at $60^{O}$ angles. The neck of the rabbit was held tightly in the triangular gap at the edge of the board and the body was fixed in such a way that the rabbit could not move during the bleeding. The hairs were removed from upper side of the ear followed by disinfection with spirit. Then 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, all precautionary measures were taken to stop the flow of the blood from the puncture. The blood sample was kept at $37^{O}$C for 1h for clotting. Then the clot was loosened with a sterile needle and antiserum was taken and clarified by centrifugation at 5,000 rpm for 10 min at room temperature. Finally, antisera were distributed in 1 ml vial and stored at $-20^{O}$C. These antisera were used for Double diffusion analysis, Dot blot analysis and Enzyme Linked Immunosorbent Assay (ELISA).

### 3.18.4. Purification of IgG

### 3.18.4.1. Precipitation

IgG was purified by affinity chromatography on a DEAE cellulose column following the method of 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 $22^{O}$C for h. Then the precipitate was dissolved in 2 ml of 0.02 M sodium phosphate buffer, pH 8.0.

### 3.18.4.2. Column preparation

Eight gram of DEAE cellulose (Sigma Co. USA) was suspended in distilled water for overnight. The water was poured off and the DEAE cellulose was suspended in 0.005 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 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.

**3.18.4.3. Fraction collection**

At the top of the column, 2ml of ammonium sulphate precipitate was applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02 M to 0.03 M. The initial elution buffer (1) was 0.02 M sodium phosphate 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 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 (DIGISPEDC 200GL).

**3.18.5. Immunodiffusion.**

**3.18.5.1. Preparation of agarose slides**

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 was prepared in 0.05 M Tris–barbiturate buffer (pH 8.6). The buffer was heated within a conical flask placed in a boiling water bath 0.9% agarose was mixed to the hot buffer and boiled for the next 15 min. The flask was repeatedly shaken thoroughly in order to prepare clean molten agarose and 0.1 % (w/v) sodium azide was added into it. For the preparation of agarose gel, the molten agarose was poured on the sterilized glass slides (10 ml slide$^{4}$) in laminar flow chamber and kept 15 min for solidification. After the 3-7 wells are cut out with a sterilized cork borer ( 6 mm diameter) at a distance of 1.5 cm – 2 cm away form central well and 2.0 – 2.5 cm from well to well ( peripheral).

**3.18.5.2. Diffusion**

Agar gel double diffusion tests were carried out using antigen and anfiserum following the method of Ouchterlony (1976). The antigen and undiluted antisera ( 50 µl/well) was pipette 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.

**3.18.5.2.1. 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 0.1% $NaN_3$) for 72 h with 6 hourly changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. Then slides were stained with Coomassic brilliant blue (R 250, Sigma; 0.25 g Coomassic blue, 45 ml methanol, 45 ml distilled water and 10 ml glacial acetic acid) for 10 min. at room temperature. After staining the slides were washed in destaining solution (methanol: distilled water: acetic acid in 45:45:10 ratio) with changes until the background became clear. Finally slides were washed with distilled water and dried in hot air oven for 3 h at 50°C.

**3.19. Determination of bacterial sustainability in soil**

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

**3.19.1. Enzyme Linked Immunosorbent assay (ELISA).**

ELISA tests as out lined by Chakraborty *et al.* (1995) were carried out using following buffers.

1. Antigen coating buffer-Carbonate-Bicarbonate buffer 0.05M pH 9.6

**Stocks**

A. Sodium carbonate-5.2995g in1000ml dist. water.

B. Sodium bicarbonate-4.2g in 1ooml dist. water.

160ml of stock A was mixed with 360ml of stock B and pH was adjusted to 9.6.

2. Phosphate buffer saline: 0.15M PBS pH-7.2

**Stocks**

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

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

280ml stock A was mixed with 720ml of stock B and the pH was adjusted to 7.2. Then 0.8% NaCl and 0.02% KCl was added to the solution.

3. 0.15M phosphate buffer saline-Tween (0.15M PBS-Tween, pH7.2)

To 0.15M PBS, 0.05% Tween-20 was added.

4. Blocking reagent (Tris buffer saline, pH 8.0)

   0.05 M Tris, 0.135M NaCl, 0.0027 M KCl

   Tris -0.657g

   NaCl-0.81g

   KCl -0.223g

Distilled water was added to make up the volume to 100ml. Then pH was adjusted to 8.0 and 0.05% Tween 20 and 1% Bovine serum albumin (BSA) were added.

5. Antisera dilution buffer (0.15M PBS-Tween, pH 7.2)

In 0.15 M PBS- Tween, pH 7.2, 0.2%BSA, 0.02% Polyvinylphyrrolidone, 10,000(PVPP 10,000) and 0.03% sodium azide ($NaN_3$) was added.

6. Substrate.

P- Nitrophenyl phosphate (Himedia) 1mg/ml dissolved in substrate buffer (1.0% w/v, diethanolamine, 3mM $NaN_3$, pH 9.8).

7. 3 N NaOH solution was used to stop the reaction.

**3.19.1.1. Direct antigen coated (DAC) ELISA.**

DAC-ELISA was performed following the method as described by Chakraborty *et al.* 1995. Antigens were diluted with coating buffer and the antigens were loaded (200 µl per well) in ELISA plate (Coaster EIA/RIA. Strip plate, USA) arranged in 12 rows in a (Cassette) ELISA plate. 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 I h. After incubation, the plate was washed as mentioned earlier, Purified IgG was diluted in antisera dilution buffer and loaded (200 µl per well) to each well and incubated at 4°C overnight. After a further washing, goat antirabbit IgG labeled with alkaline phospatase (Sigma chemicals, USA, in 1: 10,000 dilution with PBS), was added to each well (200 µl per well) and incubated at 37°C for 2 h. The plate was washed dried and loaded with 200µl of p-Nitrophenyl

Phosphate substrate in each well and kept in dark for 45-60min.Color development was stopped by adding 50µl per well of 3N NaOH solution and the absorbance was determined in an ELISA Reader (Multiskan, ThermoLabsystems) at 405 nm. Absorbance values in wells not coated with antigens were considered as blanks.

**3.19.2. Dot Blot**

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

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

(ii) Tris buffer saline (10mMpH 7.4) with 0.09% NaCI and 0.5 % Tween-20fo washing.

III Blocking solution 10 %( w/v) skim milk powder (casein hydrolysate. SLR) in TBST 0.05 Tris – HCL, 0.5

IV Alkaline phosphatase buffer (100 mM Tris HCI. 100 mM NaCI, 5mM $MgCI_2$

**Assay**

Nitrocellulose membrane (Millipore, 7cmx10cm, Lot No.: H5SMO 5255, pore size 0.45um Millipore corporation, Bedford) was first cut carefully into the required size and placed inside the template. 4µl of coating buffer was loaded into each well of the template over the NCM and was kept for 30 min. to dry. Following this 4µl 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 $4^{O}C$ for overnight. The membrane was then washed gently with running tap for 3 min. followed three time 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: 10000 in alkaline phosphatase) for 5 h at $37^{O}C$. 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.

### 3.20. 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. pumilus* 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 (pH7.2)]. 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.

#### 3.20.1. ELISA

The protein extracted from pathogen inoculated soil samples was analyzed by ELISA technique as mentioned before.

#### 3.20.2. Dot-blot

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

### 3.21. Preparation of tale – based PGPR formulation and its application in the soil

For development of bio-formulation 10 g of CMC was mixed with I 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 inocula containing $3x10^9$cfu/ml 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 tale-based formulation.

Bioformulation was also made in the tea waste. In case of tea waste bioformulation, the waste was soaked in water overnight to remove excess phenols. This was then dried and procedure as above was adopted.

## 4.1. Isolation of microorganisms from soil, screening and selection of antagonists

In order to isolate rhizosphere microorganisms, soil samples were collected from the rhizosphere of the healthy tea plants of different ages growing in different regions of the Terai and Dooars. The collected samples were brought to the laboratory and their pH was noted initially. In all cases, the soils were found to be acidic, ranging from 4.4-5.2. The microorganisms were 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 $g^{-1}$. Results revealed that the maximum population was observed in the rhizosphere of 40 years old bushes of the Terai, and minimum in 10 years old bushes of NBU Experimental Garden. The rhizosphere of the very old as well as young tea bushes had much lesser microbial population. Population did not show any consistent difference with the variety. The maximum microbial population was obtained in the hot humid months, which decreased during winter. ((Tables 3 and 4; Plates III and IV)

**Table 3**: Microbial population and number of isolates from tea rhizosphere

| Soil sample | Age (Years) | Microbial population (cfu / g soil) | No. of isolates Bacteria | Fungi |
|---|---|---|---|---|
| Hansqua Tea Estate, Terai | 90 | $14x10^7$ | 10 | 05 |
| CoochBehar Tea Estate, Dooars | 50 | $16x10^7$ | 15 | 07 |
| Kadommini Tea Estate, Dooars | 70 | $20x10^7$ | 18 | 06 |
| Margaet's Hope Tea Estate, Darjeeling | 75 | $25x10^8$ | 12 | 05 |
| Chandmoni Tea Estate, Terai | 80 | $10x10^8$ | 14 | 10 |
| Tea Research station Nagrakata Doors | 40 | $15x10^8$ | 20 | 12 |
| Tea Experimental Garden, NBU | 10 | $12x10^6$ | 10 | 08 |

The soil samples from each site were also tested for their pH. The soils were found to be acidic ranging from pH 4.2-5.2 (Table 4)

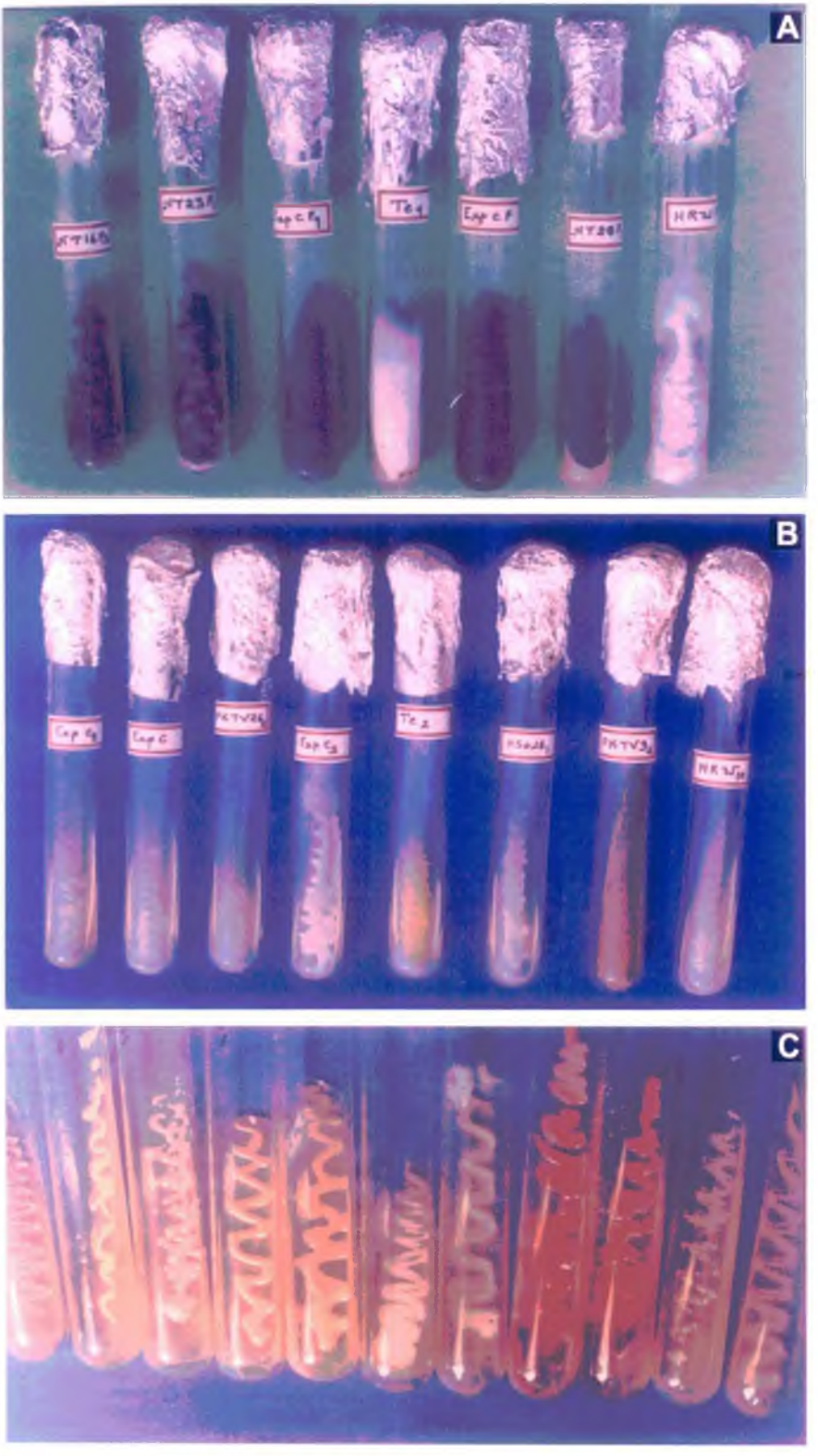


**Plate III:** Isolated soil microorganisms in test tube: (A) Fungi; (B & C) Bacteria.

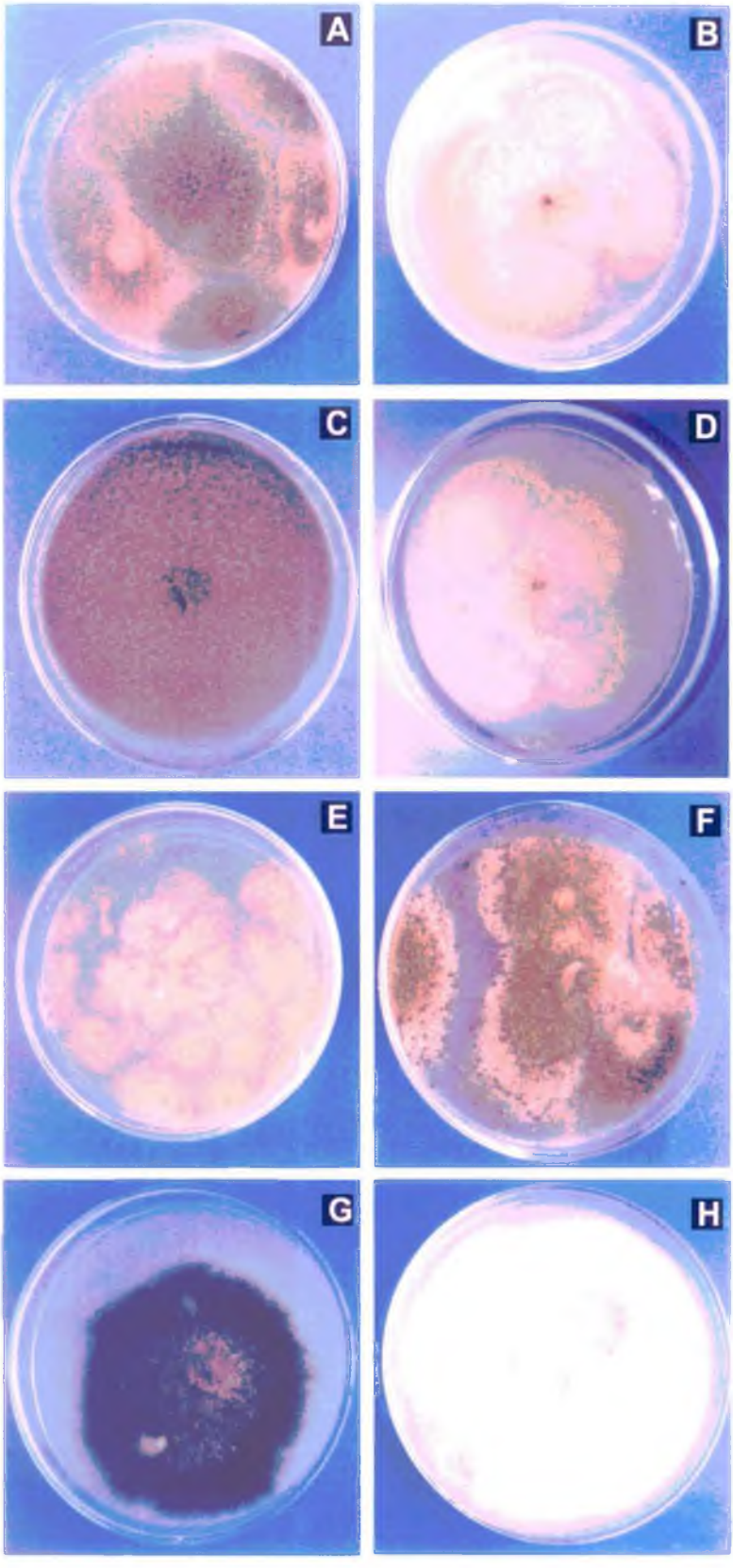


**Plate IV(A-H):** Fungal isolates obtained from tea rhizosphere grown on PDA.

**Table 4:** pH of the soil samples collected from different tea plantations

| Name of the Garden | Soil pH | Tea variety |
|---|---|---|
| Hansqua Tea Estate, Terai | 4.3 | TV-9 |
| CoochBehar Tea Estate, Dooars | 4.8 | TV-9, T-17 |
| Kadommini Tea Estate, Dooars | 4.9 | TV-26 |
| Margaet's Hope Tea Estate, Darjeeling | 5.0 | BSS-2 |
| Chandmoni Tea Estate, Terai | 5.2 | TV-18 |
| Nagrakata Tea Research station Dooars | 4.9 | TV-1,TV-9,TV-16,TV-23, Teenali17/1/54,Jorhat $S_3A_1$ |
| Tea Experimiental Garden, NBU | 4.9 | TV-18, HV-39, UP-2,UP-3, UP 26, T-17, T-78,S- 449, K-1/1, CP-1, |

The isolated bacteria were studied under microscope after suitable staining. Bacteria were classified into Gram positive and Gram negative and were taken for further biochemical tests. A large number of both Gram positive and negative bacteria were isolated.

All isolated bacteria were tested for their antagonistic activity against certain root pathogens-*Poria hypobrunnea*, *Fomes lamaoensis*, *Sphaerostilbe repens*, *Sclerotium rolfsii* and *Sclerotinia sclerotiorum in vitro* by dual pairing tests. Their interactions were categorized into the type–A: Homogenous; B: Overgrowth; C: Inhibition and D: Cessation at line of contact (Table 5)

Among all tested microorganisms, it was observed that five of the bacterial isolates viz. $HRW_9$, $HRW_{10}$, $HSoil_1$, $NT16_4$ and $KT26_2$ showed antagonistic activity against the soil borne pathogens.

**Table 5:** Interaction of soil isolates and test fungi in pairing test

(a) Bacterial isolates.

| Serial No. | Source of Soil | Isolate No. | Antagonistic test performed with | | |
|---|---|---|---|---|---|
| | | | *Fomes lamaoensis* | *Poria hypobrunnea* | *Sphaerostilbe repens* |
| 1 | Hansqua Tea Estate | H S1 | C | C | C |
| 2 | | H S 2 | B | B | B |
| 3 | | H S 3 | B | B | B |
| 4 | | H S 4 | B | B | B |
| 5 | | HRW 1 | B | B | B |
| 6 | | HRW 2 | B | B | B |
| 7 | | HRW 3 | B | B | B |
| 8 | | HRW 4 | B | B | B |
| 9 | | HRW 5 | B | B | B |
| 10 | | HRW 6 | B | B | B |
| 11 | | HRW 7 | B | B | B |
| 12 | | HRW 8 | B | B | B |
| 13 | | HRW 9 | C | C | C |
| 14 | | HRW 10 | C | C | C |
| 15 | Nagrakata Tea Research Station. TV-16 | NT16 1 | B | B | B |
| 16 | | NT16 2 | B | B | B |
| 17 | | NT16 3 | - | Partial Inhibition | - |
| 18 | | NT16 4 | C | C | C |
| 19 | | NT16 5 | B | B | B |
| 20 | | NT16 6 | B | B | B |
| 21 | | NT16 7 | B | B | B |
| 22 | | NT16 8 | B | B | B |
| 23 | | NT16 9 | B | B | B |
| 24 | | NT16 10 | B | B | B |
| 25 | | NT16 14 | B | B | B |
| 26 | $S_3A_1$ | NJ 1 | B | B | B |
| 27 | | NJ 2 | B | B | B |
| 28 | | NJ 3 | B | B | B |
| 29 | | NJ 4 | B | B | B |
| 30 | | NJ 5 | B | B | B |
| 31 | | NJ 6 | B | B | B |

| Serial No. | Source of Soil | Isolate No. | Antagonistic test performed with | | |
|---|---|---|---|---|---|
| | | | *Fomes lamaoensis* | *Poria hypobrunnea* | *Sphaerostilbe repens* |
| 32 | T-17/1/154 | Te 1 | B | B | B |
| 33 | | Te 2 | B | B | B |
| 34 | | Te 3 | - | - | - |
| 35 | | Te 4 | B | B | B |
| 36 | | Te 5 | B | B | B |
| 37 | | Te 6 | B | B | B |
| 38 | TV-1 | NT1 1 | B | B | B |
| 39 | | NT1 2 | B | B | B |
| 40 | | NT1 3 | B | B | B |
| 41 | TV-23 | NT23 1 | B | B | B |
| 42 | | NT23 2 | B | B | B |
| 43 | | NT23 3 | B | B | B |
| 44 | TV-9 | NT9 1 | B | B | B |
| 45 | | NT9 2 | B | B | B |
| 46 | | NT9 3 | B | B | B |
| 47 | | NT9 4 | B | B | B |
| 48 | | NT9 5 | B | B | B |
| 49 | | NT9 6 | B | B | B |
| 50 | | NT9 7 | B | B | B |
| 51 | | NT9 8 | - | - | - |
| 52 | TV-28 | NT28 1 | - | - | - |
| 53 | | NT28 2 | - | - | - |
| 54 | | NT28 3 | B | B | B |
| 55 | Chandmoni Tea estate TV-18 | TR/Bc 1 | B | B | B |
| 56 | | TR/Bc 2 | B | B | B |
| 57 | | TR/Bc 3 | B | B | B |
| 58 | | TR/Bc 4 | B | B | B |
| 59 | | TR/Bc 5 | B | B | B |
| 60 | | TR/Bc 6 | B | B | B |
| 61 | | TR/Bc 7 | B | B | B |
| 62 | | TR/Bc 8 | B | B | B |
| 63 | | TR/Bc 9 | B | B | B |
| 64 | | TR/Bc 10 | B | B | B |

| Serial No. | Source of Soil | Isolate No. | Antagonistic test performed with | | |
|---|---|---|---|---|---|
| | | | *Fomes lamaoensis* | *Poria hypobrunnea* | *Sphaerostilbe repens* |
| 65 | NBU Experimental Garden | $TR/B_p1$ | B | B | B |
| 66 | | $TR/B_p$ 2 | B | B | B |
| 67 | | $TR/B_p$ 3 | B | B | B |
| 68 | | $TR/B_p$ 4 | B | B | B |
| 69 | | $TR/B_p$ 5 | B | B | B |
| 70 | | $TR/B_p$ 6 | B | B | B |
| 71 | | $TR/B_p$ 7 | B | B | B |
| 72 | | $TR/B_p$ 8 | B | B | B |
| 73 | | $TR/B_p$ 9 | B | B | B |
| 74 | | $TR/B_p$ 10 | B | B | B |
| 75 | Kadommini Tea Estate TV-26 | KT26 1 | B | B | B |
| 76 | | KT26 2 | C | C | C |
| 77 | | KT26 3 | B | B | B |
| 78 | | KT26 4 | B | B | B |
| 79 | | KT26 5 | B | B | B |
| 80 | | KT26 6 | B | B | B |
| 81 | CoochBehar Tea Estate TV-9 | CT9 1 | B | B | B |
| 82 | | CT9 2 | B | B | B |
| 83 | | CT9 3 | B | B | B |
| 84 | T-17 | CT17 1 | B | B | B |
| 85 | | CT17 2 | B | B | B |
| 86 | | CT17 3 | B | B | B |

**(b)** Fungal isolates

| Serial No. | Source | Isolate No. | Antagonistic test performed with | | |
|---|---|---|---|---|---|
| | | | *Fomes lamaoensis* | *Poria hypobrunnea* | *Sphaerostilbe repens* |
| 1 | Nagrakata Tea Research Station. TV-16 | NT16F 1 | A | A | A |
| 2 | | NT16F 2 | B | B | B |
| 3 | | NT16F 3 | A | A | A |
| 4 | | NT16F 4 | B | B | B |
| 5 | | NT16F 5 | B | B | B |
| 6 | TV-9 | NT9F1 | A | A | A |
| 7 | | NT9F 2 | A | A | A |
| 8 | | NT9F 3 | B | B | B |
| 9 | | NT9F 4 | A | A | A |
| 10 | | NT9F 5 | B | B | B |
| 11 | Hansqua Tea Estate | HSF5 | A | A | A |
| 12 | | HRWF1 | B | B | B |
| 13 | | HRW F2 | B | B | B |
| 14 | | HRWF3 | B | B | B |
| 15 | | HRWF4 | A | A | A |
| 16 | | HRWF6 | A | A | A |

A: Homogenous; B: Overgrowth; C: Inhibition & D: Cessation at line of contact.

### 4.2. Characterization and identification of selected antagonists

Morphological and biochemical characteristics of the selected antagonistic bacteria were studied in the laboratory. (Table 6; Plate VI). Based on their morphology and biochemical tests, these bacteria were tentatively identified in the laboratory and their identity was confirmed at Diagnostic and Advisory Service, CABI Bioscience, U.K. These were identified as;

| | | |
|---|---|---|
| HRW9 | *Ochrobactrum anthropi* | TRS-1 |
| HRW10 | *Serratia marcescens* | TRS-2 |
| NT164 | *Bacillus megaterium* | TRS-3 |
| HSoil1 | *Bacillus pumilus* | TRS-4 |
| KT262 | *Paenibacillus lentimorbus* | TRS-5 |

Among these antagonists, $HSoil_1$ and $KT\ 26_2$ i.e. *Bacillus pumilus* and *Paenibacillus lentimorbus* were selected for further investigations. (Plate-V)

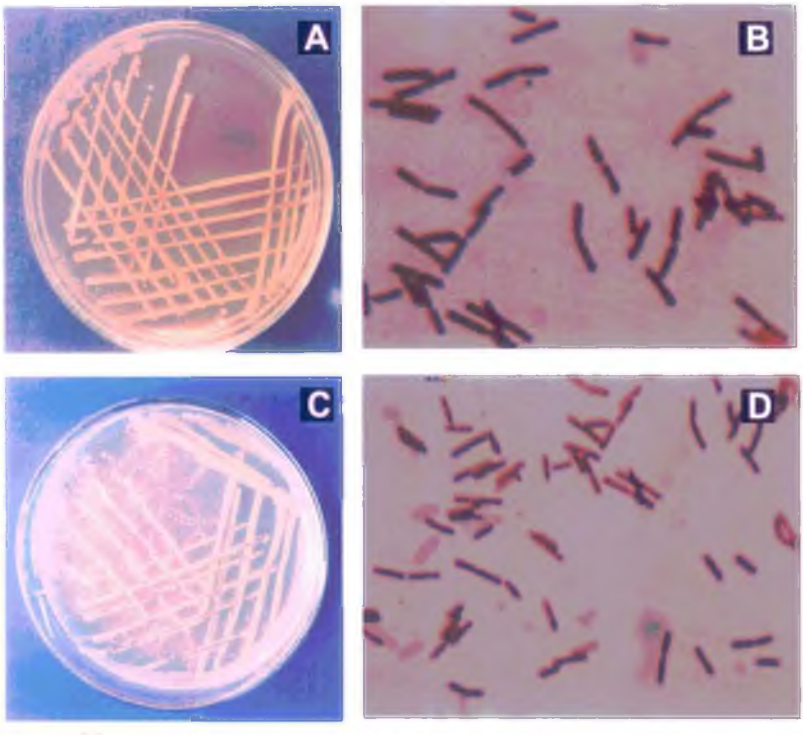


**Plate V:**Streaks of *B. pumilus* (A) and *P.lentimorbus* (C); *B. pumilus* (B) and *P. lentimorbus* (D) observed under microscope (x 100)

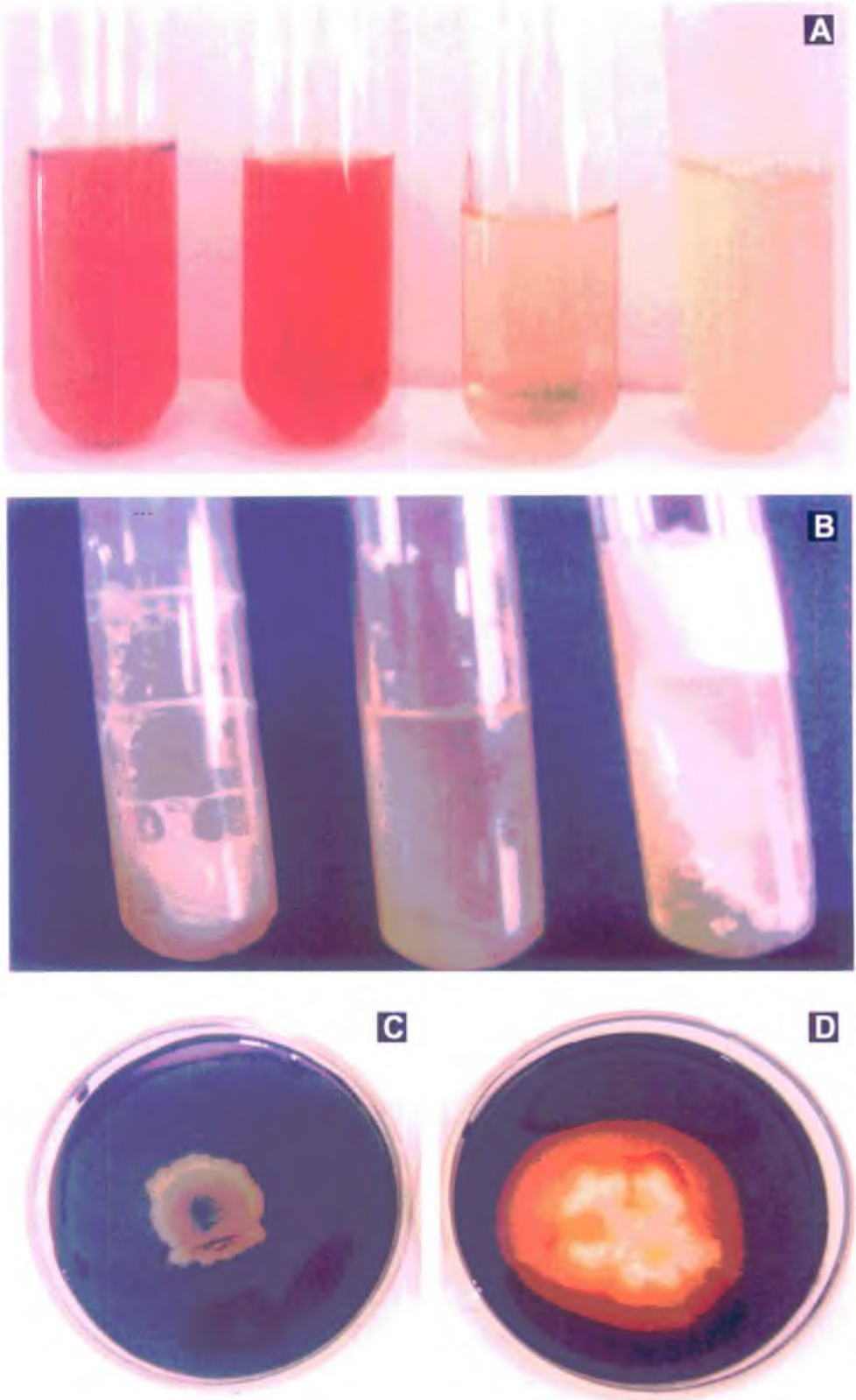


**Plate VI (A-D):** Biochemical tests of bacterial isolates (A) Carbohydrate digestion; (B) Catalase test; (C & D) Starch hydrolysis

**Table 6**: Morphological and biochemical characteristics of the selected rhizobacterial isolates.

| Characteristics | *B.pumilus* | *P.lentimorbus* |
|---|---|---|
| **Morphological** | | |
| Colony shape | circular | circular |
| Cell shape | Rod | Rod |
| Size (μm) | 2-3 x 0.7-0.8 | 2-3 x 0.5-0.6 |
| Capsule/Slime layer | - | - |
| Motility | + | + |
| Gram reaction | + | + |
| Endospore | + | + |
| Margin | + | + |
| Surface | wavy | wavy |
| Pigmentation | white | white |
| Fluorescein | – | – |
| Pyrocyanin | – | – |
| Density | opaque | opaque |
| **Biochemical** | | |
| Citrate utilization | + | + |
| Urea digestion | - | - |
| V.P.reaction | - | - |
| Catalase production | + | + |
| Oxidase production | + | + |
| Casein hydrolysis | - | - |
| Starch hydrolysis | - | + |
| Phenol Red Tartarate | - | - |
| Nitrate reduction | - | + |
| Esculine hydrolysis | + | + |
| Indole production | - | - |
| $H_2S$ production | + | + |
| $NH_3$ production | + | + |
| Gelatin liquefaction | + | + |
| Sugar utilization- | | |
| Dextose | +/- | +/- |
| Lactose | +/ - | +/- |
| Sucrose | +/ - | +/ - |
| Glycerol | +/ - | +/ - |

+ Positive reaction, - Negative reaction, +/- Weak reaction

### 4.3. Characterization and identification of fungal isolates

The isolated fungi were allowed to grow in petridishes containing sterile PDA medium for 7 days. Nature of mycelial growth, rate of growth and time of sporulation were observed. For identification, spore suspensions were prepared from individual culture. Drops of spore suspensions were placed on clean, grease free glass-slides, mounted with lacto phenol cotton blue, covered with cover slip and sealed. The slides were then observed under the microscope following which spore characteristics were determined and size of spore measured. On the basis of their morphological characters it was found that most of the fungal isolates belonged to the genera *Fusarium, Aspergillus, Curvularia, Penicillium, Alternaria* and *Colletotrichum* (Table 7).

Isolated fungi were also tested for antagonism against the test pathogens, but none of the tested fungal isolates showed antagonistic effect.

### 4.4. Cultural conditions affecting the growth of *Poria hypobrunnea*

Since further *in vivo* studies on biocontrol was undertaken against *P.hypobrunnea* causing root and stem rot of tea, initially growth conditions of this fungus was optimized. *In vitro* studies showed that variation in growth occurred depending on different factors like medium, pH, temperature and seasonal changes. The young mycelia were white or hyaline which turned gradually yellowish brown. The mycelial growth was generally submerged but sometimes superficial loose hyphal mat or rare fluffy growth was found depending on the medium. In liquid media, young white submerged mycelia grew at first slowly and then compact mycelia growth formed a plate like structure with horn-beak like edges and white hyphal growth extending a few centimeter on the wall of container. As the days passed, the white mycelial color changed to yellow brown.

#### 4.4.1. Media

*P.hypobrunnea* was grown in seven different media i.e. Potato dextrose agar (PDA), Potato sucrose agar (PSA), Richard's agar (RA),Yeast extract dextrose agar (YDA) Czapek-Dox agar (CDA), Elliot's agar (EA),Carrot juice agar (CJA).Results revealed that the fungus grew in all media. Maximum growth was recorded in PSA and minimum in EA. (Table 8; Plate VII)

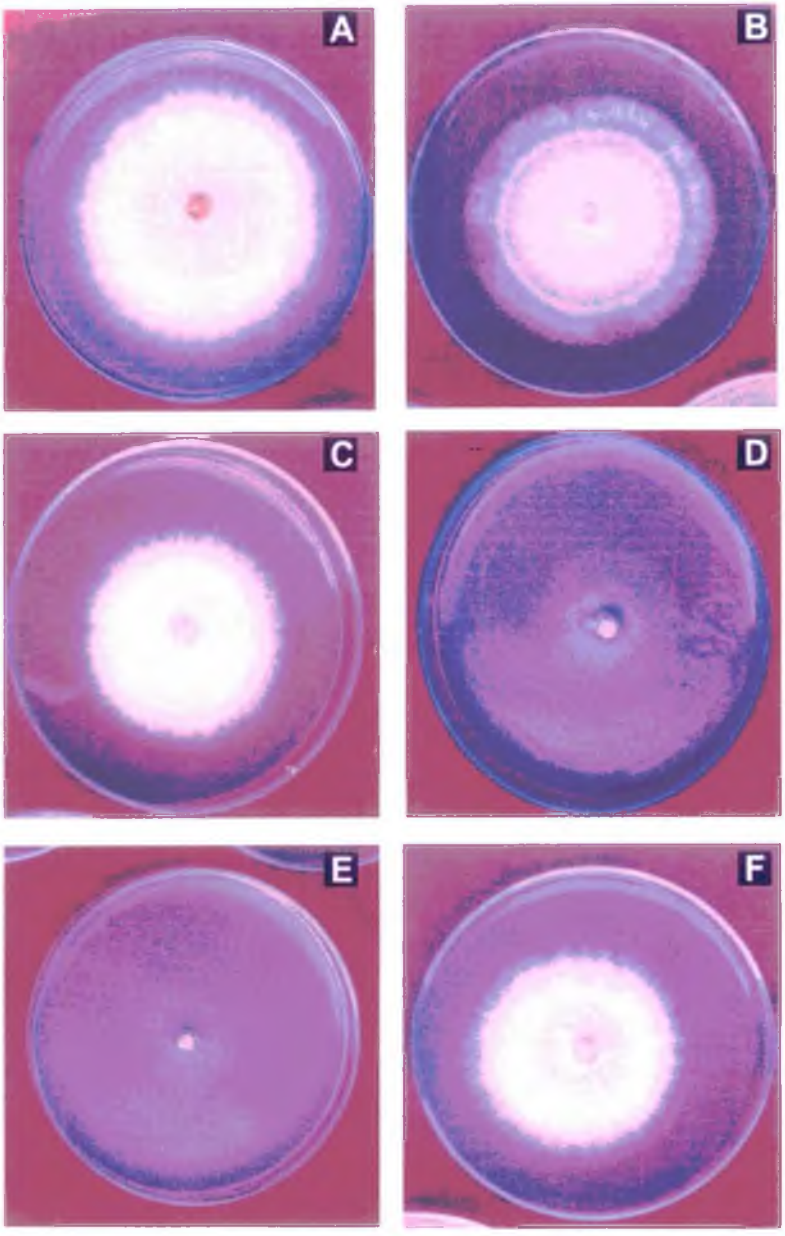


**Plate VII (A-F):** Growth of *P. hypobrunnea* in different media: (A) Potato sucrose agar; (B) Carrot agar; (C) Yeast dextrose agar; (D) Elliot's agar;(E) Richard's agar and (F) Potato dextrose agar

**Table 7**: Characterization of fungal isolates collected from tea rhizosphere

| Code name | Morphology | Name of organisms |
|---|---|---|
| NT9F-1 | Conidiophore simple, straight or curved, 1-3 septate, 5µm long, 3-6µm wide, golden brown. obclavate, pigmented conidia formed in long branching chains, ovoid, obpyriform with a short apical beak, smooth walled with 3-8 septa, base broadly round, each portion of lower part with 1-2 longitudinal septa, 18-63 x 7-18 µm. | *Alternaria alternata* |
| HRWF-1 | Conidiophores simple, become geniculated by sympodial elongation, Conidia have borne singly, muriform, beaked, dark, 5-10 transverse septa, and 120-296 x 12-20 µm. | *Alternaria solani* |
| NT16F-1 | Conidiophores hyaline, 0.4-1.0µm long and rough walled, roughened to echinucleate, on large conidiophores a layer of melulae supports the phialides. Conidial head radiating, globose to subglobose, finely roughened to echinulate, 1-3 nucleate 3.5-4.5µm. | *Aspergillus flavus* |
| NT9F-2 | Pigmented conidiophores with clavate vesicles arise from clearly differentiated thick walled foot cells. Strictly columnar conidial heads, conidia globose to subglobose, echinulate, 2.5-3.0µm, | *Aspergillus fumigatus* |
| HRWF-3 | Conidiophores arise from long broad thick walled, mostly brownish; sometimes branched foot cells about 1.5-3.0 mm. Large radiating heads, mostly globose, irregularly roughened 4.0-5.5µm. | *Aspergillus niger* |
| NT9F-3 | Conidiophores Erect, pigmented, geniculated from sympodial elongations, 3-10 septate, / Conidia olive brown, curved, ellipsoidal, 3- septate, rounded at the apex, slightly acuminate at the base, the middle septum below the centre and the third cell strongly curved, 20-30 x 9-15 µm. | *Curvularia lunata* |
| NT16F-3 | Pigmented, erect conidiophores/ conidia predominantly 4-septate, the central cell distinctly geniculated, tapering gradually towards each end, 18-37 x 8-14 µm | *Curvularia geniculatus* |
| NT9F-4 | Simple, lateral phialides short, sparsely branched/ microconidia never in chain, mostly non-septate, ellipsoidal to cylindrical, 5-12 x 2.3-3.5 µm. Macro conidia fusiform, hyaline, smooth walled, moderately curved, and pointed at both ends basal cells pedicellate, 27-46 x 3.0-4.5 µm. | *Fusarium oxysporum*. |

| Code name | Morphology | Name of organisms |
|---|---|---|
| HRWF-6 | Short, branched conidiophores 8-16 x 2-4 µm / microconidia abundant, chlamydospores borne singly, sometimes in pairs, in terminal, lateral, hyaline, smooth walled 6-10 µm. | *Fusarium solani* |
| TR/F1-1 | Conidiophores 100-200 µm long in compact columns /globose to subglobose, smooth walled, sometimes finely roughened 3.0-3.5 µm. | *Penicillium frequentans.* |
| TR/F1-2 | Conidiophores typically two-stage branched, phialides cylindrical, tip distinctly tapering. / Conidia form deep crusts which appear silky, strongly ellipsoidal, smooth-walled, 4.5-6.5 µm. | *Penicillium oxalicum* |
| TR/F1-3 | Very dense ramification of the conidiophores, conspicuously roughened stripe / Subglobose, finely echinate, long 2.5-3.0 µm. | *Penicillium simplicissimum* |
| TR/F1-4 | Long conidiophores. Grayish green, conidia globose to subglobose, slightly roughened, mostly 3.5-4.0 µm. | *Penicillium sp.* |
| NT16F-4 | Conidiophores are hyaline, short obpyriform to cylindrical producing aseptate, hyaline, oval, enteroblastic conidia. | *Macrophomina phaseolina* |
| TR/F1-4 | Sporangiophores pale to dark brown, straight, mostly 1.5 mm tall, 20-25 µm wide, each sporangiophores bear globose to subglobose multispored columellate sporangium, biconical to oval, ridged, mostly 4 nucleate 7-12 x 6-8.5 µm. | *Rhizopus stolonifer* |
| TR/F2-1 | Conidia appearing scantily on solitary phialides but normally in orange sporodochia, basal stromatic cushion covered with dense layer of cylindrical, slightly tapering phialides upto 20µm long.<br>Cylindrical with a roughed apex, slightly truncated base, hyaline, filled with granular cytoplasm forming orange-red shiny masses, 9-24 x 3-4.5µm. | *Colletotrichum gloeosporoides* |
| TR/F2-2 | Conidiophores are hyaline to brown, septate, branched at the base, smooth, formed from the upper cells of the fructification. Conidia hyaline, unicellular, falcate or lunate or cylindrical, more or less guttulate, muticate or with the apex prolonged into a simple cellular appendage, produced from phialides. | *Colletotrichum* sp |
| NT16F-5 | Oblong, sometimes two-celled, 4.0-8.5 x 2.0-3.0 µm. | *Phoma exigua* |

**Table 8**: Effect of different media on mycelia growth of *P.hypobrunnea*

| Media | Diameter of growth (cm) | | | |
|---|---|---|---|---|
| | Incubation period (days) | | | |
| | 3 | 6 | 9 | 12 |
| PDA | 1.2±0.05 | 3.5±0.11 | 6.3±0.14 | 8.2±0.08 |
| PSA | 1.3±0.11 | 4.2±0.26 | 7.1±0.18 | 9.0±0.00 |
| RA | 0.7±0.05 | 2.4±0.05 | 3.8±0.11 | 5.4±0.05 |
| YDA | 1.1±0.08 | 3.3±0.09 | 5.5±0.26 | 7.9±0.05 |
| CDA | 0.9±0.26 | 2.6±0.11 | 4.8±0.29 | 6.0±0.13 |
| EA | 0.8±0.05 | 2.3±0.11 | 4.5±0.09 | 5.8±0.14 |
| CJA | 1.1±0.11 | 3.0±0.17 | 5.9±0.05 | 7.8±0.08 |

± Standard error; Temperature 30±1$^0$C;

### 4.4.2. Incubation period

*P. hypobrunnea* was grown in Richard's medium (RM) for a period of 25 days, mycelial growth was recorded after 3, 6, 9, 12, 15, 18, 21, 24 and 25 days of growth and incubated at 30±2$^0$C.Maximum growth was recorded after 15 days of incubation after which it declined. (Fig. 1)

### 4.4.3. pH

pH of the medium plays an important role in the growth of all microorganisms. To determine the effect of pH, buffer systems have to be used to stabilize the pH. Initially buffer solution with pH values ranging from 4.0 to 8.0 were prepared by mixing $KH_2PO_4$ and $K_2HPO_4$ each at a concentration of 0.03M. The pH was finally adjusted usingN/10 HCL or N/10 NaOH in each case. Richard's medium and phosphate buffer were sterilized separately by autoclaving for 15 min. at 15 lbs. pressure. Equal parts of the buffer and medium were mixed before use in Laminar Flow Bench. After mixing flasks were inoculated and incubated for 15 days after which dry wt. was taken .Results revealed that growth of *P. hypobrunnea* was optimum in the range pH 4-7. Maximum growth was observed at pH 5.0-5.5 (Fig. 1)

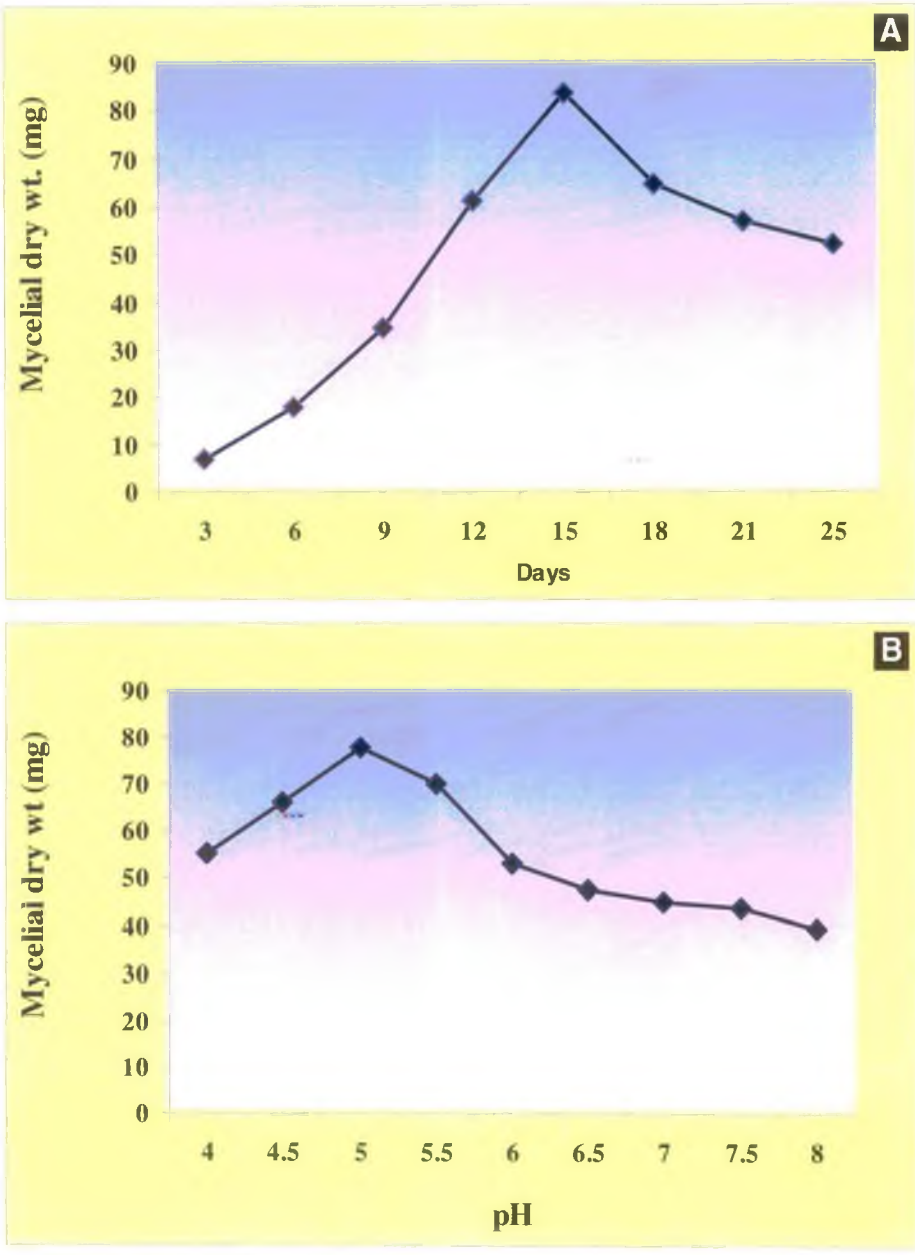


**Fig. 1:** Effect of incubation period (A) and pH (B) on mycelial growth of *P. hypobrunnea.*

### 4.4.4. Carbon source

Like the pH of the medium the growth of the fungus is greatly influenced by available nutrients. The availability of the fungi to grow in different media depends on the their capability to utilize the available nutrients, of which carbohydrates are the major ones .All carbohydrates are not utilized by the fungus in the same rate and so the growth rate varies with different carbon sources. In the present investigation, seven different carbon sources (fructose, dextrose, mannitol, maltose, sucrose, starch and lactose) were tested for their effect on the growth of *P.hypobrunnea*. These were added separately to the basal medium. Richard's medium without sugar was used as the basal medium which served as control set. Data was recorded after 15 days of incubation. Results given in table revealed maximum growth using fructose as the carbon source while minimum growth was observed in mannitol. (Table 9; Fig. 2)

**Table 9**: Effect of different carbon sources on mycelial growth of *P.hypobruunea*

| **Carbon sources** | **Dry mycelial wt. (mg)** | | | |
|---|---|---|---|---|
| | **Expt.1** | **Expt.2** | **Expt.3** | **Mean** |
| Fructose | 54.3 | 55.4 | 56.2 | 53.30±0.55 |
| Dextrose | 42.7 | 41.3 | 45.1 | 43.03±1.08 |
| Mannitol | 20.1 | 21.3 | 20.6 | 20.66±0.35 |
| Sucrose | 48.6 | 47.3 | 47.1 | 47.66±0.47 |
| Starch | 32.3 | 33.1 | 30.6 | 32.00±0.74 |
| Maltose | 30.1 | 27.5 | 28.3 | 28.63±0.77 |
| Lactose | 38.2 | 39.5 | 40.0 | 39.23±0.54 |
| Control | 5.0 | 7.0 | 5.0 | 5.66±0.66 |

± Standard error; Temperature 30±2$^0$C

### 4.4.5. Nitrogen source

The availability of nitrogen for growth of the organism depends to a great degree on the form in which it is supplied. Hence the most suitable nitrogen source for any particular microorganism can only be determined by testing a number of sources including both inorganic and organic. The effect of inorganic nitrogen sources (potassium nitrate, sodium nitrate, ammonium sulphate, calcium nitrate) as well as

complex organic sources (urea, peptone, casein, yeast extract and beef extract) on the mycelial growth of *P. hypobrunnea* was tested. A basal medium without any nitrogen source was considered as control.

After 15 days of incubation data was recorded and result revealed maximum growth in ammonium sulphate followed by potassium nitrate in inorganic nitrogen source while maximum growth in peptone and minimum in casein among organic nitrogen sources. (Table 10; Fig. 2)

**Table 10**: Effect of different nitrogen sources on mycelial growth of *P. hypobrunnea*

| Nitrogen source | Dry weight of fungal mycelium (mg) | | | |
|---|---|---|---|---|
| | Expt.1 | Expt.2 | Expt.3 | Mean |
| **Inorganic** | | | | |
| Potassium nitrate | 20.4 | 25.7 | 24.3 | 23.46±1.59 |
| Sodium nitrate | 15.3 | 16.2 | 16.0 | 15.83±0.27 |
| Ammonium sulphate | 28.9 | 27.3 | 27.5 | 27.90±0.50 |
| Calcium nitrate | 12.9 | 15.6 | 14.5 | 14.33± 0.79 |
| **Organic** | | | | |
| Urea | - | - | - | - |
| Peptone | 520.2 | 531.3 | 528.3 | 526.60±3.22 |
| Casein | 100.2 | 117.3 | 115.8 | 111.11±5.47 |
| Yeast extract | 420.3 | 426.4 | 427.3 | 424.67±2.20 |
| Beef extract | 342.6 | 355.6 | 348.3 | 348.83±3.77 |
| Control | 8.0 | 6.0 | 5.0 | 6.33±0.88 |

± Standard error; Temperature 30±2$^0$C; Incubation period 15 days

### 4.5. Cultural characteristic of bacterial antagonists

To determine the effect of different factors on growth of *B. pumilus* and *P. lentimorbus* both bacteria were grown in different pH, temperature and medium

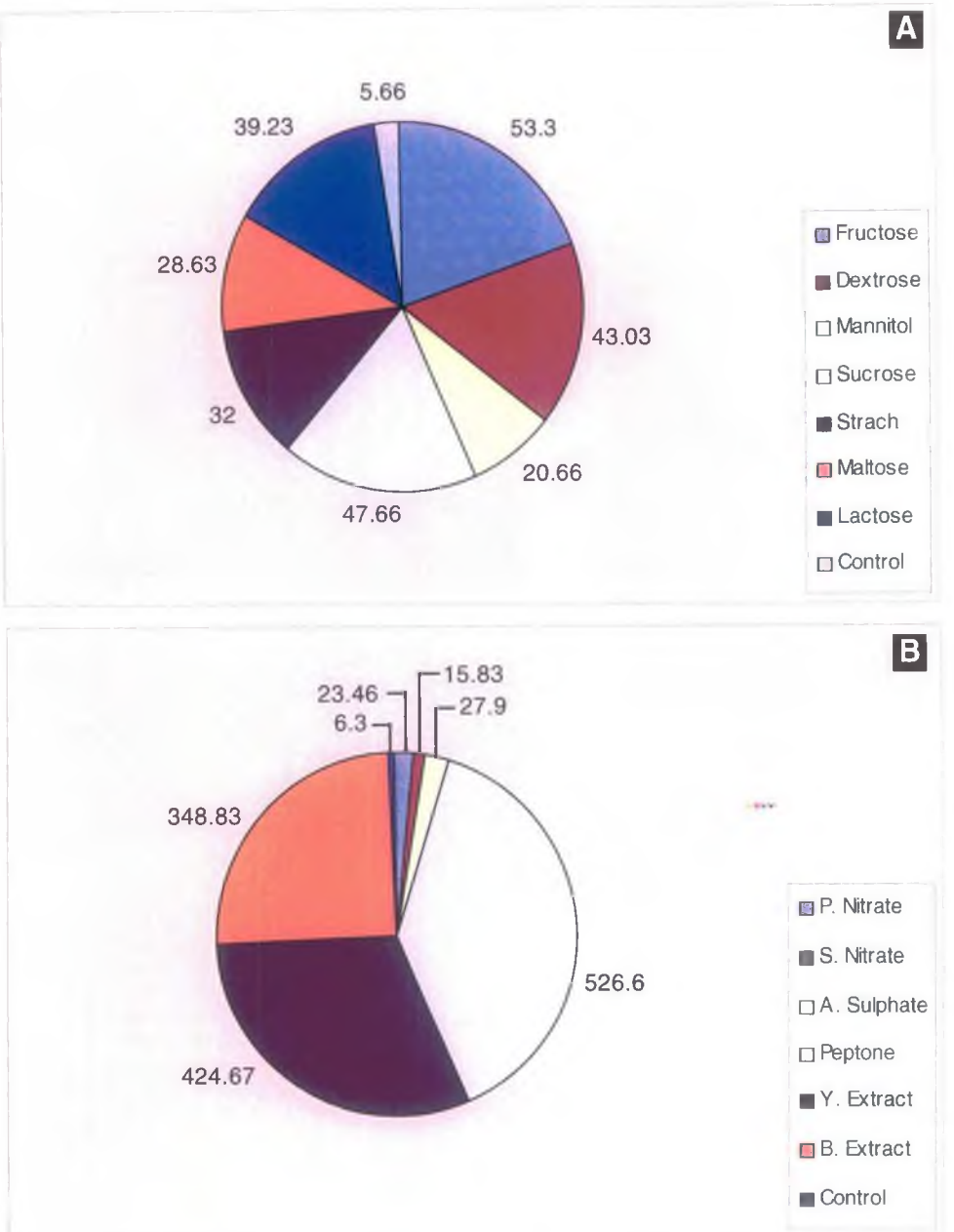


**Fig. 2:** Effect of Carbon sources (A) and Nitrogen sources (B) on mycelial growth of *P. hypobrunnea.*

**Table11**: Effect of antagonistic rhizobacterial isolates on mycelial growth of test pathogens.

| Fungal Pathogen | *B. pumilus* | *P.lentimorbus* |
|---|---|---|
| | MDW* (g) | MDW*(g) |
| *P.hypobrunnea* | 0.101 | 0.120 |
| *F.lamaoensis* | 0.125 | 0.140 |
| *S.repens* | 0.387 | 0.400 |
| *S.rolfsii* | 0.105 | 0.130 |
| *S.sclerotirum* | 0.130 | 0.150 |

*Average mycelial dry weight of three replicates; after 7 days of growth in PDB.

**4.5.1. pH**

*B.pumilus* and *P.lentimorbus* grew best at pH 6.0 and did not grow well below pH 4.0 and above pH 8.0. Results are given in Fig. 3

**4.5.2. Temperature**

The growth of *B.pumilus* and *P.lentimorbus* were observed at different temperatures ranging from 20 to $50^0$C.NB medium was inoculated with the bacteria and the flasks were incubated at 20, 25, 30, 35, 40, 45 and $50^0$C.Both the bacteria grew well within these ranges of temperature but grew best at $35^0$C. (Fig. 3)

**4.5.3. Media**

Six different media (PDB, NB, KB, LB, NSB and GYP) were selected to assess the growth of the rhizobacteria and it was recorded that Nutrient Broth is the best medium for growth of *B.pumilus* and *P. lentimorbus*. (Table 12)

**Table 12**: Effect of media on the growth of *B.pumilus* and *P. lentimorbus*

| Name of medium | Cfu/ml | |
|---|---|---|
| | *B.pumilus** | *P.lentimorbus** |
| PDA | $3.31 \times 10^7$ | $1.81X10^6$ |
| NB | $6.02X10^{13}$ | $3.38X10^{12}$ |
| KB | $1.90X10^{11}$ | $2.60X10^9$ |
| LB | $5.88X10^8$ | $1.38X10^8$ |
| NSA | $1.81X10^6$ | $1.81X10^6$ |
| GYP | $2.60X10^9$ | $3.31X10^7$ |

Average of three replicates; Incubation period-4 days

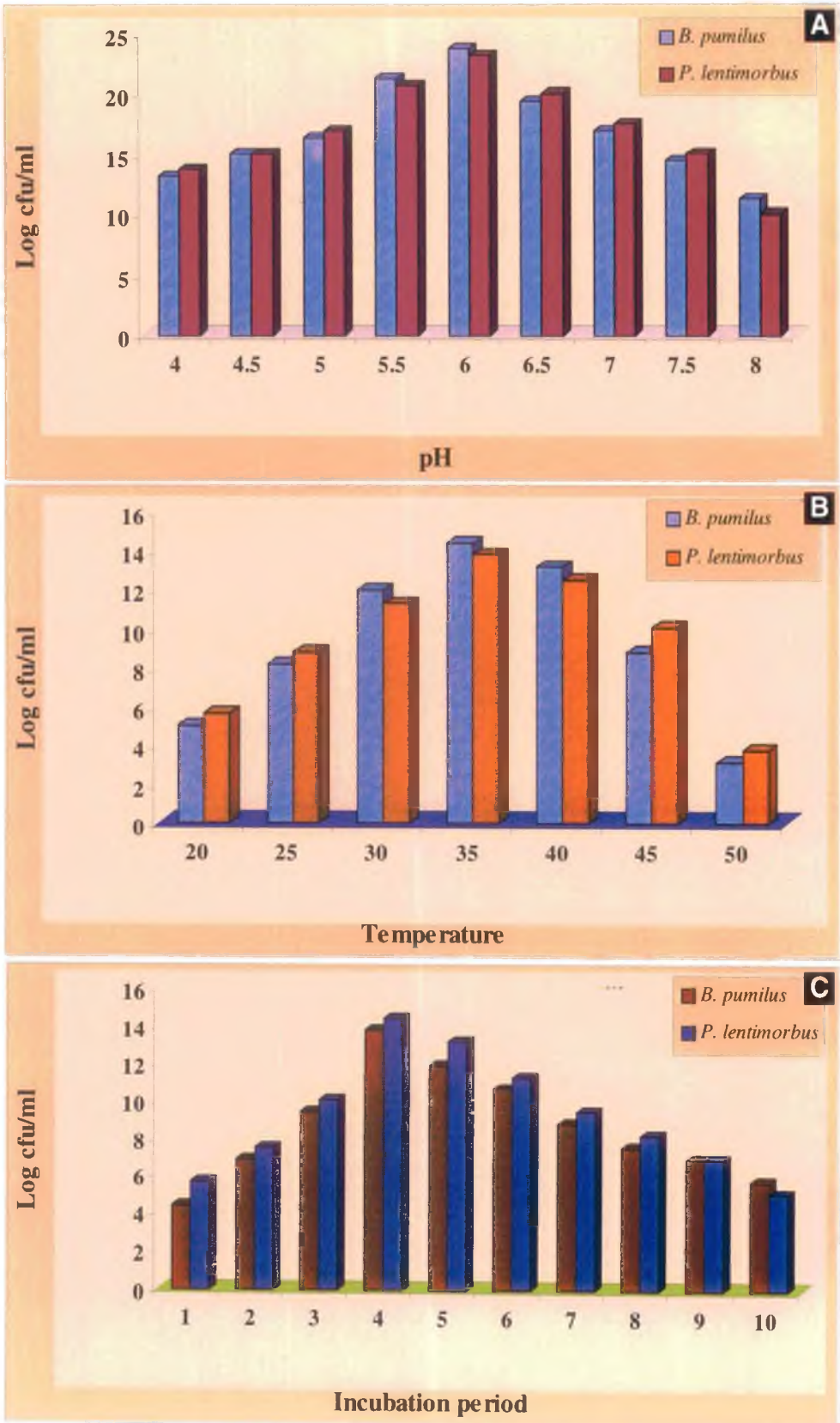


**Fig. 3:** Effect of pH (A), temperature (B) and incubation period (C) on growth of *B. pumilus* and *P. lentimorbus*.

### 4.5.4. Incubation period

*B.pumilus* and *P.lentimorbus* were grown in Nutrient Broth (NB) for a period of 10 days with growth being recorded after 1, 2, 3, 4, 5, 6, 7, 8, 9 and10 days of growth at temperature of 35±2$^0$C. Maximum growth was recorded after 4 days and then growth gradually decreased. (Fig. 3)

### 4.5.5. Antibiotic sensitivity

Antibiotic sensitivity test were performed to know the strength of rhizobacteria against particular antibiotic. Antibiotics were mixed at rate of 15µg/ml in Nutrient Agar medium; Results showed that *B.pumilus* and *P.lentimorbus* were highly sensitive to amoxicillin, streptomycin, gentamycin and norfloxacin. (Table 13)

**Table13**: Antibiotic sensitivity of the rhizobacterial isolates

| Antibiotics | *B.pumilus* | *P.lentimorbus* |
|---|---|---|
| Amoxicillin | HS | HS |
| Penicillin | PS | PS |
| Chloramphenical | HS | HS |
| Streptomycin | HS | HS |
| Gentamycin | HS | HS |
| Norfloxacin | HS | HS |
| Tetracycline | HS | MS |
| Kanamycin | MS | MS |

PS-Partially sensitive; HS-Highly sensitive; MS-Moderately sensitive

### 4.6. *In vitro* tests of antagonists against test fungi

Antagonistic activity of *B.pumilus* and *P.lentimorbus* were confirmed by dual culture method both in solid and liquid media.

### 4.6.1. Solid media

In case of solid medium tests, diameter of fungal growth alone or with antagonistic bacteria and zone of inhibition were noted. The results revealed that both the bacteria inhibited *F.lamaoensis* most, followed by *P.hypobrunnea*, *S.repens*, *S.sclerotiorum*, and *S. rolfsii* and percentage of inhibition was maximum in *P.hypobrunnea*. (Table 14, Plates VIII and IX)

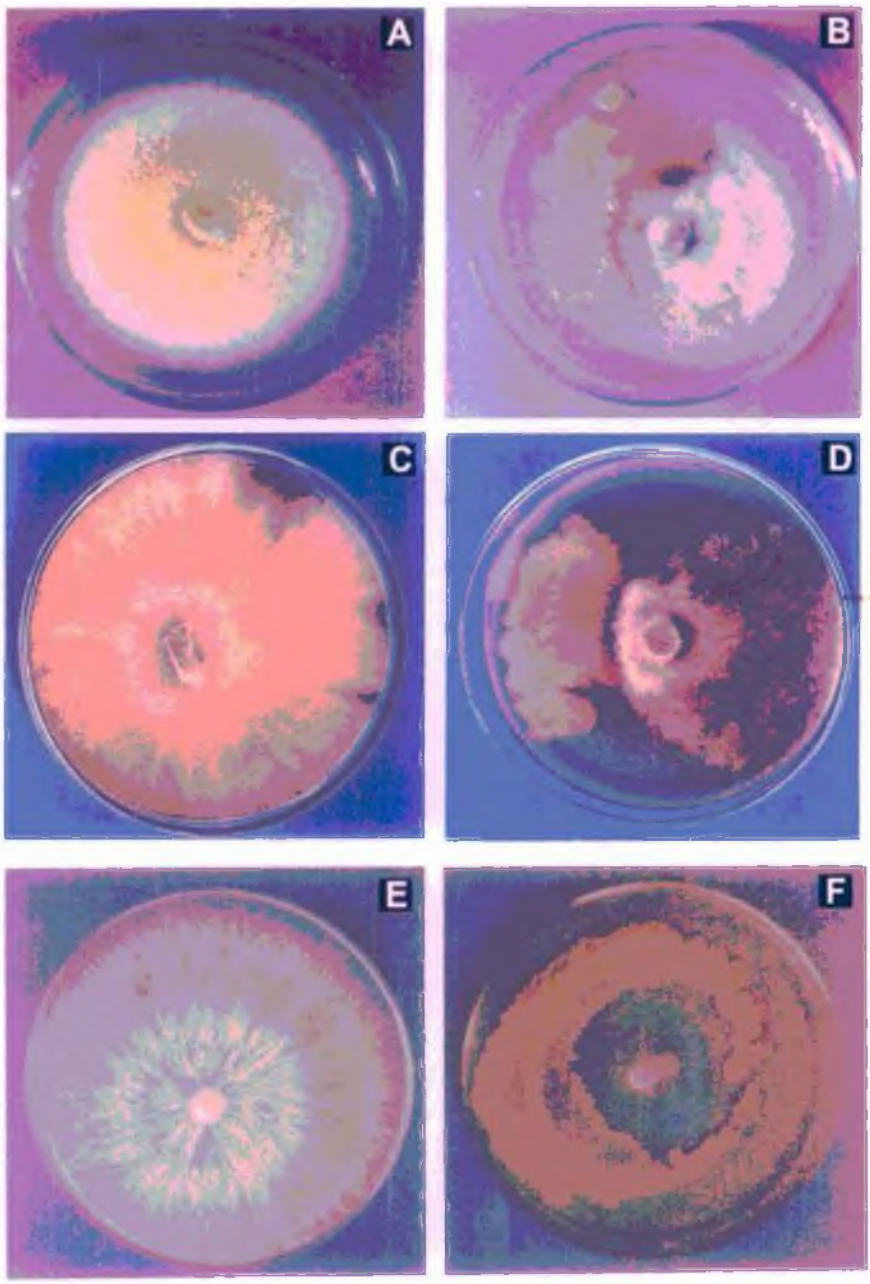


**Plate VIII (A-F):** Antagonistic activity of *B. pumilus* against *P. hypobrunnea* (B); *F. lamaoensis* (D); *S. rolfsii* (F); A,C and E are respective controls

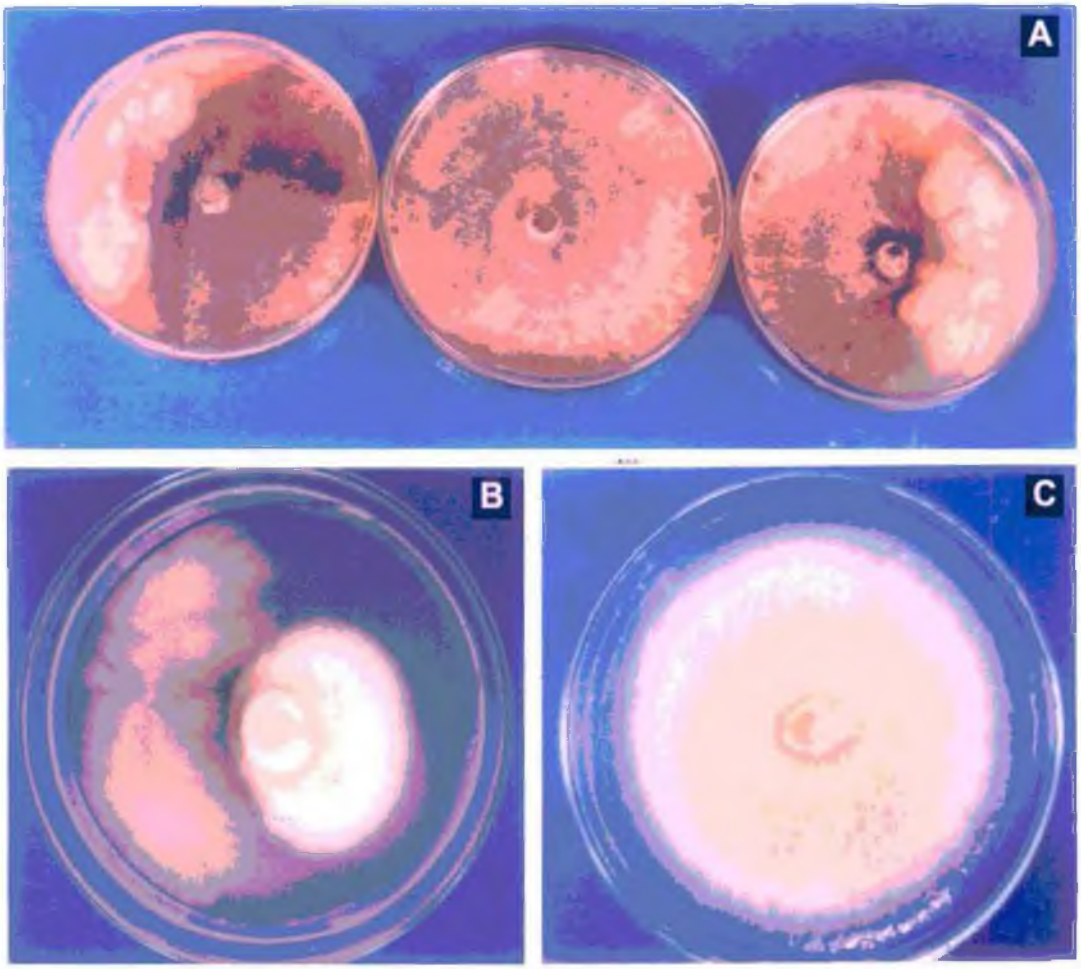


**Plate IX:** Pairing test of *P. lentimorbus* against (A) *F. lamaoensis* and (B) *P. hypobrunnea* (C) Control *P. hypobrunnea*.

**Table 14**: Effect of rhizobacterial isolates against the test pathogen in solid medium

| Test pathogen | Dia. of inhibition Zone (cm) | | % of inhibition | |
|---|---|---|---|---|
| | *B.pumilus* | *P.lentimorbus* | *B.pumilus* | *P.lentimorbus* |
| *P.hypobrunnea* | 1.4 ± 0.03 | 1.1 ± 0.04 | 50.33 | 48.11 |
| *F.lamaoensis* | 1.6 ± 0.08 | 1.3 ± 0.05 | 43.67 | 42.44 |
| *S.repens* | 1.0 ± 0.01 | 0.9 ± 0.02 | 33.22 | 28.89 |
| *S.rolfsii* | 0.6 ± 0.05 | 0.3 ± 0.04 | 22.71 | 21.33 |
| *S.sclerotiorum* | 0.9 ± 0.06 | 0.8 ± 0.07 | 27.78 | 24.71 |

± Standard error; Average of three replicates; Temperature 30±2$^0$C; after 7 days of incubation

### 4.6.2. Liquid media

The bacteria were also tested for their inhibitory activity against the test fungi in liquid medium. After 7 days of growth, mycelia were harvested and dried and mycelial dry weigh was taken. Lowest mycelial dry weight was recorded in *P. hypobrunnea* and percentage of reduction was less in *S. repens* as compared to others (Table 12; Fig. 4).

## 4.7. Effect of application of rhizobacteria on the growth of plants

### 4.7.1. Chickpea and Mungbean

Before applying to perennial plants experiments were conducted on some annual plants i.e. chickpea and mungbean. Seeds of chickpea and mungbean were bacterized and sown. After that, germination percentage and vigour index of the plants were calculated. Both the bacterial isolates enhanced the germination percentage and vigour index markedly as compared to non-inoculated control seeds. In both seeds, *B.pumilus* was more effective than *P.lentimorbus* (Table 15; Plate X).

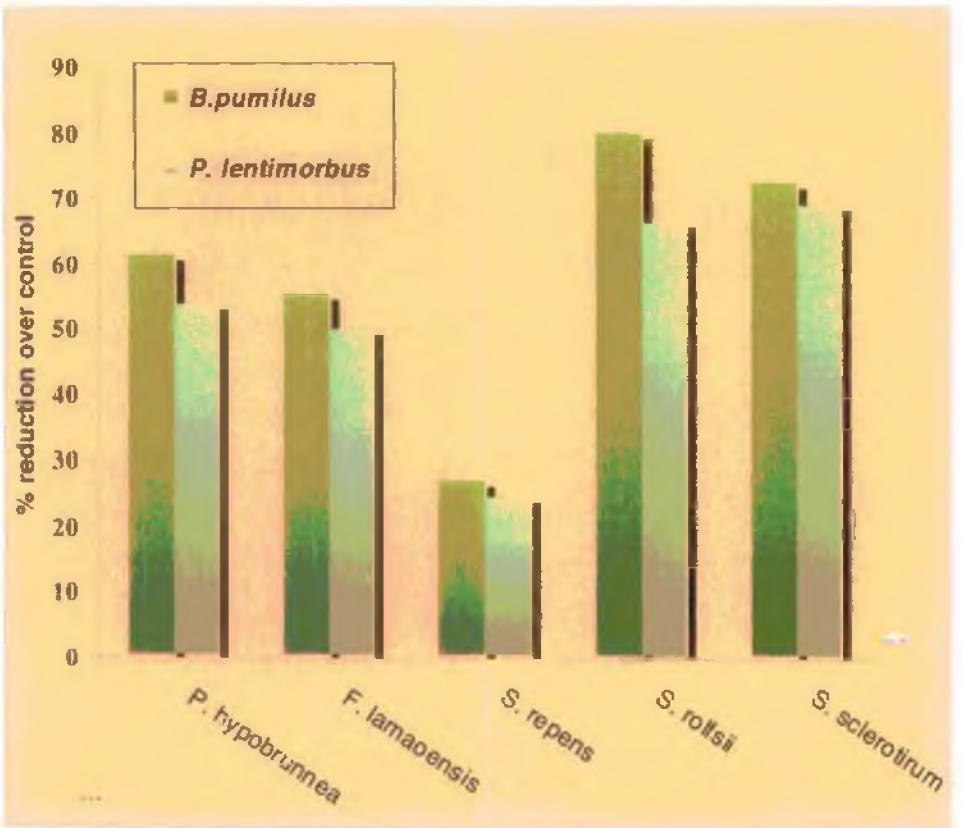


**Fig. 4:** Reduction of mycelial growth of *P. hypobrunnea* by antagonistic bacteria.

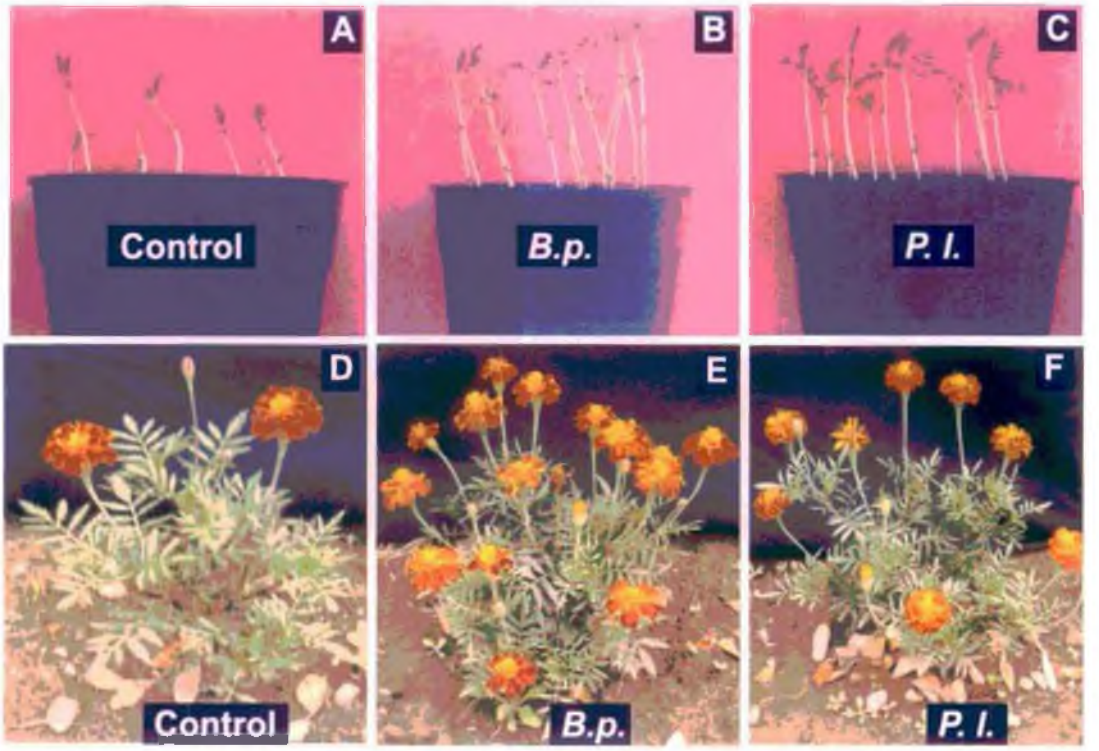


**Plate X :** Effect of *Bacillus pumilus* (B.p.) and *Paenibacillus lentimorbus* (P.l.) on chickpea (A-C) and marigold (D-F) plants.

**Table15**: Effect of seed bacterisation with rhizobacteria on vigour index of seedling.

| Treatment | % germination | Mean root length+ Mean shoot length (cm) | Vigour Index |
|---|---|---|---|
| *Cicer arientinum* | | | |
| Control | 80 | 4.2 | 409.2 |
| *B.pumilus* | 98 | 5.5 | 472.5 |
| *P.lentimorbus* | 93 | 5.2 | 433.3 |
| *Vigna radiata* | | | |
| Control | 84 | 6.3 | 629.9 |
| *B.pumilus* | 97 | 8.1 | 810.0 |
| *P.lentimorbus* | 96 | 7.9 | 750.2 |

Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants.

### 4.7.2. Marigold

Besides chickpea and mungbean, effect of the PGPR on growth and flowering of marigold, a garden plant, was also tested. The bacterial suspensions of both the bacteria enhanced height and number of flower in marigold plants compared to untreated control (Table 16; Plate X)

**Table 16:** Effect of bacterial soil drench on Marigold

| Treatment | After 30 days | |
|---|---|---|
| | % Increase in height (cm) | % Increase in flower no. |
| Control | 40.5 | 79.3 |
| *B.pumilus* | 492.4 | 692.4 |
| *P.lentimorbus* | 468.7 | 574.7 |

Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants.

### 4.7.3. Tea

#### 4.7.3.I. Seedling

As the selected bacteria enhanced the growth promotion in annual plants these rhizobacteria were applied to perennial plant i.e. in tea seedlings. The growth promotion of different varieties of tea seedling 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 seedling in relation to untreated control. Percentage increases in height of plants as well as number of leaves after two and four months of application of bacteria to the soil were calculated. The results showed that both *B.pumilus* and *P.lentimorbus* efficiently promoted growth in tea plants irrespective of their variety. (Tables 17 and 18; Plate XI; Fig. 5)

**Table17**: Effect of rhizobacteria on growth of tea seedlings

| Tea varieties | Treatment | 2 months after treatment | | | |
|---|---|---|---|---|---|
| | | Height of seedling (cm) | | No. of Leaves of seedling | |
| | | Initial | Final | Initial | Final |
| CP-1 | Control | 12.7±0.7 | 15.5±1.2 | 7±0.6 | 9±0.5 |
| | *B. pumilus* | 12.7±0.9 | 20.8±1.5 | 8±0.5 | 18±1.2 |
| | *P.lentimorbus* | 12.7±0.6 | 19.6±1.4 | 8±0.5 | 15±1.2 |
| TV-20 | Control | 14.0±1.2 | 15.2±1.3 | 8±0.4 | 12±0.9 |
| | *B. pumilus* | 14.0±1.1 | 23.1±2.1 | 8±0.4 | 14±1.1 |
| | *P.lentimorbus* | 14.0±1.4 | 22.4±2.0 | 9±0.6 | 15±1.3 |
| T-17 | Control | 12.7±1.0 | 14.0±0.9 | 7±0.5 | 8±0.6 |
| | *B.pumilus* | 12.7±0.9 | 19.1±1.1 | 7±0.3 | 12±0.8 |
| | *P.lentimorbus* | 12.7±1.0 | 19.8±1.0 | 7±0.7 | 13±1.2 |
| K-1/1 | Control | 15.2±1.7 | 17.0±1.4 | 8±0.6 | 10±0.8 |
| | *B. pumilus* | 15.2±1.6 | 19.8±1.6 | 8±0.4 | 13±1.1 |
| | *P.lentimorbus* | 15.2±1.1 | 19.3±1.9 | 9±0.6 | 13±1.0 |
| UP-3 | Control | 14.0±1.0 | 15.5±1.6 | 8±0.5 | 10±0.9 |
| | *B.pumilus* | 14.0±0.7 | 21.1±2.7 | 7±0.5 | 11±1.1 |
| | *P.lentimorbus* | 14.0±0.9 | 20.1±2.5 | 8±0.6 | 12±1.0 |

Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded 2 months following the bacterial inoculation; ± S.E.

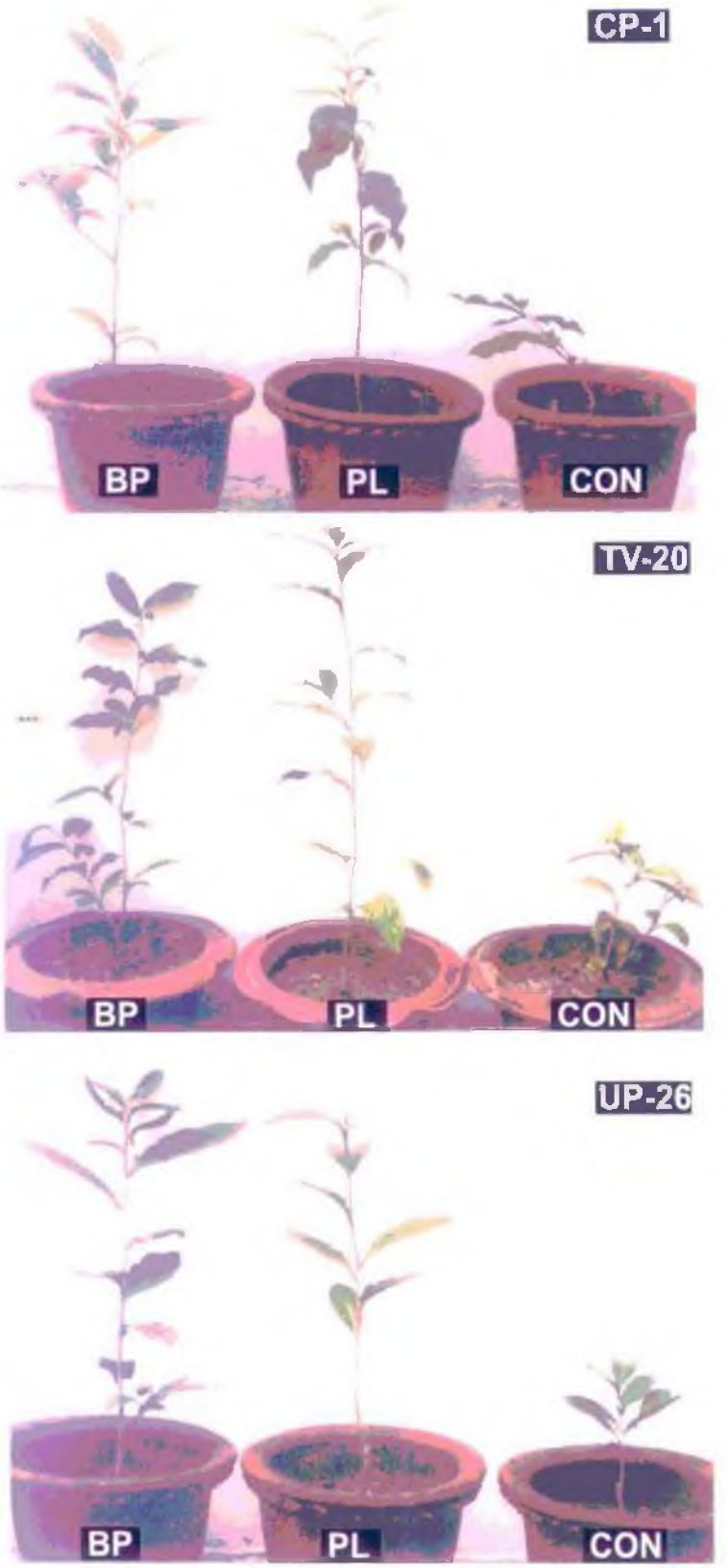


**Plate XI:** Growth promotion of different varieties of tea seedling following inoculation with *B. pumilus* (BP) and *P. hypobrunnea* (PL).

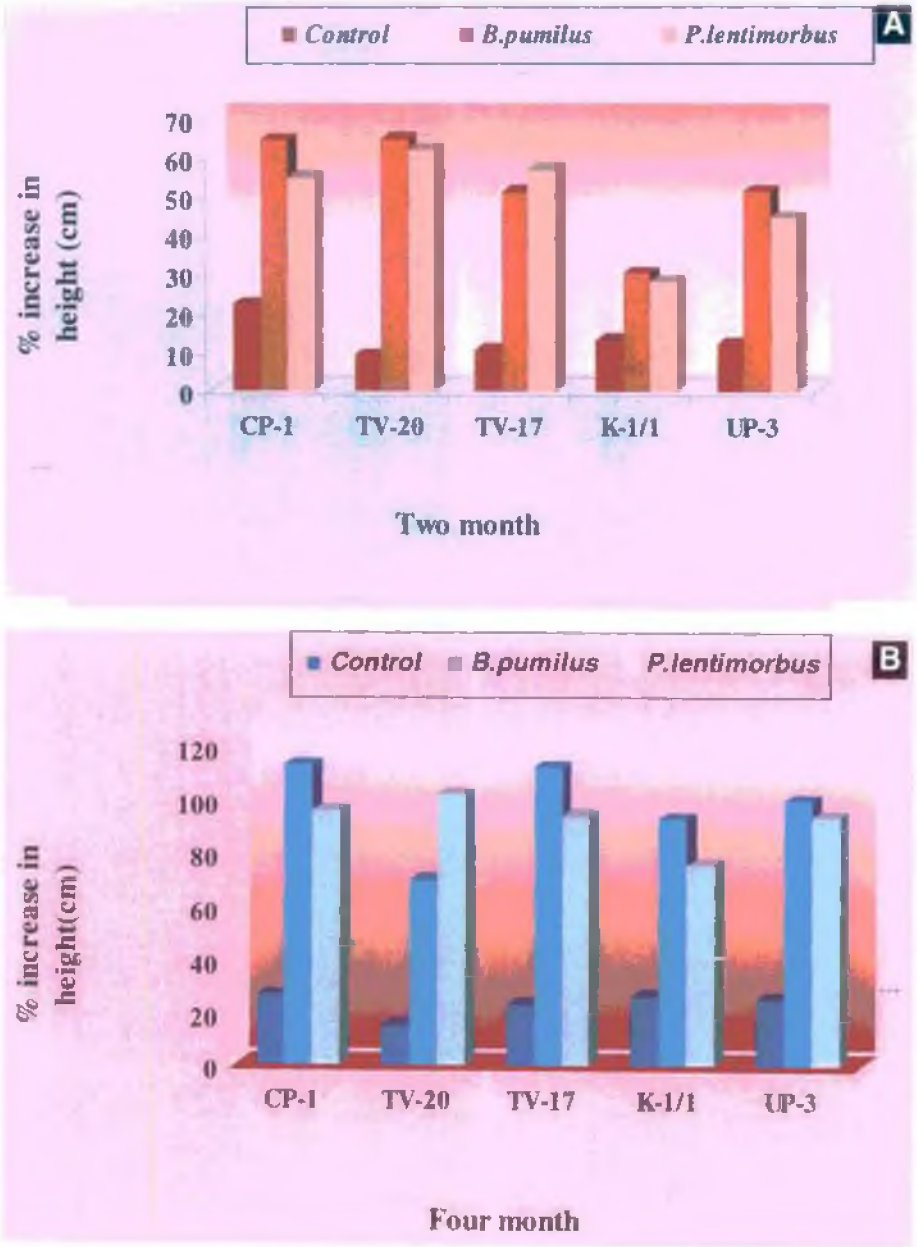


**Fig. 5:** Effect of PGPRs on growth of tea seedlings of different varieties.

**Table 18**: Effect of rhizobacteria on leaf development of tea seedlings

| Tea Variety | Treatment | % Increase in no. of leaves | |
|---|---|---|---|
| | | 2 months* | 4 months* |
| CP 1 | Control | 28.6± 1.71 | 75.0± 1.95 |
| | *B.pumilus* | 125.0± 1.75 | 175.0±1.20 |
| | *P.lentimorbus* | 87.5± 2.11 | 167.0±1.00 |
| TV-20 | Control | 50.0±1.92 | 51.0± 2.05 |
| | *B.pumilus* | 75.0±1.82 | 133.3±1.65 |
| | *P.lentimorbus* | 66.6± 2.11 | 123.0±1.65 |
| T-17 | Control | 14.3 ±1.46 | 52.0± 1.73 |
| | *B.pumilus* | 65.5±0.78 | 130.3 ± 1.43 |
| | *P.lentimorbus* | 84.6 ±3.22 | 142.0± 4.20 |
| K-1/1 | Control | 25.0± 1.73 | 60.5± 2.02 |
| | *B.pumilus* | 62.5 ± 2.31 | 168.4± 4.56 |
| | *P.lentimorbus* | 44.4 ± 6.01 | 178.5 ±1.00 |
| UP-26 | Control | 25.0± 2.31 | 60.0± 2.31 |
| | *B.pumilus* | 57.1± 2.31 | 165.7± 3.32 |
| | *P.lentimorbus* | 50.0 ±1.64 | 128.4±1.20 |

Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants; ± S.E; Results were recorded 2 and 4 months following the bacterial inoculation.

#### 4.7.3.2. Two years old plants

Bacterial suspensions of *B.pumilus* and *P.lentimorbus* were also applied to the rhizosphere of two years old potted plants at a regular interval of 20-25 days under same environment and physical conditions. Growth promotion of different varieties by individual bacteria was noted as compared to untreated control. The results showed that both *B. pumilus* and *P. lentimorbus* significantly increased height, lateral branches and leaves in all tested varieties. (Table 19; Plate XII; Fig. 6)

#### 4.7.3.3. Tea bushes

In order to further confirm the ability of the bacteria to promote growth, aqueous suspensions of these bacteria were made and sprayed on the tea bushes after pruning. It was observed that spraying with the suspension led to significantly better growth of the shoot. More luxuriant growth was obtained. It was also observed that certain infections which normally occur in natural conditions were also not present following spraying with the bacterial suspension (Plate XIII).

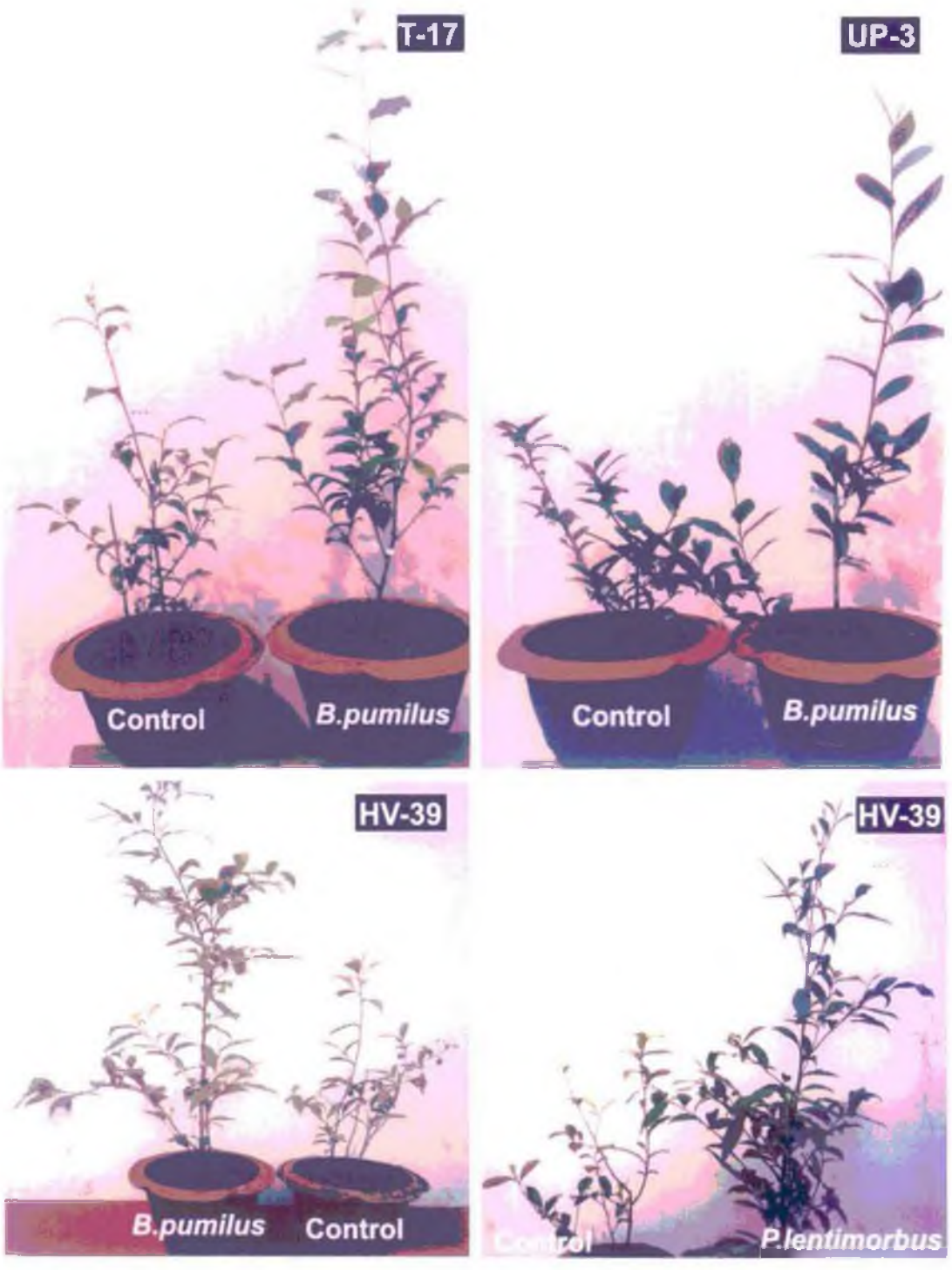


**Plate XII:** Effect PGPRs on growth of two year old potted tea plants of different varieties.

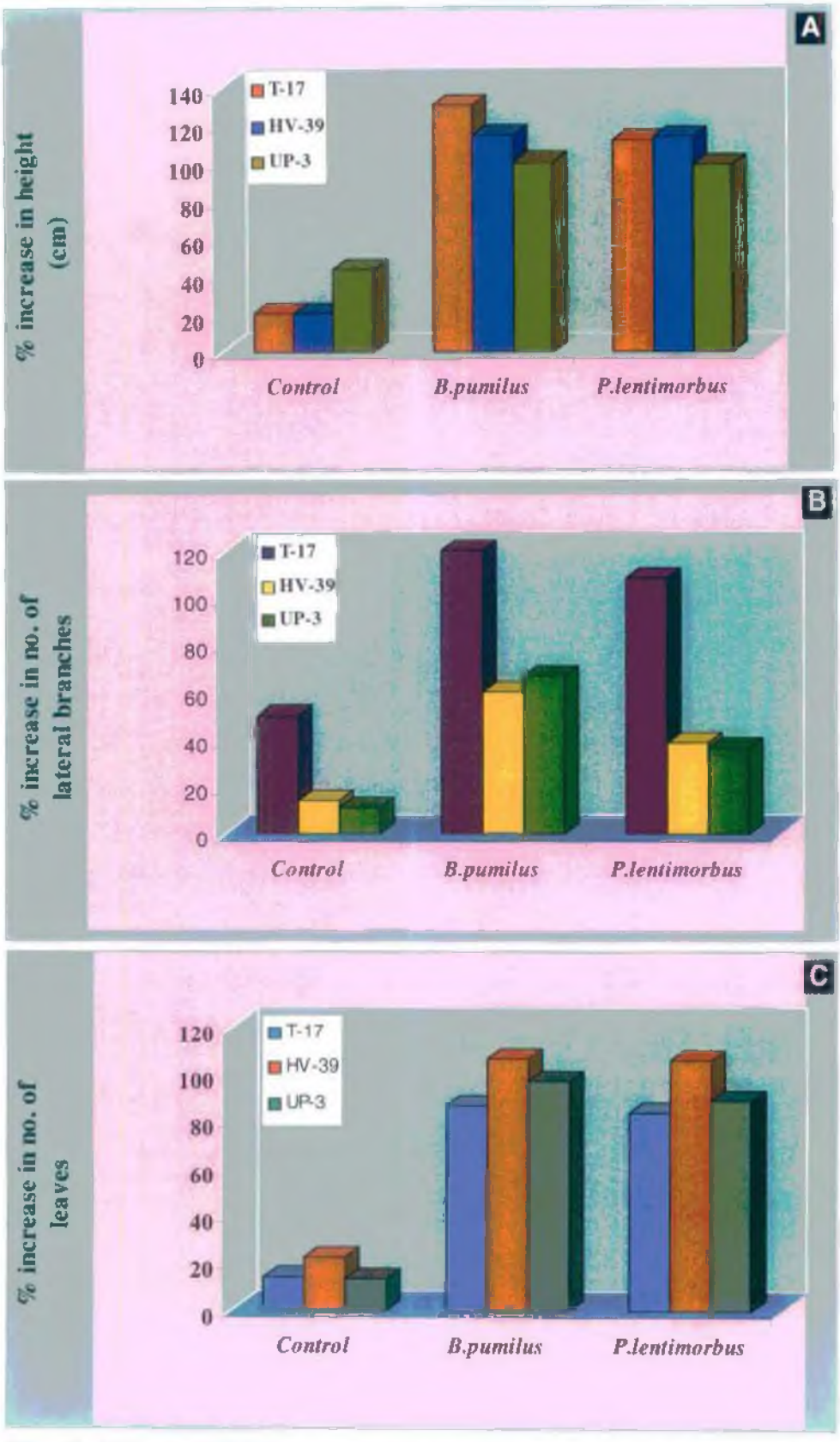


**Fig. 6:** Effect of PGPRs on two year old tea plants of three varieties (2 months after application).


**Plate XIII:** Foliar application of *B.pumilus* on potted plants and tea bushes. Control-(A) Potted plant and (C & D) Tea bush; (B) Potted plant sprayed with *B.pumilus*; (E & F) Tea bush sprayed with *B.pumilus*.

**Table 19**: Growth of 2 year old tea plants after treatment with bacterial isolates

| Tea Variety | Treatment | Height of Plants (cm) | | No. of lateral Branches | | No. of leaves | |
|---|---|---|---|---|---|---|---|
| | | Initial | After 2 months | Initial | After 2 months | Initial | After 2 months |
| T-17 | Control | 31.2±2.1 | 37.6±2.3 | 10±0.6 | 15±1.0 | 43±2.3 | 49±2.8 |
| | *B.pumilus* | 34.3±1.8 | 78.7±1.8 | 10±0.5 | 22±1.2 | 44±1.8 | 82±3.4 |
| | *P.lentimorbus* | 33.0±2.1 | 70.0±3.1 | 12±0.4 | 25±0.9 | 43±1.7 | 79±3.2 |
| HV-39 | Control | 37.1±1.8 | 44.7±2.5 | 14±0.7 | 16±0.8 | 60±3.1 | 73±2.9 |
| | *B.pumilus* | 35.6±2.0 | 76.2±3.2 | 10±0.4 | 16±0.7 | 33±1.9 | 68±2.5 |
| | *P.lentimorbus* | 38.6±2.2 | 82.5±3.5 | 13±0.6 | 18±0.9 | 37±1.8 | 76±3.1 |
| UP-3 | Control | 31.2±1.7 | 44.7±1.9 | 9±0.2 | 10±0.4 | 45±1.9 | 51±2.3 |
| | *B.pumilus* | 40.4±1.6 | 81.2±3.6 | 9±0.6 | 15±0.8 | 32±1.2 | 63±2.5 |
| | *P.lentimorbus* | 38.6±1.3 | 76.7±3.1 | 11±0.7 | 15±0.7 | 35±1.4 | 66±2.3 |

Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded 2 months following the bacterial inoculation; ± S.E.

### 4.8. Effect of co-inoculation with bacteria on growth of tea seedlings

Since it was proved that both the bacteria had the power to promote plant growth 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 applied individually as well as in different combinations to the young seedlings of 6 varieties. 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. (Table 20 and 21; Plate XIV)

**Table 20**: Effect of joint application of bacteria in soil on growth of tea seedlings

| Treatment | 2 months after application | | | | | |
|---|---|---|---|---|---|---|
| | % Increase in height of seedling* | | | % Increase in no. of leaves* | | |
| | T-17 | K-1/1 | T-78 | T-17 | K-1/1 | T-78 |
| Control | 10.0 | 13.0 | 16.7 | 14.3 | 20.0 | 25.0 |
| *B.pumilus* | 50.0 | 33.8 | 50.0 | 53.0 | 33.0 | 50.0 |
| *P.lentimorbus* | 43.3 | 37.5 | 41.1 | 49.7 | 32.2 | 48.4 |
| *B.pumilus*+*P.lentimorbus* | 62.5 | 44.3 | 47.0 | 64.6 | 48.5 | 52.3 |

Each treatment consisted of 10 plants, in triplicate and the values are an average of 30 plants. Results were recorded 2 months following the bacterial inoculation.


**Plate XIV:** Effect of coinoculation with PGPRs on growth of tea seedlings of two varieties (P.l. - *P.lentimorbus*; B.p - *B.pumilus*).

**Table 21**: Effect of bacterial consortia on the growth of tea seedling (after 10 months)

| Treatment | % increment in shoot length | | |
|---|---|---|---|
| | T-17 | K-1/1 | T-78 |
| Control | 85.7 | 94.6 | 53.2 |
| *B.pumilus* | 213.8 | 220.8 | 91.6 |
| *P.lentimorbus* | 207.1 | 209.3 | 78.8 |
| *B.pumilus*+*P.lentimorbus* | 360.3 | 345.5 | 122.5 |

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.

### 4.9. *In vitro* determination of mechanism of action of selected antagonists

The growth promotion of plants may be achieved by the ability of bacteria to fixor sequester iron, facilitate phosphorus uptake or produce phytohormones (IAA), HCN, volatiles and chitinase that trigger responses in a growing plants. To determine the mechanisms of action of two selected rhizobacteria following experiments were conducted. Results are summarized below.

#### 4.9.1. IAA production

IAA production by the selected rhizobacteria was assessed for their ability to produce Indole Acetic Acid (IAA) by growing them in Tripticase Soya agar supplemented with tryptophane (0.1 mM). *B. pumilus* recorded the IAA production of 42mg/L and *P.lentimorbus* was found to produce 46 mg/L.

#### 4.9.2. Phosphate solubilization

Formation of clear zone around the colony grown in Pikovskaya's medium is an indication of phosphate solubilisation by rhizobacteria. In Pikovskaya's medium both *B.pumilus* and *P. lentimorbus* produced clear zone of diameter 2.65cm and 2.15cm after 5 days of incubation which indicated that both the isolates could solubilise the insoluble phosphate into simpler substances.

#### 4.9.3. Siderophore production

To assess the siderophore production by the antagonistic rhizobacteria bacteria, these were inoculated into Chrome Azurol Sugar agar plates and were incubated for 10-15 days. The appearance of yellow halo region was observed around both *B. pumilus* and *P. lentimorbus* which indicated the both bacterial isolates were able to chelate $Fe^{3+}$ from Chrome Azurol Sugar. The diameter of halo region was 1.6

cm and 1.5 cm for *B. pumilus* and *P. lentimorbus* respectively after 12 days of incubation.

Production of siderophore was further confirmed by $Fe^{+}$ chelate test. Both *B.pumilus* and *P.lentimorbus* showed reduction in their ability to inhibit the mycelial growth of *Poria hypobrunnea* with increasing concentration on iron supplemented in the medium. The inhibition was maximum in case of 150µg/ml and minimum in case of 600µg/ml iron concentration.

### 4.9.4 HCN production

To determine the ability of *B.pumilus* and *P.lentimorbus* 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 paraflim.Results were observed after 4-7 days. Both *B.pumilus* and *P.lentimorbus* were found to be non-cyanogenic in nature. This suggests that compound other than HCN may be associated in the inhibition of *P. hypobrunnea* in dual plate inverted chamber.

### 4.9.5. Chitinase production

To determine chitinase production by the antagonistic rhizobacteria bacteria spot inoculation was made in the 5 % colloidal chitin amended minimal medium and incubated at $28^{0}$C for 6-7 days. It was observed that no extracellular chitinase was secreted by *B.pumilus* and *P.lentimorbus* even when grown on chitin amended media.

### 4.9.6. Volatile production

Volatile compound production by the antagonistic rhizobacteria was assessed 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 showed that both the antagonists produced volatile compounds. The maximum inhibition of *F.lamaoensis* was exhibited by *B.pumilus* where as the maximum inhibition of *P.hypobrunnea* was exhibited by *P. lentimorbus*

Further, when the effect of age of bacteria on the growth inhibition of pathogen was compared maximum inhibition was observed on the 4th day of incubation and one day old inocula as compared to same age, 2 and 3days old inocula (Table 22 and 23; Plate XV).

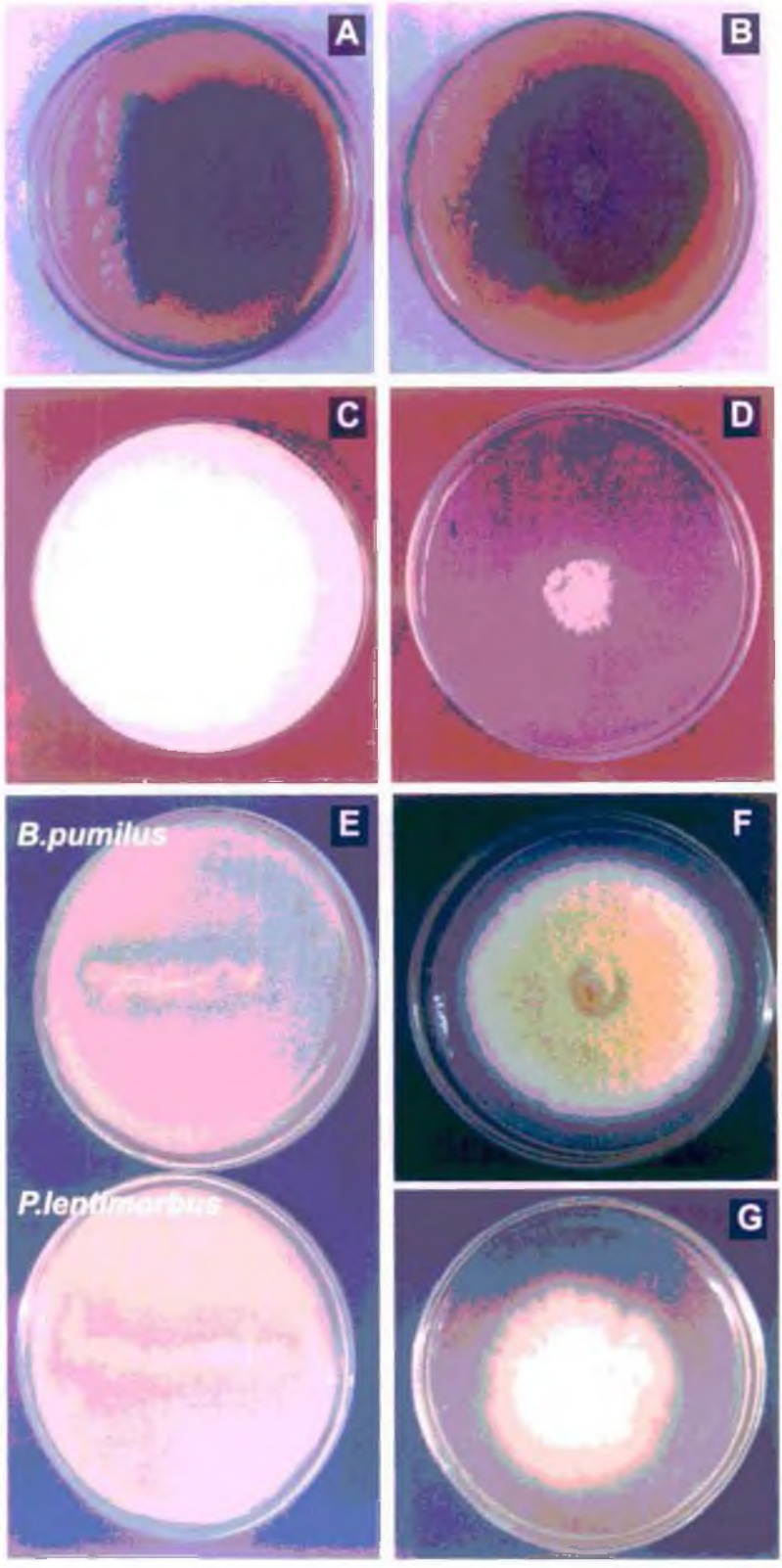


**Plate XV(A-G):** *In vtro* PGPR activities of *B.pumilus* and *P.lentimorbus.* Siderophore production of *B.pumilus* (A) and *P.lentimorbus* (B); Radial growth of *S.rolfsii* (D) and *P.hypobrunnea* (G) inhibited by volatiles from *B.pumilus;* C and F-respective controls; E-Phosphate solubilization by two bacteria.

**Table22:** Effect of volatile compounds on growth inhibition of test pathogens

| Pathogens | *B.pumilus* | | *P.lentimorbus* | |
|---|---|---|---|---|
| | RMG (cm) | GI (%) | RMG (cm) | GI (%) |
| *P.hypobrunnea* | 3.3 | 63.33 | 3.2 | 64.44 |
| *F.lamaoensis* | 3.0 | 66.76 | 3.9 | 56.66 |
| *S.repens* | 4.3 | 52.22 | 4.7 | 47.77 |
| *S.rolfsii* | 5.2 | 44.22 | 5.5 | 38..89 |
| *S.sclerotirum* | 5.0 | 44.45 | 5.2 | 42.21 |
| Control | 9.0 | - | 9.0 | - |

RMG-Radial mycelial growth; GI- Growth inhibition; Average of three replicates; 1 day old inocula; 4 day of incubation.

**Table 23**: Growth inhibition (%) of *P.hypobrunnea* by volatile compounds released by *B.pumilus*

| Antagonist | Grwoth inhibition (%) | | | | |
|---|---|---|---|---|---|
| | Days | Same age | 1 day old | 2 day old | 3 day old |
| *B.pumilus* | 1 | 16.25 | 10.70 | 00.00 | 00.00 |
| | 2 | 30.45 | 40.23 | 37.61 | 41.26 |
| | 3 | 39.71 | 52.55 | 40.25 | 46.83 |
| | 4 | 47.78 | 63.33 | 45.56 | 49.63 |

Results of all the above tests showed positive reaction; negative reactions were obtained incase of HCN production and chitinase production. (Table 24; Plate XV)

**Table 24**: PGPR activity of selected rhizobacteria

| Mechanism of action | *B.pumilus* | *P.lentimorbus* |
|---|---|---|
| IAA production | + | + |
| Phosphate solubilisation | + | + |
| Siderophore production | + | + |
| Volatile production | + | + |
| HCN production | - | - |
| Chitinase production | - | - |

Average of three replicates; + Positive reaction; - Negative reaction.

### 4.10. Bioassy of active principle from *B.pumilus* and *P.lentimorbus* against test fungi

#### 4.10.1. Cell free culture filtrate

Since the bacteria inhibited the growth of the pathogens both in solid and liquid medium, it was decided to determine whether the culture filtrates could inhibit the growth of the pathogens. Cell free culture filtrate of both the bacterial strains *B.pumilus* and *P.lentimorbus* were prepared and sterilized either by autoclaving (15 lbs. for 15 min.) or by passing through sterilized G-5 filter. These were then mixed with the medium in different proportions and the test pathogen (*P. hypobrunnea)* was inoculated into it. Results revealed that both autoclaved and cold-sterilized cell free culture filtrates significantly restricted the growth of *P. hypobrunnea in vitro*. As the ratio of culture filtrate in broth increased, a marked decrease in mycelial growth of the pathogen was recorded. (Table 25)

The active principle present in the culture filtrate was found to be heat stable. Autoclaved culture filtrate retained full inhibitory activity against the mycelial growth of the pathogens. There were no significant differences in the percentage of inhibition of pathogen growth due to the autoclaved culture filtrate and cold sterilized culture filtrate.

**Table 25**: Effect of Autoclaved culture filtrate (ACF) on the mycelial growth of *Poria hypobrunnea*

| Treatment | Mycelial dry wt. (mg) | |
|---|---|---|
| | *B.pumilus* | *P.lentimorbus* |
| PDB | 285.1 | 289.2 |
| ACF | 0 (100) | 0 (100) |
| ACF+10%PDB | 040.4 (85.8) | 043.3 (85.0) |
| ACF+30%PDB | 090.2 (68.4) | 101.0 (65.1) |
| ACF+50% PDB | 124.7 (56.3) | 138.6 (52.1) |

PDB- Potato Dextrose Broth; Figures in parenthesis indicate percentage (%) of inhibition over control

#### 4.10.2. Solvent extraction

Results of previous experiments showed that both the rhizobacteria secreted antifungal substances into the medium. For the characterization of these antifungal metabolites, the cell-free culture filtrates were extracted separately with five solvents-benzene, hexane, chloroform, diethylether, petroleum ether or ethylacetate. The solvent extracts were dried in vacuum and were finally dissolved in methanol as described earlier.

**4.10.2.1. Bioassay of solvent extracts from *B.pumilus* and *P.lentimorbus***

The different solvent extracts were bioassayed against *P.hypobrunnea* to determine the fraction containing the active principle.

**4.10.2.1.1. Spore germination**

For spore germination bioassay a drop of each solvent extract was placed on clean, grease-free glass slide and allowed to evaporate following which drop of spore suspension of *P.hypobrunnea* was placed over it. Percentage spore germination was determined after 24 h. For control set drop of methanol was used. Results showed spores germination of most of the test pathogens totally inhibited by diethylether fraction of *B. pumilus* and benzene fraction of *P.lentimorbus*. Partial inhibitions were found in other fraction. (Table 26; Fig. 7)

**Table 26:** Effect of solvent extracts from culture filtrate of *P.lentimorbus* on spore germination of different fungal spores.

| Fungal species | % spore germination | | | | | | |
|---|---|---|---|---|---|---|---|
| | Control | fr.1 | fr.2 | fr.3 | fr.4 | fr.5 | fr.6 |
| *C.lunata* | 97.9 ±4.1 | 03.5 ±0.3 | 08.3 ±0.6 | 01.5 ±0.8 | 12.4 ±0.9 | 13.2 ±1.2 | 10.7 ±0.6 |
| *F.oxysporum* | 98.3 ±2.7 | 04.6 ±0.4 | 08.7 ±0.5 | 01.0 ±0.2 | 13.4 ±0.8 | 11.2 ±0.6 | 14.4 ±1.1 |
| *A.niger* | 98.4 ±1.2 | 04.2 ±0.2 | 09.5 ±1.2 | 00.0 | 14.8 ±1.1 | 13.2 ±1.2 | 15.7 ±1.1 |
| *S.repens* | 98.0 ±1.2 | 06.3 ±0.5 | 15.4 ±1.3 | 02.5 ±0.1 | 17.3 ± 1.7 | 18.7 ±1.9 | 23.8 ±2.2 |
| *S.rolfsii* | 98.4 ±2.4 | 07.2 ±0.2 | 17.7 ±1.5 | 03.3 ±0.2 | 15.8 ±1.4 | 20.5 ±2.0 | 21.2 ±2.1 |
| *F.lamaoensis* | 97.6 ±2.3 | 05.3 ±0.8 | 08.1 ±0.7 | 00.0 | 08.3 ±0.8 | 09.4 ±0.7 | 11.5 ±0.8 |
| *P.hypobrunnea* | 97.2 ±4.5 | 06.4 ±0.6 | 09.8 ±0.5 | 00.0 | 07.9 ±0.7 | 09.6 ±0.5 | 12.3 ±0.7 |

Fr.1 – Diethyl ether extract; Fr.2- Hexane extract; Fr.3- Benzene extract; Fr.4- Petroleum ether; Fr.5- Ethyacetate extract; Fr.6– Chloroform extract; [A] Average of 200 spores/treatment; [B] Average of 50 germ lings/treatment.

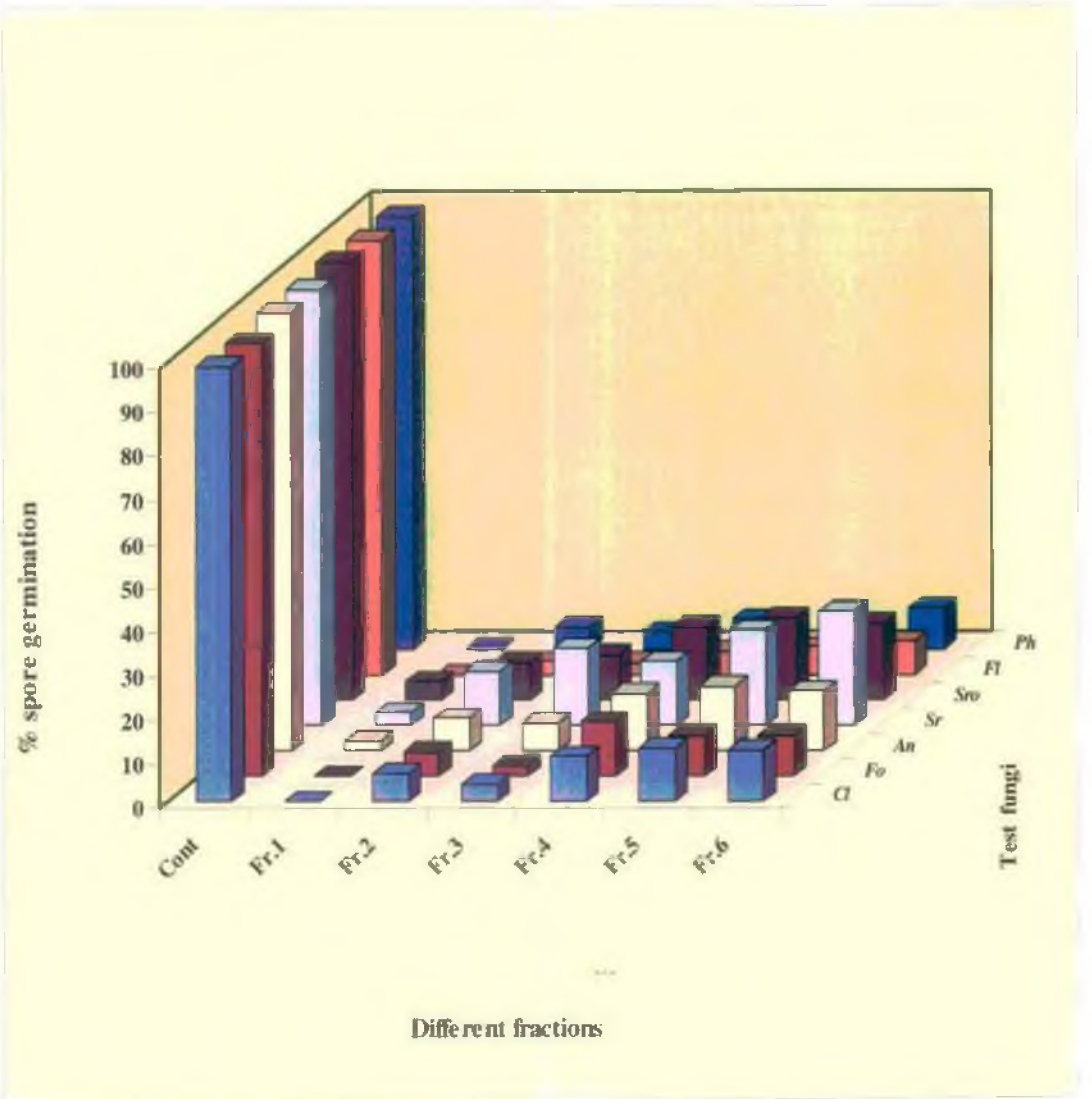


**Fig. 7:** Effect of solvent extracts from *B. pumilus* on germination of different fungal spores; Cl - *C. lunata*; Fo - *F. oxysporum*; An - *A. niger*; Sr - *S. repens*; Sro - *S. rolfsii*; Fl - *F. lamaoensis* and Ph - *P. hypobrunnea*. Fr.1 – Diethyl ether extract; Fr.2- Hexane extract; Fr.3- Benzene extract; Fr.4- Petroleum ether; Fr.5- Ethyacetate extract and Fr.6– Chloroform extract.

**4.10.2.1.2. Radial growth**

The effect of the different extracts on radial growth of *P. hypobrunnea* was tested on solid medium. After 4 days of growth in the control sets the mycelia completely covered the petridishes while varying degrees of inhibition in mycelial growth was noted in the different extracts. (Table 27; Plates XVI & XVII) In the diethyl ether fraction of *B. pumilus* maximum (82.7%) inhibition was recorded whereas maximum inhibition (79.1%) showed by benzene fraction of *P. lentimorbus*.

**Table 27:** Effect of solvent extract of *B.pumilus* against *P.hypobrunnea*

| Solvent | *B.pumilus* | | *P.lentimorbus* | |
|---|---|---|---|---|
| | **Mycelia Diameter (cm)** | **Reduction of mycelial growth (%)** | **Mycelia Diameter (cm)** | **Reduction of mycelialgrowth(%)** |
| Control | 7.0 | — | 7.0 | |
| Benzene | 1.6 ± 0.2 | 71.6 ± 2.4 | 1.7 ± 0.4 | 79.1 ± 3.2 |
| Hexane | 2.0 ± 0.4 | 75.4 ± 1.7 | 2.5 ± 0.5 | 64.2 ± 1.6 |
| Diethyl ether | 1.4 ± 0.1 | 82.7 ± 3.8 | 1.9 ± 0.7 | 75.5 ± 2.8 |
| Petroleum ether | 2.4 ± 0.3 | 65.7 ± 1.8 | 2.1 ± 0.4 | 70.0 ± 3.6 |
| Ethyl acetate | 2.6 ± 0.2 | 62.9 ± 3.4 | 2.8 ± 0.5 | 60.0 ± 3.1 |
| Chloroform | 2.2 ± 3.2 | 68.6 ± 1.5 | 2.7 ± 0.3 | 61.4 ± 1.8 |

Average of three replicates; Cork Borer size-0.6 cm; Petri dish size -7.0 cm.

**4.11. Optimization of active principle production by antagonists**

Results of the previous experiments have established that bacteria were antagonistic to *P.hypobrunnea* both *in vitro* and *in vivo*. So, another experiment was conducted to optimize the active principle produced by antagonists. The production of antifungal metabolites by bacteria in culture is influenced by a number of factors including available carbon and nitrogen sources, pH of the medium, temperature, size of inoculums and time period. Maximum production of the antifungal principle will be at the optimum combinations. Considering the above, it was decided to study the effect of the important factors on production of active principle by *B.pumilus*. Incubation period, pH, different media and carbon sources were considered.

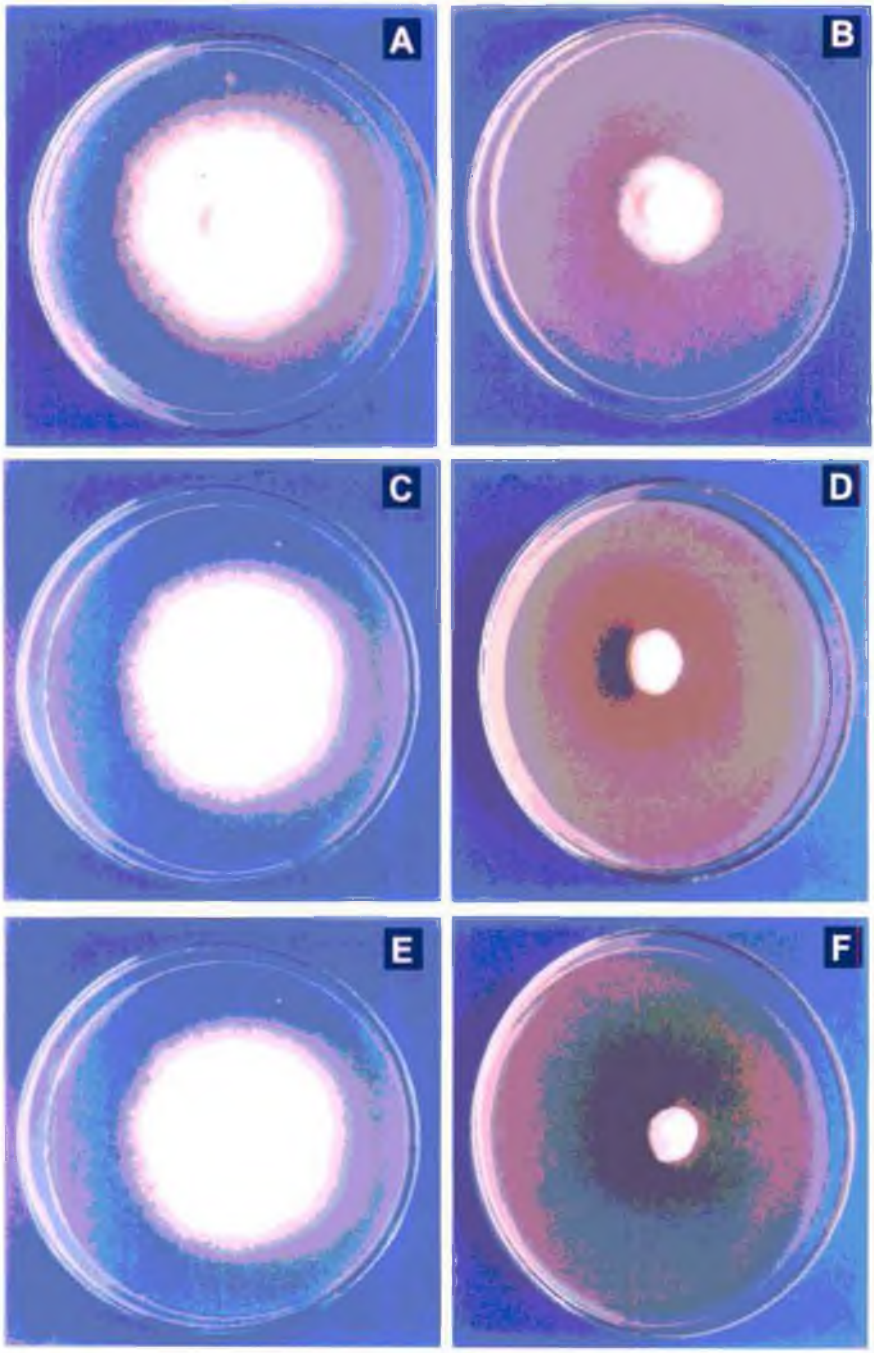


**Plate XVI:** Radial growth bioassay of solvent extracts from *B.pumilus*.Hexane (B); Benzene (D); Di-ethylether (F); Respective controls - A,C and E.

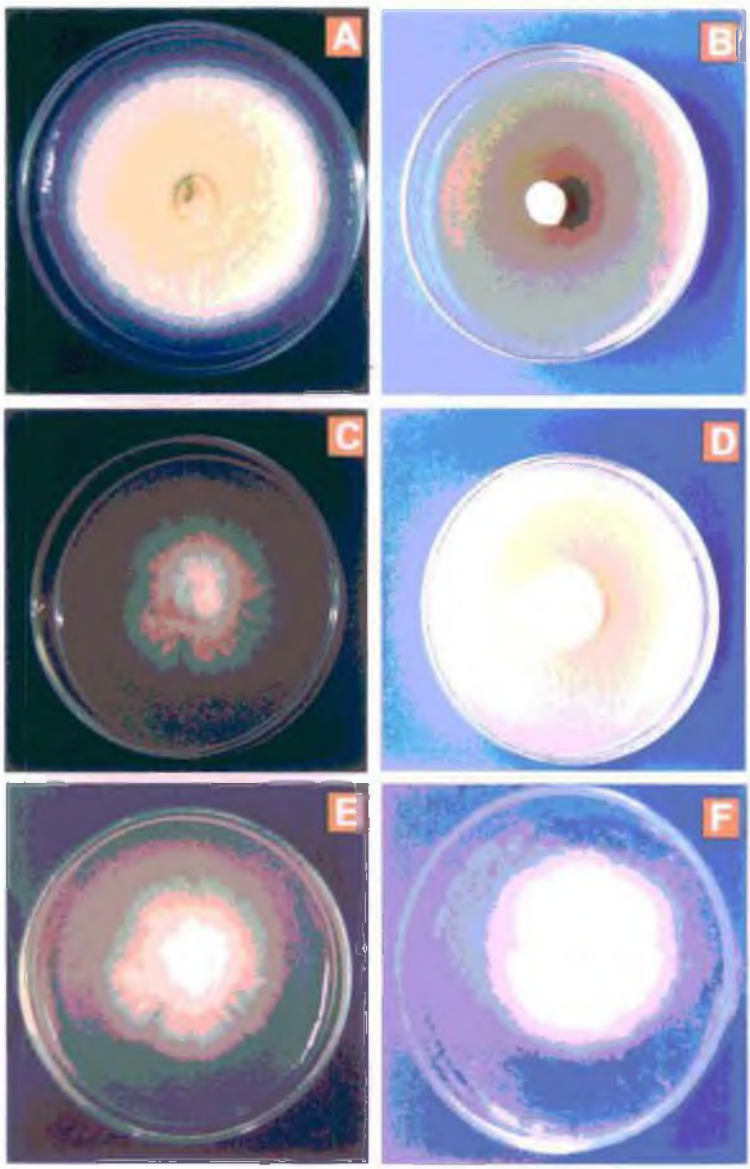


**Plate XVII:** Radial growth bioassay of solvent extracts from *P.lentimorbus*. Control (A); Benzene (B); Hexane (C); Diethyl ether (D); Ethyl acetate (E) and Chloroform (F).

### 4.11.1. Effect of incubation period

To determine the optimum incubation period for the production of antifungal compound by *B. pumilus*, nutrient broth (50 ml/250 ml flask) was inoculated with 0.5 ml inoculums and kept for 7 days at 35$^0$C in shaking condition. The culture filtrate was collected at intervals of 24h upto 168h. The culture filtrates were extracted with diethyl ether as described earlier. The extracts were bioassayed by radial growth inhibition and agar cup methods. The results revealed that maximum production of antifungal compound was at 96h of incubation as evidenced by minimum radial growth (17mm) and maximum inhibition zone (25mm). Production of the metabolite increased from 24h to 96h and there was a steady decline after 96h. Results, therefore, indicate that 96h is the optimum incubation period for production of antifungal metabolite by *B .pumilus*. (Table 28; Fig. 8)

**Table 28**: Effect of different incubation time on production of antifungal compound by *B.pumilus*

| **Growth period of *B.pumilus* (h) [a]** | **Inhibition zone in Agar cup assay (mm) [b]** |
|---|---|
| 24 | 19.5 ± 1.5 |
| 48 | 21.5 ± 1.6 |
| 72 | 22.8 ± 1.4 |
| 96 | 25.0 ± 1.4 |
| 120 | 23.3 ± 1.2 |
| 140 | 18.6 ± 2.2 |
| 168 | 16.4 ± 1.4 |

Average of three replicate/ treatment; [a] *B. pumilus* grown in Nutrient Broth
[b] Growth measured after 4 days; Petridish size –7 cm; Cork borer size- 0.6cm

### 4.11.2. Effect of different media

Four different media (Nutrient Broth, Nutrient Sucrose Broth, Glucose Yeast Peptone Broth and Luria Broth) were considered to select the most suitable one for production of antifungal compound. After 96h of growth in the different media the bacterial culture filtrates were collected and extracted as before. Activities of the extracts were tested by radial growth inhibition and agar cup bioassays. It was observed that among the tested media, Nutrient Sucrose Broth was the most effective followed by Glucose Yeast Peptone Broth. (Table 29)

**Table29:** Effect of different media on production of antifungal compound of *B.pumilus*

| Media | Radial growth of *P.hypobrunnea*(mm)[a] | Inhibition zone in Agar cup assay(mm)[a] |
|---|---|---|
| Nutrient Sucrose Broth | 17.3 ± 2.1 | 28.4 ± 2.3 |
| Nutrient Broth | 22.2 ± 1.6 | 24.3 ± 2.2 |
| Glucose Yeast Peptone Broth | 19.7 ± 1.3 | 26.6 ± 1.5 |
| King's B | 26.1 ± 2.4 | 23.6 ± 1.7 |

Average of three replicate/ treatment; [a]Growth measured after 4 days; Petri dish size – 7 cm; Cork borer size- 0.6cm

### 4.11.3. Effect of carbon sources

Since nutrient sucrose broth was most effective among all four tested media, it was selected as the basal medium for further studies. To determine the effect of carbon sources on production of antifungal compound, the original carbon source of the medium (sucrose) was replaced by fructose, maltose, dextrose, starch, galactose, or mannitol. In all cases the medium was supplemented with 0.1% w/v C- source to maintain the same concentration as in the original medium. Productions of antifungal metabolites in the above cases were tested by radial growth inhibition and agar cup bioassay techniques. Both bioassays confirmed maximum production of antifungal compound in the medium containing sucrose as carbon source, followed by dextrose. (Table 30)

**Table 30**: Effect of different carbon sources on the production of antifungal compound by *B.pumilus*

| Carbon sources[a] | Radial growth of *P.hypobrunnea*(mm)[b] | Inhibition zone in Agar cup assay(mm)[b] |
|---|---|---|
| Dextrose | 23.7 ± 1.9 | 23.0 ± 2.1 |
| Fructose | 25.6 ± 2.0 | 21.4 ± 2.0 |
| Maltose | 27.3 ± 1.9 | 20.6 ± 2.3 |
| Sucrose | 16.3 ± 1.1 | 25.1 ± 1.7 |
| Starch | 27.1 ± 1.6 | 19.7 ± 2.1 |
| Galactose | 28.2 ± 1.7 | 19.1 ± 1.1 |
| Mannitol | 29.3 ± 2.1 | 18.8 ± 1.3 |

Average of three replicate/ treatment; [a]Basal medium Nutrient Sucrose Broth.
[b] Growth measured after 4 days; Petridish size – 7 cm; Cork borer size- 0.6cm.

### 4.11.4. Effect of different pH

As pH of the medium is known to influence the production of metabolites by microorganisms, it was one of the factors considered here. For this nutrient sucrose nroth was adjusted to different pH (5.5, 6.0, 6.5, 7.0, and 7.5) with HCl or NaOH. Culture filtrates obtained from each pH was extracted separately with diethyl ether and tested for their activity. Results revealed that 6.0 was the optimum pH for production of antifungal compound from *B. pumilus*. (Fig. 8)

### 4.12. Effect of *B.pumilus* on disease development in tea

As the bacteria inhibited the growth of the pathogens *in vitro* studies were conducted to determine the effectiveness of these bacteria in controlling root rot diseases. For this experiment, an important root rot diseases caused by *Poria hypobrunnea* was selected. It was observed that *B. pumilus* was successful in reducing intensity of root rot disease. (Table 31; Plate XVIII)

**Table 31**: Effect of *B. pumilus* on development Poria root rot disease of tea

| Varieties | Treatment | Root rot index Days after inoculation | | |
|---|---|---|---|---|
| | | 15 | 30 | 45 |
| HV-39 | *Poria hypobrunnea* | 1.55 | 3.10 | 5.80 |
| | *Poria hypobrunnea*+ *B.pumilus* | 0.25 | 1.15 | 2.45 |
| UP-3 | *Poria hypobrunnea* | 1.35 | 2.45 | 4.85 |
| | *Poria hypobrunnea*+ *B.pumilus* | 0.35 | 2.45 | 4.85 |
| T-17 | *Poria hypobrunnea* | 1.10 | 2.50 | 4.75 |
| | *Poria hypobrunnea*+ *B.pumilus* | 0.40 | 0.95 | 2.10 |

Age of the plant 2yr.; Average of 10 separate inoculated plants; Rot 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% roots affected; 4- shoot tips also withering; 60-70% roots affected; 5- shoots withered with defoliation of lower withered leaves, 80% roots affected; 6- whole plants die, with upper withered leaves still remaining attached; roots fully rotted.

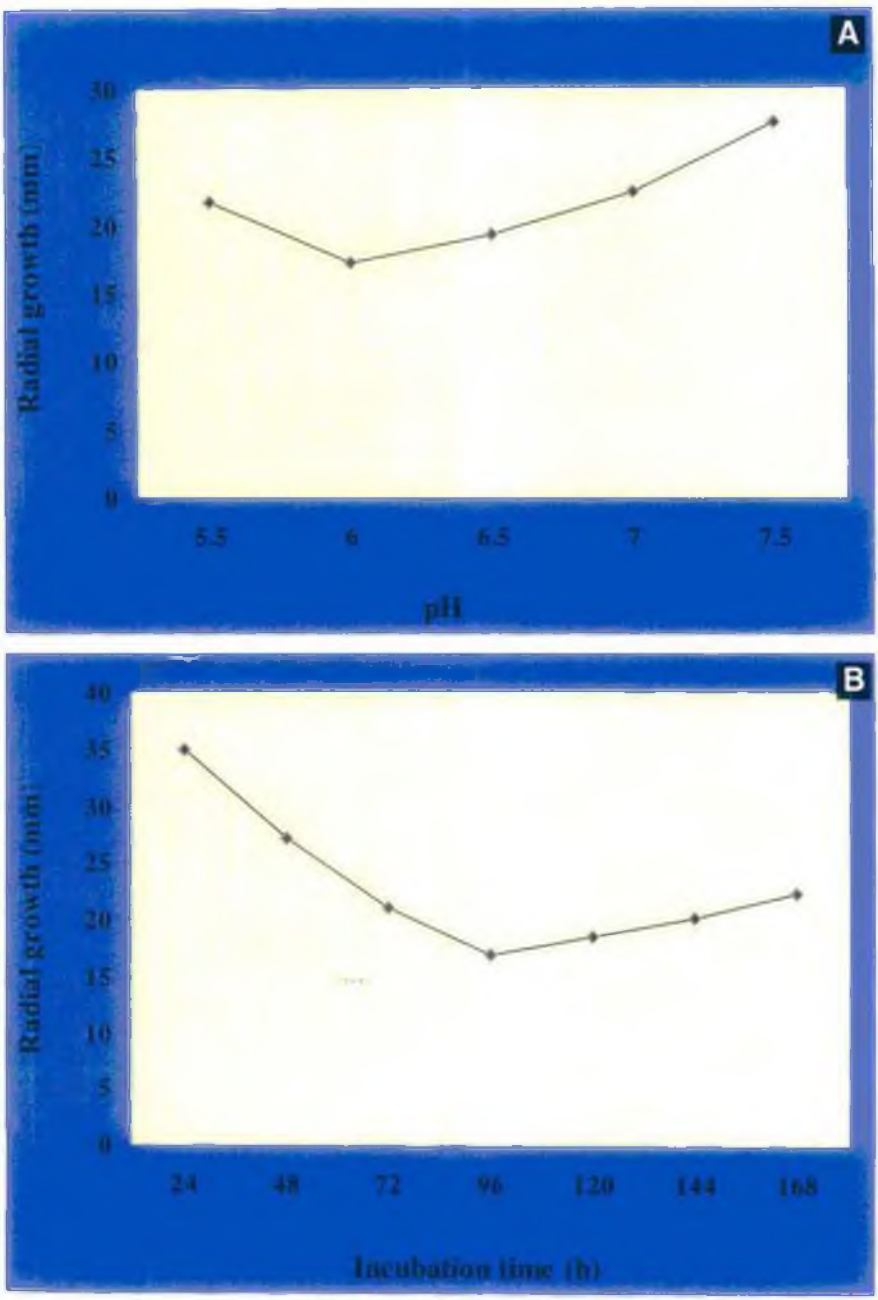


**Fig. 8:** Optimization of active principle production by *B. pumilus* - A: pH; B: Incubation time.

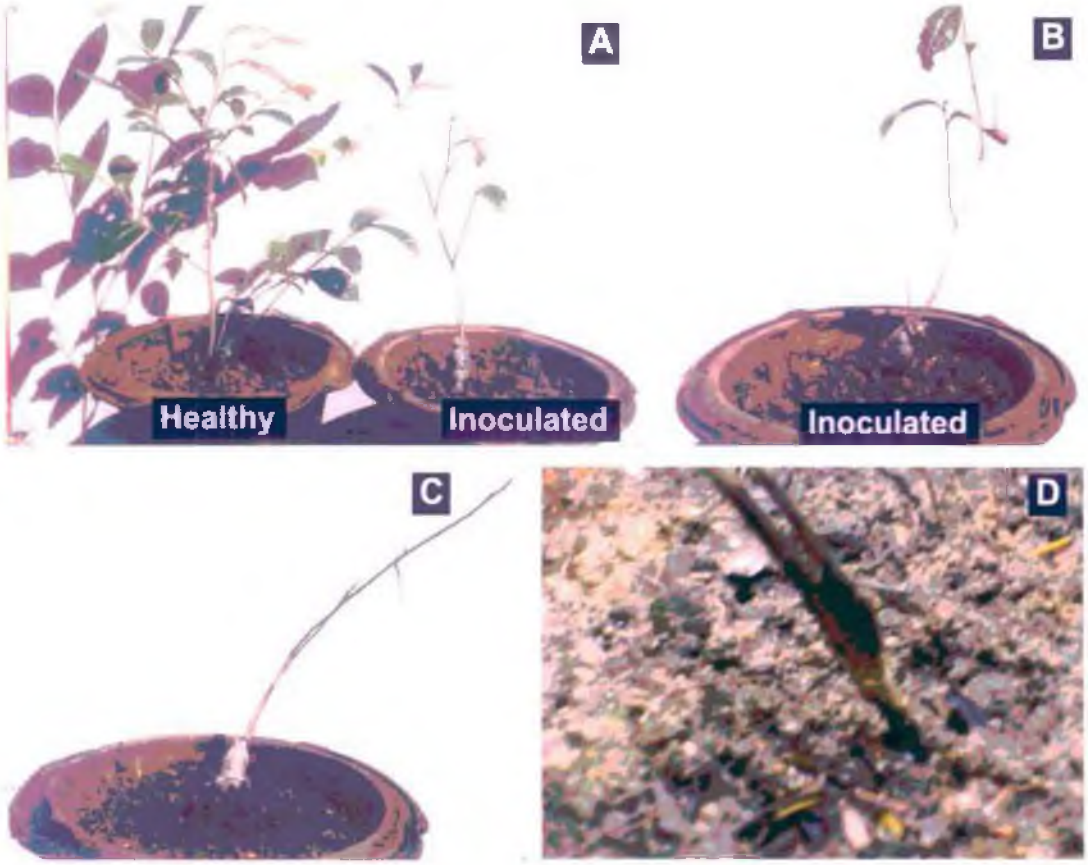


**Plate XVIII (A-D):** Disease symptoms in tea plants inoculated with *P.hypobrunnea*.

### 4.13. Biochemical changes in Tea leaves induced by application of *B.pumilus*

Since plant growth promotion could also be due to induction of biochemical responses within the host, experiments were conducted to assess the effect of *B.pumilus* on biochemical components of tea leaves. Polyphenols are major constituents of tea leaves and hence phenol contents were determined. In order to determine the effect on photosynthetic apparatus changes in chlorophyll content were also analyzed. Besides, catechins the flavonoid flavour component of tea leaves are extremely important and changes in these were also analyzed by HPLC. Activities of some of the enzymes which are involved in phenol metabolism as well as in defense- i.e., peroxidase, phenylalanine ammonia lyase, chitinase, β-1, 3 glucanase were also determined.

#### 4.13.1. Chlorophyll

The quantitive analysis revealed that all treatments led to an increase in chlorophyll content both total Chlorophyll as well as Chl-a and Chl-b. (Table 32)

**Table 32**: Effect of *B.pumilus* on chlorophyll contents of tea leaves

| Treatment | Varieties | Total Chlorophyll (mg/gm tissue) | Chlorophyll a (mg/gm tissue) | Chlorophyll b (mg/gm tissue) |
|---|---|---|---|---|
| Control | HV-39 | 1.493±0.06 | 0.582±0.02 | 0.923±0.08 |
| *B.pumilus* | | 1.695±0.16 | 0.831±0.01 | 0.864±0.15 |
| Control | T-17 | 1.233±0.03 | 0.604±0.03 | 0.606±0.08 |
| *B.pumilus* | | 1.937±0.06 | 0.829±004 | 1.112±0.03 |
| Control | S-449 | 0.820±0.03 | 0.395±0.01 | 0.425±0.02 |
| *B.pumilus* | | 1.321±0.03 | 0.612±0.03 | 0.709±0.05 |
| Control | BSS-2 | 1.270±0.04 | 0.474±0.03 | 0.797±0.18 |
| *B.pumilus* | | 1.364±0.06 | 0.587±0.05 | 0.778±0.01 |
| Control | UP-3 | 0.838±0.05 | 0.385±0.07 | 0.565±0.05 |
| *B.pumilus* | | 0.987±0.03 | 0.386±0.02 | 0.601±0.03 |
| Control | TV-18 | 1.069±0.29 | 0.485±0.01 | 0.554±0.05 |
| *B.pumilus* | | 1.403±0.02 | 0.549±0.02 | 0.854±0.18 |

#### 4.13.2. Catechin analysis

Catechins derived from leaves of 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 few new peaks indicating new isomers. (Fig. 9, 10 and 11; Table 33, 34 and 35)

**Table 33:** HPLC analysis of catechin extracts from leaves of tea plants (cv. BSS-2) grown in untreated and treated (with *B. pumilus*) soil

| Peak No. | | Ret. Time | Area (mv.S) | Height (mv) | Area (%) | Height (%) |
|---|---|---|---|---|---|---|
| 1 | Control | 2.480 | 475.3894 | 24.629 | 0.089 | 0.311 |
| 2 | | 3.120 | 17218.8853 | 997.074 | 3.228 | 12.577 |
| 3 | | 3.990 | 3604.7871 | 260.989 | 0.676 | 3.292 |
| 4 | | 4.610 | 5859.0140 | 212.786 | 1.098 | 2.684 |
| 5 | | 5.950 | 41722.2667 | 541.317 | 7.822 | 6.828 |
| 6 | | 7.800 | 20455.8179 | 305.019 | 3.835 | 3.847 |
| 7 | | 9.550 | 51121.9293 | 712.538 | 9.584 | 8.988 |
| 8 | | 12.370 | 128895.6883 | 980.621 | 24.164 | 12.369 |
| 9 | | 14.070 | 37264.4918 | 977.556 | 6.986 | 12.330 |
| 10 | | 16.050 | 34571.3428 | 974.012 | 6.481 | 12.286 |
| 11 | | 17.220 | 52108.0618 | 971.946 | 9.769 | 12.260 |
| 12 | | 18.550 | 140120.2215 | 969.566 | 26.268 | 12.228 |
| 1 | Treated with *B.pumilus* | 2.680 | 5778.0166 | 467.597 | 0.890 | 5.137 |
| 2 | | 3.240 | 31952.9386 | 1009.981 | 4.921 | 11.096 |
| 3 | | 4.000 | 15194.7796 | 1009.398 | 2.340 | 11.089 |
| 4 | | 4.660 | 15925.3609 | 676.130 | 2.453 | 7.428 |
| 5 | | 5.110 | 3749.7174 | 222.369 | 0.577 | 2.443 |
| 6 | | 5.930 | 37916.0413 | 738.907 | 5.839 | 8.118 |
| 7 | | 8.110 | 32205.1821 | 327.824 | 4.960 | 3.602 |
| 8 | | 9.790 | 26807.4281 | 640.050 | 4.128 | 7.032 |
| 9 | | 12.660 | 128246.5328 | 1004.046 | 19.750 | 11.031 |
| 10 | | 14.270 | 48861.9702 | 1003.015 | 7.525 | 11.019 |
| 11 | | 15.200 | 32948.8814 | 1002.400 | 5.074 | 11.012 |
| 12 | | 17.960 | 269758.6119 | 1000.690 | 41.543 | 10.993 |

**Table 34:** HPLC analysis of catechin extracts from leaves of tea plants (cv. HV-39) grown in untreated and treated (with *B. pumilus*) soil

| Peak No. | | Ret. Time | Area (mv.S) | Height(mv) | Area(%) | Height(%) |
|---|---|---|---|---|---|---|
| 1 | Control | 2.150 | 32.8467 | 4.299 | 0.010 | 0.065 |
| 2 | | 2.620 | 884.8221 | 23.468 | 0.283 | 0.355 |
| 3 | | 3.890 | 7656.5542 | 601.786 | 2.447 | 9.095 |
| 4 | | 4.500 | 2747.9053 | 111.081 | 0.878 | 1.679 |
| 5 | | 5.890 | 5808.0080 | 154.650 | 1.856 | 2.337 |
| 6 | | 7.680 | 4301.7337 | 198.085 | 1.375 | 2.994 |
| 7 | | 8.360 | 2121.0916 | 76.457 | 0.678 | 1.156 |
| 8 | | 9.060 | 5868.1514 | 210.666 | 1.875 | 3.184 |
| 9 | | 10.120 | 782.7649 | 31.727 | 0.250 | 0.479 |
| 10 | | 10.520 | 956.4171 | 34.915 | 0.306 | 0.528 |
| 11 | | 11.790 | 58392.0233 | 986.706 | 18.660 | 14.912 |
| 12 | | 13.790 | 24063.2510 | 984.191 | 7.690 | 14.874 |
| 13 | | 14.690 | 6993.7403 | 258.686 | 2.235 | 3.910 |
| 14 | | 16.120 | 32019.2394 | 981.295 | 10.232 | 14.830 |
| 15 | | 16.920 | 50890.1126 | 980.351 | 16.263 | 14.816 |
| 16 | | 18.480 | 109406.4298 | 978.423 | 34.962 | 14.786 |
| 1 | Treated with *B.pumilus* | 2.560 | 97.9829 | 4.912 | 0.034 | 0.071 |
| 2 | | 3.060 | 173.7390 | 7.621 | 0.059 | 0.110 |
| 3 | | 3.440 | 1031.4689 | 76.106 | 0.353 | 1.094 |
| 4 | | 3.860 | 4814.5449 | 506.681 | 1.649 | 7.285 |
| 5 | | 4.440 | 3601.7054 | 205.404 | 1.233 | 2.953 |
| 6 | | 5.460 | 1056.3126 | 43.626 | 0.362 | 0.627 |
| 7 | | 5.790 | 3673.4167 | 218.680 | 1.258 | 1.014 |
| 8 | | 6.320 | 1020.7940 | 70.522 | 0.350 | 0.685 |
| 9 | | 6.630 | 1341.4991 | 47.621 | 0.459 | 0.459 |
| 10 | | 7.560 | 4890.0459 | 257.035 | 1.674 | 3.695 |
| 11 | | 8.170 | 3015.3947 | 118.047 | 1.033 | 1.697 |
| 12 | | 8.840 | 5425.3266 | 239.111 | 1.858 | 3.438 |
| 13 | | 9.390 | 1415.5217 | 63.003 | 0.485 | 0.906 |
| 14 | | 10.330 | 1675.8800 | 41.837 | .574 | 0.620 |
| 15 | | 11.550 | 62544.8490 | 992.681 | 21.417 | 14.272 |
| 16 | | 13.670 | 29526.6908 | 991.149 | 10.111 | 14.250 |
| 17 | | 14.950 | 5863.7555 | 152.196 | 2.008 | 2.188 |
| 18 | | 15.710 | 32905.7934 | 976.823 | 11.268 | 14.044 |
| 19 | | 16.790 | 58495.6025 | 988.943 | 20.030 | 14.218 |
| 20 | | 18.390 | 69464.6642 | 953.401 | 23.785 | 13.707 |

**Table 35:** HPLC analysis of catechin extracts from leaves of tea plants (cv. S-449) grown in untreated and treated (with *B. pumilus*) soil

| Peak No. | | Ret. Time | Area (mv.S) | Height(mv) | Area(%) | Height(%) |
|---|---|---|---|---|---|---|
| 1 | | 2.810 | 473.3173 | 12.257 | 0.144 | 0.150 |
| 2 | | 3.410 | 868.7875 | 65.448 | 0.264 | 0.803 |
| 3 | | 3.870 | 7682.3199 | 857.601 | 2.331 | 10.523 |
| 4 | | 4.420 | 4259.0464 | 197.280 | 1.292 | 2.421 |
| 5 | | 5.420 | 1044.2576 | 57.297 | 0.317 | 0.703 |
| 6 | | 5.710 | 6447.4943 | 358.672 | 1.956 | 4.401 |
| 7 | | 6.260 | 2041.9531 | 128.611 | 0.619 | 1.578 |
| 8 | | 6.620 | 575.5837 | 41.603 | 0.175 | 0.510 |
| 9 | | 6.890 | 493.4300 | 40.140 | 0.150 | 0.493 |
| 10 | | 7.450 | 5257.1728 | 271.776 | 1.595 | 3.335 |
| 11 | | 8.010 | 4513.3562 | 174.622 | 1.369 | 2.143 |
| 12 | Control | 8.630 | 4563.9448 | 188.224 | 1.385 | 2.309 |
| 13 | | 9.170 | 2978.0295 | 142.535 | 0.903 | 1.749 |
| 14 | | 9.880 | 2265.8441 | 54.195 | 0.687 | 0.665 |
| 15 | | 11.360 | 67655.9305 | 990.888 | 20.525 | 12.158 |
| 16 | | 12.780 | 4809.0689 | 109.204 | 1.459 | 1.340 |
| 17 | | 13.400 | 12530.5400 | 474.985 | 3.801 | 5.828 |
| 18 | | 14.460 | 4986.1522 | 202.144 | 1.513 | 2.480 |
| 19 | | 15.220 | 8726.9136 | 269.448 | 2.648 | 3.306 |
| 20 | | 15.630 | 26869.0730 | 988.138 | 8.151 | 12.124 |
| 21 | | 16.720 | 46211.7512 | 987.483 | 14.020 | 12.116 |
| 22 | | 17.490 | 10799.8614 | 551.024 | 0.276 | 6.761 |
| 23 | | 18.280 | 103569.5928 | 986.499 | 31.420 | 12.104 |
| 1 | Treated with *B.pumilus* | 3.000 | 533.9653 | 19.365 | 0.172 | 0.236 |
| 2 | | 3.420 | 951.7176 | 64.073 | 0.306 | 0.779 |
| 3 | | 3.860 | 6133.5504 | 621.490 | 1.974 | 7.559 |
| 4 | | 4.440 | 3776.2338 | 135.153 | 1.215 | 1.644 |
| 5 | | 5.730 | 10168.8955 | 408.078 | 3.273 | 4.963 |
| 6 | | 6.730 | 1036.6882 | 52.698 | 0.334 | 0.641 |
| 7 | | 7.500 | 4255.8639 | 147.582 | 1.370 | 1.795 |
| 8 | | 8.100 | 3996.8123 | 141.639 | 1.286 | 1.723 |
| 9 | | 8.760 | 4912.9617 | 123.339 | 1.581 | 1.500 |
| 10 | | 10.040 | 2694.0107 | 59.404 | 0.867 | 0.722 |
| 11 | | 11.520 | 64728.0004 | 992.513 | 20.833 | 12.071 |
| 12 | | 13.090 | 3469.2315 | 91.235 | 1.117 | 1.110 |
| 13 | | 13.630 | 7343.2052 | 171.530 | 2.363 | 2.086 |
| 14 | | 14.590 | 5020.4748 | 204.134 | 1.616 | 2.483 |
| 15 | | 15.680 | 28249.8214 | 989.352 | 9.092 | 12.033 |
| 16 | | 16.740 | 35009.4416 | 988.600 | 11.268 | 12.023 |
| 17 | | 18.150 | 35306.9967 | 952.921 | 11.364 | 11.590 |
| 18 | | 18.440 | 43797.5820 | 987.336 | 14.096 | 12.008 |
| 19 | | 19.630 | 40439.0283 | 920.235 | 13.015 | 11.192 |
| 20 | | 21.260 | 8875.1528 | 151.601 | 2.858 | 1.842 |

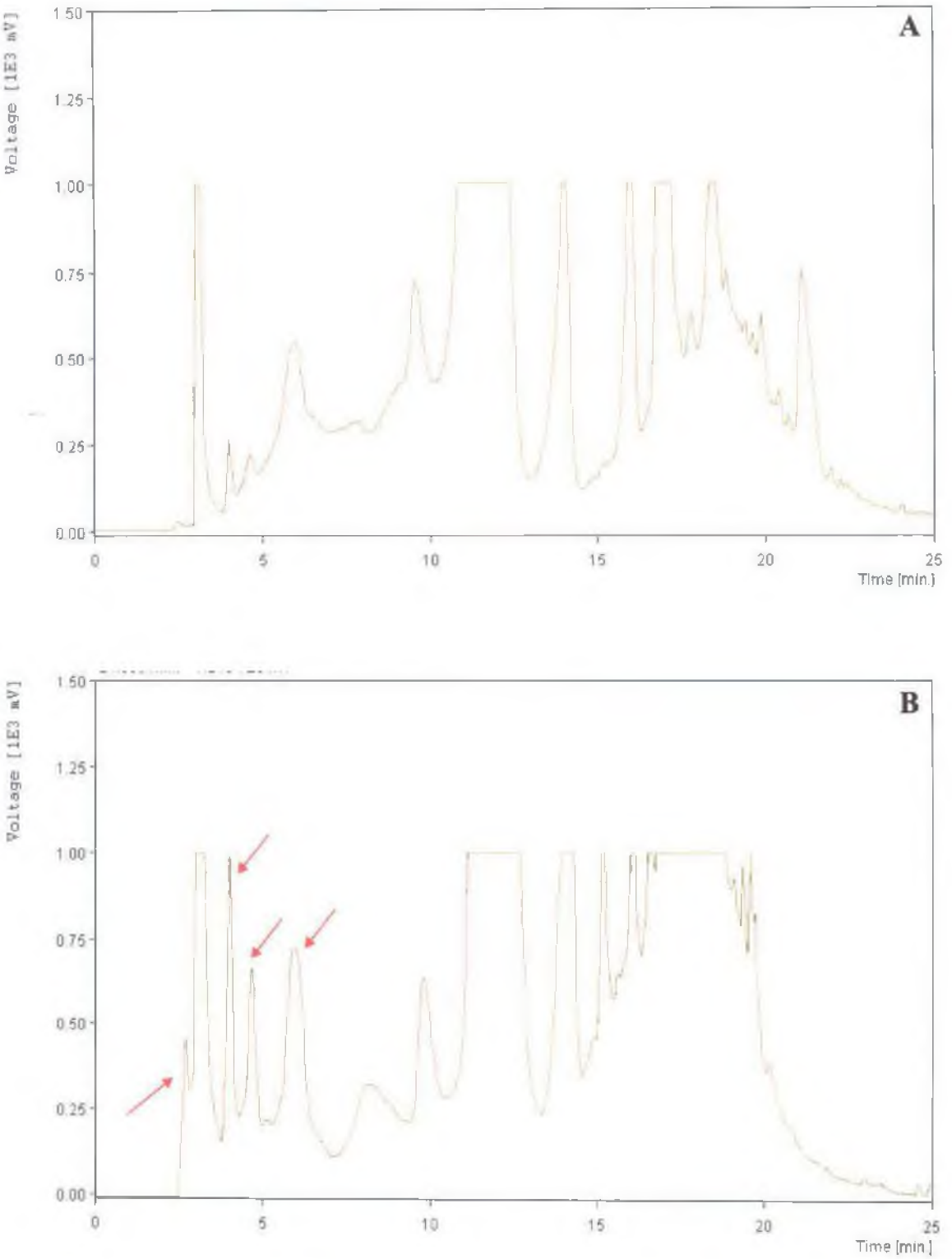


**Fig. 9:** HPLC analysis of catechin extracts from leaves of tea plants (BSS-2). A: Control; B: Soil treated with *B.pumilus*.

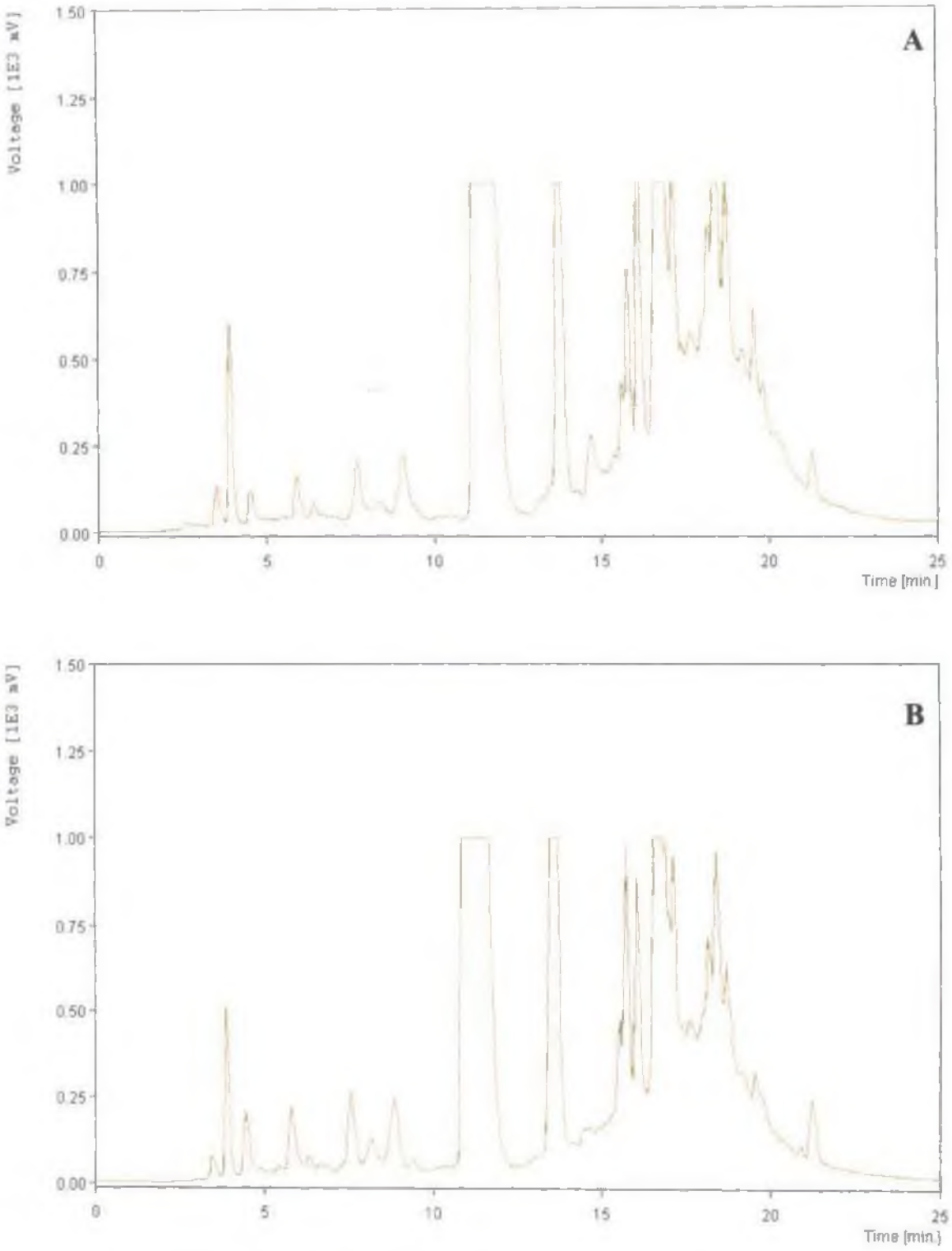


**Fig. 10:** HPLC analysis of catechin extracts from leaves of tea plants (HV-39). A: Control; B: Soil treated with *B.pumilus*.

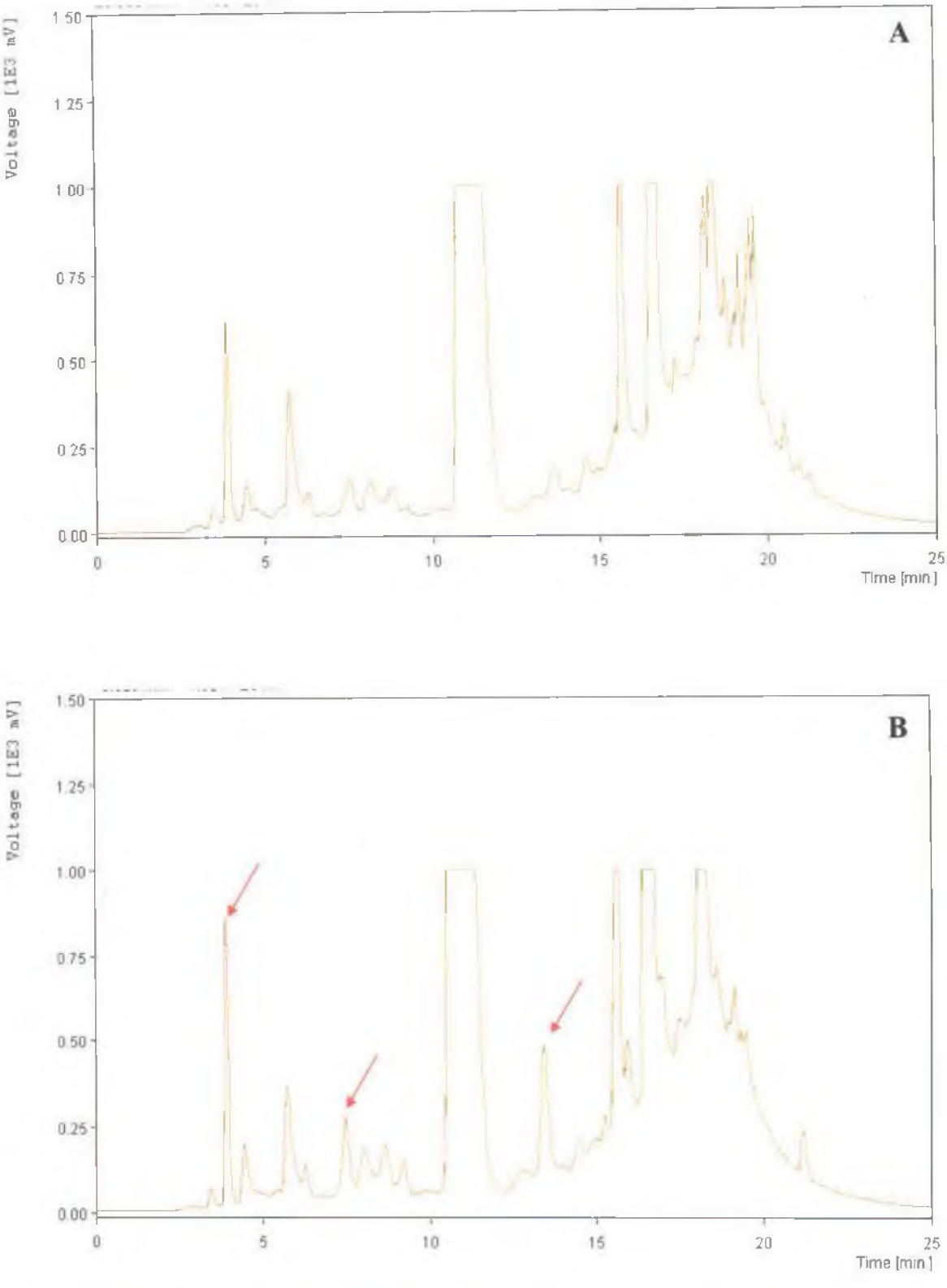


**Fig. 11:** HPLC analysis of catechin extracts from leaves of tea plants (S-449). A: Control; B: Soil treated with *B.pumilus*.

**4.13.3. Phenols and Defense enzymes**

Since phenols and defense enzymes are known to be involved in defense of plants against pathogen, analysis of these were done not only after bacterial inoculation, but also with pathogen inoculation.

**4.13.3.1. Phenol content**

Both the Total and O-dihydroxy phenol contents of the tea leaves were increased significantly after application of *B.pumilus* as compared to untreated control in different varieties of tea. Results revealed that maximum accumulation occurred when there was joint inoculation by PGPR and pathogen. (Table 36)

**Table 36**: Phenol contents in the tea leaves following treatments in the rhizosphere

| Varieties | Treatment | Phenol content (mg/g tissue) | |
|---|---|---|---|
| | | Total | O-dihydroxy |
| T-17 | Control | 30.2±3.2 | 8.2±1.4 |
| | *P.hypobrunnea* | 33.4±2.1 | 8.6±0.8 |
| | *B.pumilus* | 42.5±2.2 | 11.3±1.1 |
| | *P.hypobrunnea*+ *B.pumilus* | 44.6±3.6 | 11.5±1.0 |
| HV-39 | Control | 28.2±1.6 | 7.5±1.4 |
| | *P.hypobrunnea* | 34.1±2.9 | 8.9±1.3 |
| | *B.pumilus* | 36.4±3.9 | 10.1±0.6 |
| | *P.hypobrunnea*+ *B.pumilus* | 43.4±3.8 | 13.2±1.5 |
| UP-3 | Control | 34.4±0.4 | 6.5±1.3 |
| | *P.hypobrunnea* | 39.1±0.6 | 7.8±0.8 |
| | *B.pumilus* | 46.0±2.5 | 8.3±1.0 |
| | *P.hypobrunnea*+ *B.pumilus* | 49.5±3.9 | 14.2±1.6 |
| TV-18 | Control | 30.4±1.4 | 7.9±4.1 |
| | *P.hypobrunnea* | 34.7±3.9 | 9.1±2.9 |
| | *B.pumilus* | 41.5±2.8 | 9.8±1.8 |
| | *P.hypobrunnea*+ *B.pumilus* | 45.3±1.1 | 11.3±2.1 |
| S-449 | Control | 24.1±0.8 | 7.6±1.0 |
| | *P.hypobrunnea* | 38.1±0.6 | 9.2±1.2 |
| | *B.pumilus* | 41.2±0.4 | 10.4±2.1 |
| | *P.hypobrunnea*+ *B.pumilus* | 45.6±0.9 | 11.8±1.7 |
| BSS-3 | Control | 22.5±1.2 | 7.8±1.8 |
| | *P.hypobrunnea* | 33.7±1.3 | 9.2±1.4 |
| | *B.pumilus* | 45.0±1.1 | 14.2±1.6 |
| | *P.hypobrunnea*+ *B.pumilus* | 48.1±2.2 | 14.8±2.3 |

Sampling done after a period of two month of treatments; Average of three replicates; ± S.E.

**4.13.3.2.Enzymes activities.**

Application of *B. pumilus* to the tea plants resulted increase in activities of all the four tested enzymes in the leaves of the bacteria treated plants compared with untreated plants. However, further enhancement was observed when *P.hypobrunnea* was also inoculated. (Table 37; Fig. 12 and 13)

**Table 37:** Enzyme activities in leaves of different tea varieties grown in soil following treatments in the rhizosphere

| Varieties | Treament | Enzyme activities | | | |
|---|---|---|---|---|---|
| | | POX[a] | PAL[b] | CHT[c] | GLU[d] |
| T-17 | Control | 3.7±0.2 | 66±0.5 | 12.5±0.9 | 360±2.0 |
| | *P.hypobrunnea* | 4.8±0.1 | 95±0.8 | 17.5±0.4 | 443±5.1 |
| | *B.pumilus* | 6.0±0.4 | 97±0.7 | 22.2±1.6 | 465±3.8 |
| | *P.hypobrunnea+ B.pumilus* | 8.2±0.2 | 141±0.3 | 26.4±1.0 | 532±1.2 |
| HV-39 | Control | 1.7±0.1 | 96±0.4 | 12.5±1.1 | 336±3.9 |
| | *P.hypobrunnea* | 2.4±0.2 | 101±0.4 | 13.2±0.4 | 356±2.0 |
| | *B.pumilus* | 3.8±0.2 | 110±0.6 | 18.0±1.8 | 476±5.2 |
| | *P.hypobrunnea+ B.pumilus* | 5.2±0.3 | 173±0.7 | 30.0±2.2 | 542±2.4 |
| TV-18 | Control | 3.8±0.1 | 65±0.2 | 16.4±1.4 | 340±1.8 |
| | *P.hypobrunnea* | 4.4±0.7 | 73±0.6 | 17.3±1.2 | 492±0.8 |
| | *B.pumilus* | 5.3±0.3 | 75±0.8 | 20.1±1.7 | 560±2.4 |
| | *P.hypobrunnea+ B.pumilus* | 5.8±0.6 | 132±0.4 | 29.2±1.1 | 610±2.1 |
| UP-3 | Control | 5.3±0.2 | 60±0.4 | 14.3±2.1 | 340±1.8 |
| | *P.hypobrunnea* | 6.2±0.2 | 69±0.8 | 17.7±1.1 | 489±1.2 |
| | *B.pumilus* | 7.1±0.5 | 82±0.3 | 20.4±1.0 | 520±3.7 |
| | *P.hypobrunnea+ B.pumilus* | 9.3±0.3 | 137±0.5 | 29.2±2.3 | 556±2.9 |
| S-449 | Control | 4.5±0.3 | 84±0.5 | 18.3±1.7 | 410±2.9 |
| | *P.hypobrunnea* | 5.6±0.7 | 97±0.8 | 26.5±1.8 | 470±2.3 |
| | *B.pumilus* | 6.1±0.4 | 130±0.3 | 32.0±1.4 | 510±5.2 |
| | *P.hypobrunnea+ B.pumilus* | 7.4±0.5 | 139±0.9 | 35.2±2.1 | 560±4.2 |
| BSS-2 | Control | 4.9±1.0 | 64±0.8 | 13.8±1.3 | 450±3.9 |
| | *P.hypobrunnea* | 5.8±1.1 | 68±3.4 | 17.4±3.1 | 490±4.2 |
| | *B.pumilus* | 6.4±0.3 | 82±0.4 | 25.0±1.8 | 560±4.1 |
| | *P.hypobrunnea+ B.pumilus* | 7.1±0.7 | 96±0.6 | 29.6±1.7 | 580±4.4 |

[a] POX activity assayed as Δ A465 $min^{-1}$ 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] β 1,3- GLU activity assayed as µg glucose released by enzyme from 1 g tissue $min^{-1}$

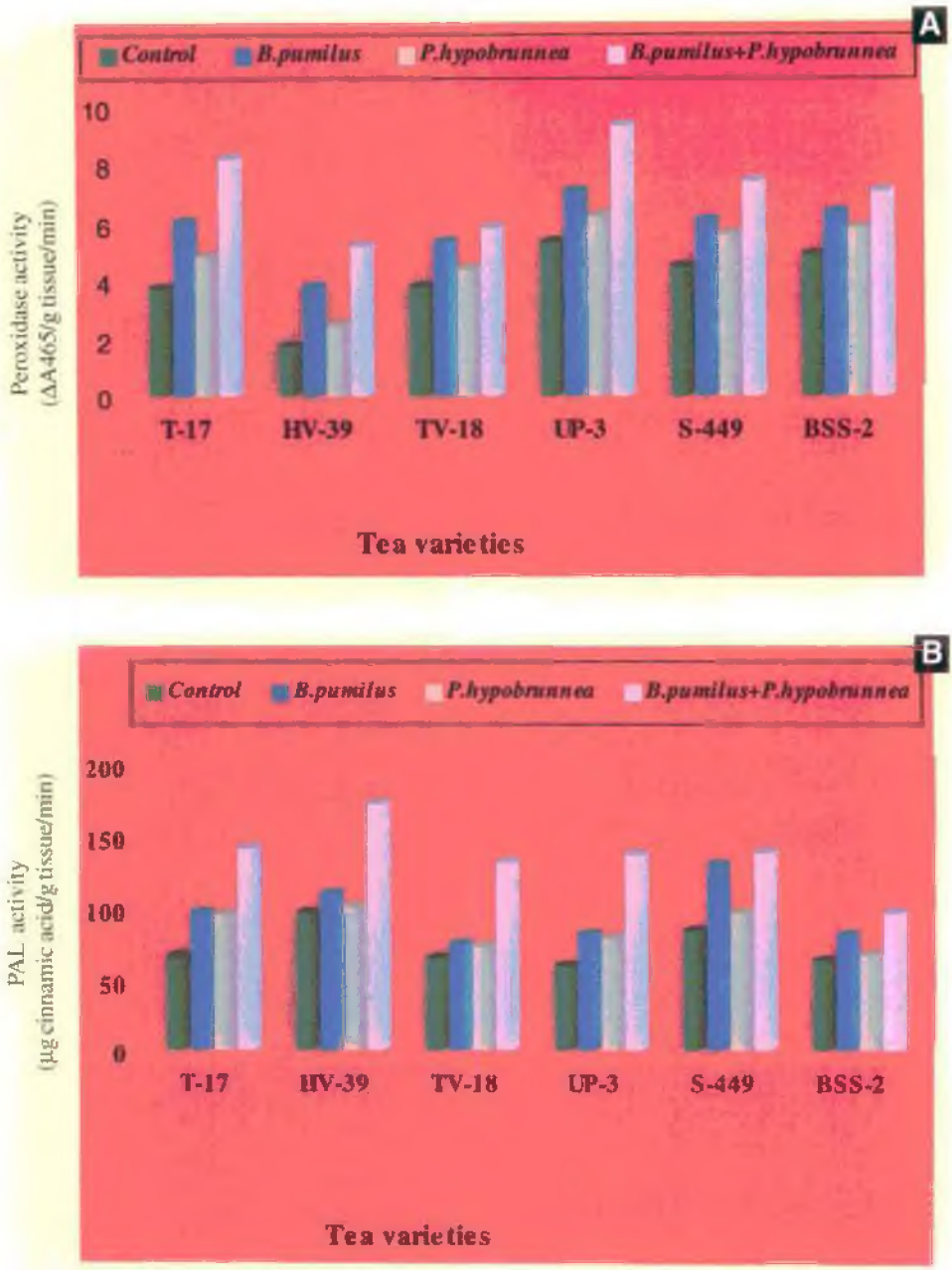


**Fig. 12:** Peroxidase (A) and PAL activity (B) in tea leaves of different varieties following treatment in the rhizosphere.

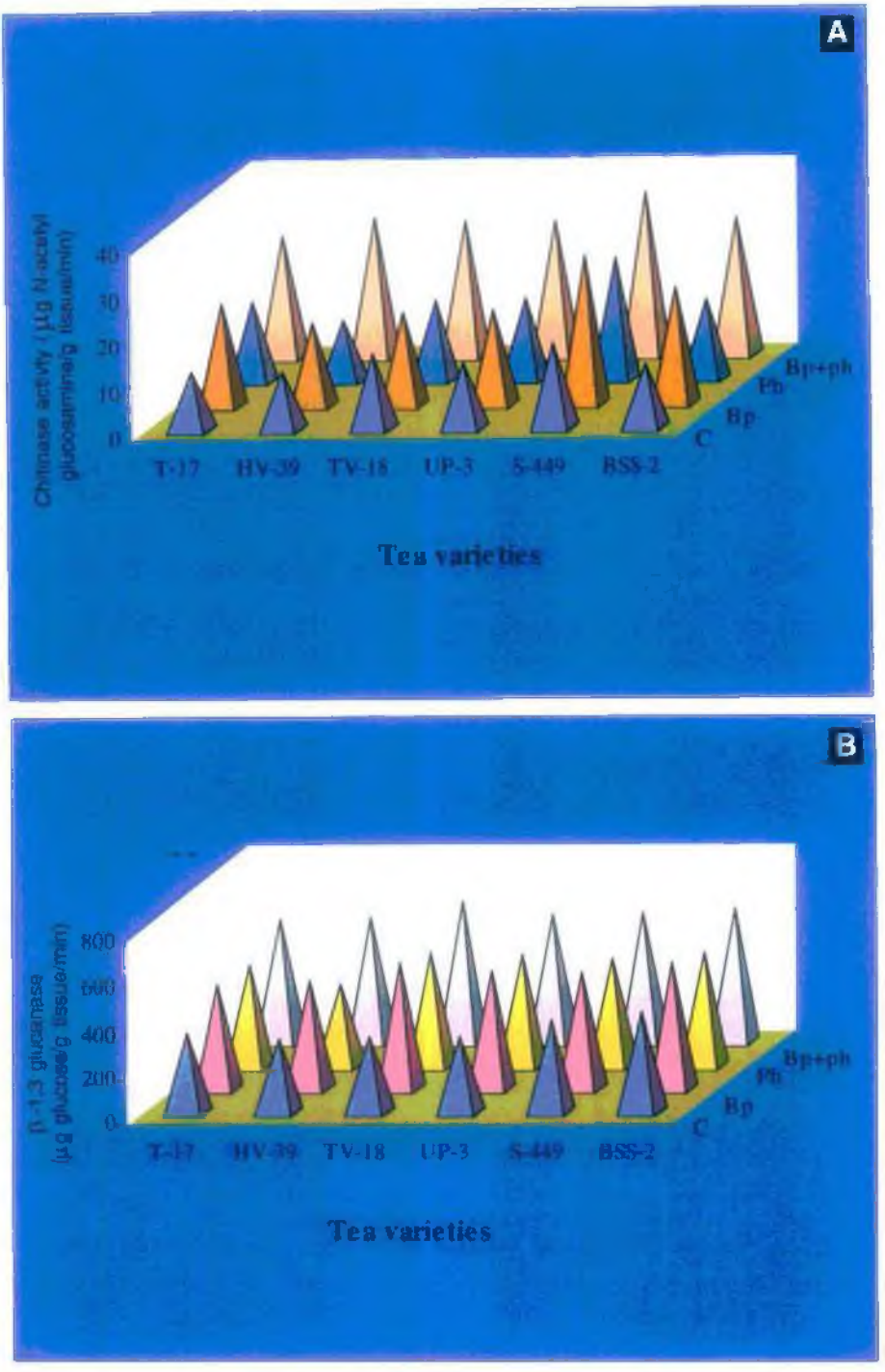


**Fig. 13:** Chitinase (A) and β-1,3 glucanase activity (B) in tea leaves of different varieties following treatment in the rhizosphere. C-Control; Bp-*B. pumilus;* Ph-*P. hypobrunnea.*

### 4.14. Immunodetection of *P. hypobrunnea* and *B. pumilus* in soil

Polyclonal antibodies (PAbs) were raised in rabbits against mycelial antigen of *P. hypobrunnea* as well as from bacterial antigen of *B.pumilus* as described previously. Prior to injection, the quantity of protein was measured and these were also analyzed by SDS-PAGE. Good bands were evident in all cases indicating the yield of bacterial protein and fungal proteins (Plate XIX). Antisera were collected for 6 bleeds and IgG purified from these. For each antigen source normal serum were collected before immunization. These were used for further experimental purposes.

#### 4.14.1. Immunodiffusion

The effectiveness of antigen preparations from bacterial cell and fungal mycelia in raising PAbs was checked by homologous cross reaction following agar gel double diffusion tests. Control sets involving normal sera and antigen of bacteria and fungus were all negatives. Strong precipitin reaction was occurred when PAbs reacted with its own antigen and good titre was obtained in $2^{nd}$ and $5^{th}$ bleedings with maximum reaction occurring in $3^{rd}$ and $4^{th}$ bleed. For subsequent immunoassays, $3^{rd}$ /$4^{th}$ bleed PAbs were used. Only weak precipitin bans were observed in $1^{st}$ and $6^{th}$ bleedings. (Plate XIX)

#### 4.14.2. ELISA

The observed reduction in disease intensity could be either due to reduction of pathogen population in soil or by induction of resistance; or by a combination of both. Pathogen population can be specifically determined in soil by immuno-detection techniques using PAbs raised against the specific pathogens. PAbs were used for detection of pathogens both by ELISA and Dot-blot.

##### 4.14.2.1. For pathogen

Fungal root rot pathogen, *P. hypobrunnea* in the soil was detected by ELISA using specific PAbs of *P. hypobrunnea*. Antigens were prepared from treated and untreated soil and tested by ELISA, using PAbs of specific pathogens. The results showed that population of *P.hypobrunnea* in soil were reduced when PGPR was also applied to soil. It was also shown that when soil was inoculated with bacteria and pathogen lower A405 values were obtained as compared to antigens from soil treated only with *P.hypobrunnea* (Table 38)

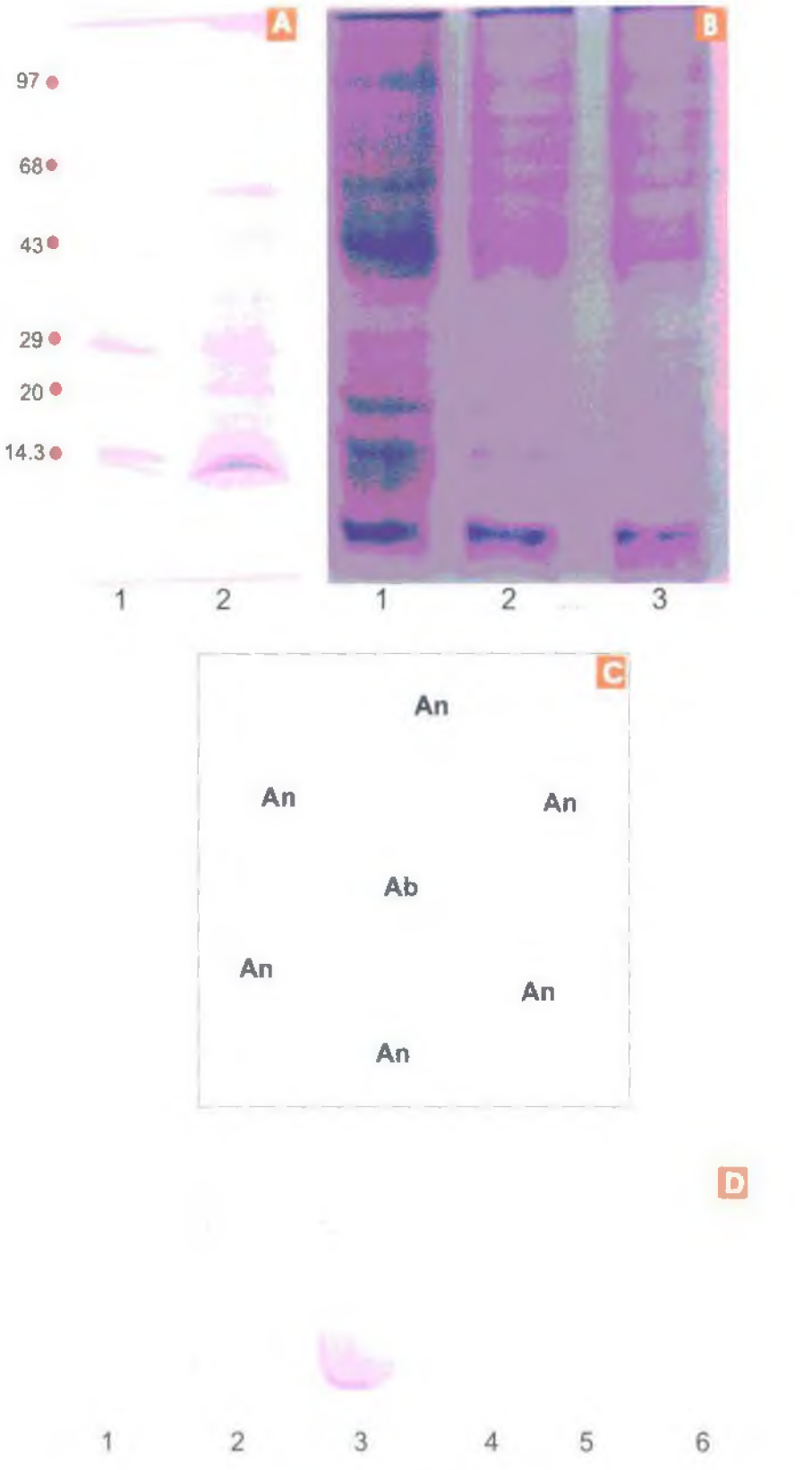


**Plate XIX:** A & D - SDS PAGE analysis of proteins from *B.pumilus* (Lane 1- marker; 2-protein) and *P.hypobrunnea* (Lanes 1,2 and 3); C-Immunodiffusion of antigens from *B.pumilus* reacted with homologus PAb; D-Dot blot assay of antigens from *B.pumilus* reacted with homologus PAb (Lanes 1-6: PAbs from 1-6 bleedings).

**Table 38:** ELISA and Dot-blot values of soil antigens from different treatments after reaction with PAbs of *P.hypobrunnea*

| Soil antigen* | ELISA A 405 values | Dot-blot Colour intensity** |
|---|---|---|
| Uninfested soil | 0.320±0.06 | - |
| Treatment | | |
| *P.hypobrunnea* | 0.986±0.04 | ++ |
| *B.pumilus* | 0.005±0.00 | - |
| *P.hypobrunnea*+ *B.pumilus* | 0.490±0.07 | + |

Average of 3 replicates; PAb dilution: 1: 500; * Sample collected 30 days after inoculation with pathogen; ** Fast red colour intensity: Pinkish red: ++++; Bright pink: +++; Pink: ++; Light pink: +; No colour: -;

**4.14.2.2. For bacteria**

The sustainability of applied bacteria in the rhizosphere was determined by ELISA. Different soil antigens were tested against PAbs of *B. pumilus*. The results revealed that bacteria could successfully survive in the tea rhizosphere even after 3 months of inoculation. Maximum A405 values were obtained from the soils collected soon after application of bacteria. The values reduced to some extent with time, though even after 3 months, these were still detectable at fairly high concentrations. It was also noted that bacteria survived equally well in rhizosphere when applied individually or in combination. (Table 39)

**Table 39**: Detection of survival of *B. pumilus* in soil after direct application

| Antigen source: Rhizosphere Soil from | Absorbance at 405 nm | | | |
|---|---|---|---|---|
| | Uninoculated | Time after bacterial application | | |
| | | Immediate | 15 days | 3 months |
| TV-18 | 0.350±0.06 | 1.031±0.03 | 1.023±0.07 | 1.006±0.06 |
| TV-26 | 0.442±0.08 | 1.074±0.09 | 1.052±00.9 | 1.011±0.02 |
| S-449 | 0.245±0.16 | 1.233±0.06 | 1.198±0.05 | 1. 095±0.05 |
| T-78 | 0.340±0.03 | 1.165±0.08 | 1.112±0.17 | 0.998±0.11 |
| HV-39 | 0.520±0.09 | 1.310±0.07 | 1.123±0.20 | 1.078±0.08 |

Average of 3 replicates; PAb dilution: 1: 500; ± S.E.

### 4.14.3. Dot-blot

Dot immunobloting technique is rapid and sensitive method for detection of survival of bacteria the in soil. The presence of bacteria in the soil was detected by this technique using the antigen from rhizosphere soil inoculated with bacteria and PAb raised against the bacterium. Results revealed that when antigen dots reacted with PAb of *B.pumilus*, colour intensity was highest when soil antigens were prepared soon after bacterial application. With time, the intensity decreased, though not significantly.

Dot-blot was also used for detection of pathogen in the soil. When the antigen prepared from the rhizosphere soil amended with pathogen alone and from soil treated with PGPR followed by pathogen inoculation was probed with PAbs of *P. hypobrunnea*, violet coloured dots were visible in pathogen alone treated samples indicating the survival of the *P. hypobrunnea* in the rhizosphere of the plant whereas the samples from both PGPR and pathogen treated showed very faint colour indicating the inability of pathogen to survive in presence of PGPR.

### 4.15. Studies on talc- based formulation of PGPR

*B.pumilus* as soil drench, foliar spray and seed bacterization promoted growth significantly; it was further decided to prepare formulation of this bacterium in a suitable carrier and determine the efficiency of the formulation. Formulation was prepared using carboxy methyl cellulose with talcum powder as carrier material and tested under green house conditions for their effect on growth promotion of tea. Two g of talc- based formulation was applied per pot in the rhizosphere of 3 tea varieties UP-3, TV-18 and BSS-2, and growth of seedlings were observed and compared with control. The percentage of increase in plant height and no. of leaves were significantly enhanced on application of the talcum formulation in comparison to control. (Table 40; Plate XX)

### 4.15.1. Detection of PGPRs in bioformulation by ELISA

The viability of formulation was tested during storage period of 60 days and 90 days by ELISA. The results showed that bacterial population declined gradually during storage but when applied in the field the bacteria could successfully establish themselves in the rhizosphere. (Table 41)

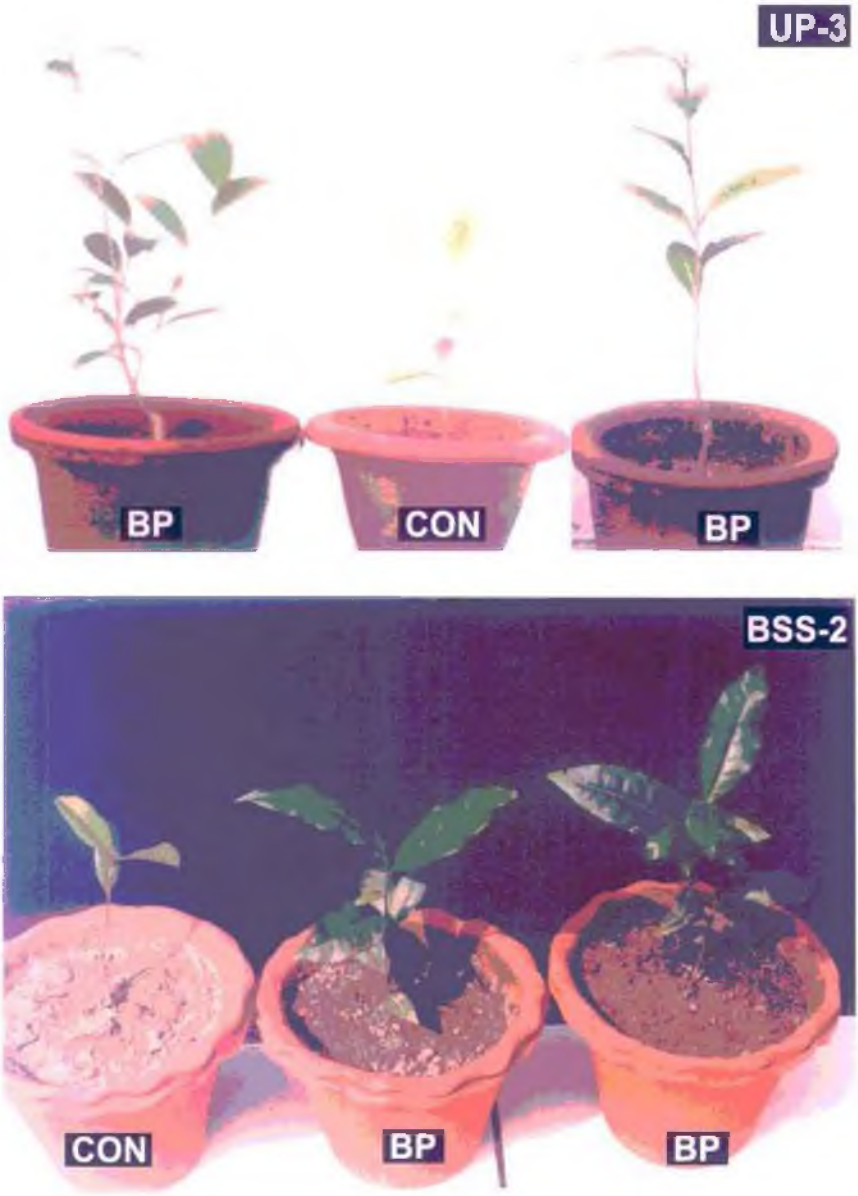


**Plate XX**: Growth promotion in tea seedling by application of PGPR based talcum formulation; BP = *Bacillus pumilus;* CON = Control.

**Table 40**: Growth promotion in Tea plants by Talcum based formulation

| Varieties | Treament | 2 months | | 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±0.00 | 0.7±0.03 | 1.0±0.00 |
| | *B.pumilus* | 3.0±0.06 | 3.0±0.29 | 4.5±0.33 | 5.0±0.58 |
| TV-18 | Control | 0.5±0.00 | 1.0±0.00 | 1.5±0.11 | 2.0±0.02 |
| | *B.pumilus* | 3.0±0.46 | 3.0±1.16 | 6.0±1.33 | 5.0±1.167 |
| BSS-2 | Control | 0.5±0.00 | 1.0±0.00 | 1.5±0.23 | 1.0±0.00 |
| | *B.pumilus* | 3.0±0.11 | 4±0.58 | 4.5±1.7 | 7.0±0.58 |

Average of 10 replicate plants per treatment; ± S.E.

**Table 41**: Immunodetection of *B.pumilus* in bioformulation after application in rhizosphere

| Antigen source | Absorbance at 405 nm* | |
|---|---|---|
| | **2 months** | **3 months** |
| Talcum | 1.435±0.02 | 0.876±0.03 |
| UP-26 | 1.023±0.02 | 1.011±0.04 |
| UP-3 | 1.005±0.08 | 0.941±0.05 |
| CP-1 | 1.098±0.01 | 0.974±0.06 |
| HV-39 | 1.120±0.03 | 1.026±0.09 |

Average of 3 replicates; PAb dilution: 1: 500; ± S.E.; Antigens reacted with PAb of *B.pumilus*

The region around the root, the rhizosphere, supports large and active microbial populations capable of exerting beneficial, neutral, or detrimental effects on plant growth. The importance of rhizosphere microbial populations for maintenance of root health, nutrient uptake, and tolerance of environmental stress is now recognized (Basnayake and Birch, 1995; Benhamou *et al.* 1996)). These beneficial microorganisms can be a significant component of management practices to achieve the attainable yield, which has been defined as crop yield limited only by the natural physical environment of the crop and its innate genetic potential (Benhamou *et al.* 1996). 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).

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). Colonizing 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. The prospect of manipulating crop rhizosphere microbial populations by inoculation of beneficial bacteria to increase plant growth has shown considerable promise in laboratory and greenhouse studies, but responses have been variable in the field (Basnayake and Birch, 1995). This approach has potential environmental benefits of leading to a reduction in the use of agricultural chemicals making it suitable for sustainable management practices.

The present study was undertaken in order select potential microorganisms from tea rhizosphere and make detailed studies so that one or more of such microorganisms could be used as PGPRs. At the onset, 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- *Poria hypobrunnea, Fomes lamaoensis, Sphaerostilbe repens* and *Sclerotium rolfsii* 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 pumilus* TRS4 and *Paenibacillus lentimorbus* TRS5. In earlier studies, Gardener (2004) described recent advances in understanding of the ecology of *Bacillus* and *Paenibacillus spp.* and how different subpopulations of these two genera can promote crop health. The abundance, diversity, and distribution of native populations and inoculants strains in agricultural fields have been characterized using a variety of methods. While native populations of these genera occur abundantly in most agriculture soils, plant tissues are differentially colonized by distinct subpopulations. Screening of rhizosphere microflora for antagonism against pathogenic fungi in order to select suitable biocontrol agents has been reported by a large number of workers. 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, Bacillus 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. In another study, 11 *Bacillus pumilus* isolates were evaluated by Bargabus *et al.* (2004), of which 2 strains were found to be most effective against *Cercospora beticola.* The potential of various isolates of *Bacillus pumilus* has thus been recorded previously also.

In the present study, the two selected PGPRs were tested for their plant growth promoting activity and also biocontrol activity against one of the important root rot diseases of tea caused by *Poria hypobrunnea.* Several *in vitro* tests were conducted prior to the *in vivo* tests. Initially, the optimum conditions of the growth of the test pathogen and selected bacteria were determined. The fungus showed optimum growth in Potato Sucrose medium, at pH 5.0-5.5, with incubation period of 15 days, with fructose and peptone as carbon and nitrogen sources, respectively. Both the bacteria grew best between $35^0C$ 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 order to determine the potential of the two bacterial isolates in plant growth promotion and disease suppression, *in vivo* experiments were next carried out. Experiments were carried out on tea plants which are perennial as well as on annuals i.e., chickpea, mungbean and marigold. 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. Most of the reported works on plant growth promotion by PGPRs is on annuals with relatively few on perennials. Enebak *et al.* (1998) obtained both positive and negative result, using 12 rhizobacterial strains 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. In alder, Ramos *et al.* (2003) reported that *Bacillus licheniformis* increased growth when applied at the seedling stage. On studying changes in microbial communities, they suggested that increases in plant growth could also be attributed to changes in the rhizosphere microbial communities. Plant growth promotion in tea, *Camellia sinensis* (L.) O.Kuntze by *Bacillus megaterium*, *B. pumilus*, *Ochrobactrum anthropi* and *Serratia marcescens* was reported by Chakraborty *et al.* (2004). Besides tea, in the present study, both *B.pumilus* and *P.lentimorbus* could increase growth in chickpea and mungbean, as well as growth and flowering in marigold. Results thus show that plant growth is promoted by the rhizobacteria in both annuals and perennials. 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 m 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. It was reported by Chakraborty *et al.* (2007) that dual application of *Bacillus megaterium* and *Ochrobactrum anthropi* was more effective than either of the single applications in plant growth promotion. 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 nitrogenase activity by various degrees. They reported that the combination of *Rhizobium* sp. with *Azotobacter chroococcum* or *Azospirillum brasilens* registered a marginal but non significant increase over inoculation of *Rhizobium* alone. Most of the previous work in plant growth promoting rhizobacteria had focused on two genera- *Pseudomonas* and *Bacillus* and hence several reports are available regarding the PGPR activity of these two bacteria. Inoculation of sunflower seeds and soil with a strain of *Rhizobium* was observed to cause a significant increase in root dry mass, 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 a study conducted to examine the effect of PGPR inoculation alone and in combination with three levels of mineral nitrogen fertilizer (0-56-60, 56-56-60 and 112-56-60 kg NPK/ha) on cotton (cv.MNH-552), the bacterial inoculum (50 g / kg of seed) significantly increased seed cotton yield (21%), plant height (5%) and microbial population in soil (41 %) over their respective controls while boll weight, GOT and staple length remained statistically unaffected (Anjum *et al.* 2007).

Besides plant growth promotion, the ability of *B.pumilus* was also tested in reducing root rot disease. It could reduce Poria root rot intensity caused by *P.hypobrunnea* effectively. In a previous work, it was shown that *Bacillus megaterium* could effectively control brown root rot of tea caused by *Bacillus megaterium* (Chakraborty *et al.* 2006). *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). *Pseudomonas 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 agent's *P. fluorescens, B. subtilis* and *Trichoderma viride* 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 *Helminthsporium oryzae, Saroclavum 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. *Paenibacillus polymyxa* was reported to protect *Arabidopsis thaliana* against both biotic and abiotic stresses (Timmusk, 2003; Timmusk *et al.* 2005).

Thus, it is clear from the present work as also those of previous workers that several rhizobacteria have the ability of plant growth promotion as well as disease suppression. PGPRs enhance plant growth by direct and indirect means, but the specific mechanisms involved have not all been well-characterized (Castillo *et al.* 2002; Chanway *et al.* 2000). Direct mechanisms of plant growth promotion by PGPRs can be demonstrated in the absence of plant pathogens or other rhizosphere microorganisms, while indirect mechanisms involve the ability of PGPR to reduce the deleterious effects of plant pathogens on crop yield. PGPRs have been reported to directly enhance plant growth by a variety of mechanisms: fixation of atmospheric nitrogen that is transferred to the plant, production of siderophores that chelate iron and make it available to the plant root, solubilization of minerals such as phosphorus, and synthesis of phytohormones (Castillo *et al.* 2002). Direct enhancement of mineral uptake due to increases in specific ion fluxes at the root surface in the presence of PGPR has also been reported (Ait Barka *et al.* 2000; Bais *et al.* 2004). PGPR strains may use one or more of these mechanisms in the rhizosphere. In the present study, among the direct mechanisms tested, both *B.pumilus* and *P.lentimorbus* could solubilize phosphate, produce siderophores and volatiles as well as sufficient amounts of IAA. However, no HCN was produced. 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 isolates of the species *Pseudomonas fluorescens* showed the maximum properties related to plant growth promotion, ACC deaminase activity, phytohormone 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.

In greenhouse experiments 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. jirmis* 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.

Since the two isolated bacteria *B.pumilus* and *P.lentimorbus* 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. 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, Monilinia 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 compound from *Proteus* strain. This was shown to inhibit growth of several *Fusarium* sp. as well as tea root rot pathogens *F. lamaoensis* and *U. zonata.* It was also reported by Kobayashi *et al. (2000)* that inhibition of *R. solani* by *P.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 *el 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.

PGPRs that indirectly enhance plant growth via suppression of phytopathogens do so by a variety of mechanisms. These include the ability to produce siderophores that chelate iron, making it unavailable to pathogens; the ability to synthesize anti-fungal metabolites such as antibiotics, fungal cell wall-lysing enzymes, or hydrogen cyanide, which suppress the growth of fungal pathogens; the ability to successfully compete with pathogens for nutrients or specific niches on the root; and the ability to induce systemic resistance (Bashan and Holguin, 1998; Castillo *et al.*, 2000; Cornelis and Matthijs 2002). Biochemical and molecular approaches are providing new insight into the genetic basis of these traits, the biosynthetic pathways involved, their regulation, and importance for biological control in laboratory and field studies (Bashan and Holguin, 1998; Basnayake and Birch, 1995; Castillo *et al.*, 2000; Cornelis and Matthijs, 2002).

Biopriming plants with some PGPRs can also provide systemic resistance against a broad spectrum of plant pathogens. Diseases of fungal, bacterial, and viral origin, and in some instances even damage caused by insects and nematodes, can be reduced after application of PGPR (Kerry, 2000; Sturz, 2000; Ramamoorthy *et al.*, 2001; Ping and Boland, 2004; Ryu *et al.* 2004). Certain bacteria trigger a phenomenon known as ISR phenotypically similar to systemic acquired resistance (SAR). SAR develops when plants successfully activate their defense mechanism in response to primary infection by a pathogen. ISR is effective against different types of pathogens but differs from SAR in that the inducing PGPR does not because visible symptoms on the host plant (Van Loon *et al.*, 1998). PGPR-elicited ISR was first observed on carnation (*Dianthus caryophillus*) with reduced susceptibility to wilt caused by *Fusarium* sp. (Van Peer *et al.*, 1991) and on cucumber (*Cucumis sativus*) with reduced susceptibility to foliar disease caused by *Colletotrichum orbiculare* (Wei *et al.*, 1991). Manifestation of ISR is dependent on the combination of host plant and bacterial strain (Van Loon *et al.*, 1998; Kilic-Ekici *et al.*2004). Most reports of PGPB-mediated ISR involve free-living rhizobacterial strains, but endophytic bacteria have also been observed to have ISR activity. For example, ISR was triggered by *P. fluorescens* EP1 against red rot caused by *Colletotrichum falcatum* on sugarcane (Viswanathan and Samiyappan, 1999), *Burkholderia phytofirmans* PsJN against *Botrytis cinerea* on grapevine (Ait Barka *et al.* 2000; Ait Barka *et al.* 2002) and *Verticllium dahliae* on tomato (Sharma and Nowak, 1998), *P. denitrificans* 1-15 and *P. putida* 5-48 against *Ceratocystis fagacearum* on oak (Brooks *et al.* 1994), *P. fluorescens* 63-28 against *F. oxysporum* f. sp. *radicis-lycopersici* on tomato (M'Piga et al., 1997) and *Pythium ultimum* and *F. oxysporum* f. sp. *pisi* on pea roots (Benhamou *et al.* 1994), and *Bacillus pumilus* SE34 against *F. oxysporum* f. sp. *pisi* on pea roots (Benhamou *et al.*, 1996) and *F. oxysporum* f. sp. *vasinfectum* on cotton roots (Conn *et al.*, 1997).

In order to determine whether *B.pumilus* elicits ISR in tea plants, several biochemical analyses were done. 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), as well as phenolics increased significantly, especially in presence of the pathogen. In a similar study with 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 □ -1, 3-GLU but not of CHT activity. Ramanathan *et al.* (2003) and Bargabus *et al,* (2004) also obtained systemic resistance elicitation by *B. pumilus* in sugar beet which was marked by increase in CHT, 3-1 ,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.*

In a 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. 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.

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. 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.

Since both *B.pumilus* 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 1M 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 log10 cfu $g^{-1}$ respectively for 1year of storage compared to 3.5 log10 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.

Population of *P.hypobrunnea* was also determined in the soil using dot using PAbs raised against *P.hypobrunnea* the causal agent of root-stem rot of tea. It was shown that the population of the pathogen reduced significantly in the bacteria 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. In an earlier study, Mohandas *et al.* (2005) reported immunodetection of *Phytophthora parasitica* var. *nicotianae* in papaya root pretreated with biocontrol agents. In plant treated with biocontrol agents and *Phytophthora* there was a considerable decrease in the *Phytophthora* population as seen by the number of fluorescent colonies as compared to plants treated with *Phytophthora* alone. A maximum reduction in *Phytophthora* population was observed in VAM+ *Trichoderma* (89.6%) treated plants followed by *Trichoderma* (86.2%) and *Pseudomonas* (79.3%) treated plants

The overall results of the present study have shown that two rhizobacteria isolated from tea rhizosphere, *B.pumilus* and *P.lentimorbus* 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. 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 which 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.

In conclusion, it may be stated that the application of PGPRs for control of fungal pathogens in greenhouse systems shows considerable promise (Corbell and Loper, 1995), due in part to the consistent environmental conditions and high incidence of fungal disease in greenhouses. Achieving consistent performance in the field where there is heterogeneity of abiotic and biotic factors and competition with indigenous organisms is more difficult. Knowledge of these factors can aid in determination of optimal concentration, timing and placement of inoculant, and of soil and crop management strategies to enhance survival and proliferation of the inoculant (Basnayake and Birch, 1995; Conn *et al.* 1997). The concept of engineering or managing the rhizosphere to enhance PGPR function by manipulation of the host plant, substrates for PGPR, or through agronomic practices, is gaining increasing attention (Basnayake and Birch, 1995; Chin-A-Woeng *et al.*2001). Development of better formulations to ensure survival and activity in the field and compatibility with chemical and biological seed treatments is another area of focus; approaches include optimization of growth conditions prior to formulation and development of improved carriers and application technology.

1. A review of literature has been presented to focus plant growth promotion and disease control by biological agents.

2. Materials used and methods followed have been presented in Materials and Methods.

3. Microorganisms were isolated from rhizosphere of tea in different regions of Darjeeling and Dooars. Screening of rhizobacterial was carried out against root rot pathogens- *Poria hypobrunnea, Fomes lamaoensis, Sphaerostilbe repens and Sclerotium rolfsii* by dual pairing test.

4. Bacterial and fungal cultures were identified by microscopic observation and biochemical tests.

5. Five bacteria were found antagonistic to test pathogens out of which two were selected for further experimental purposes. These were identified as *Bacillus pumilus* and *Paenibacillus lentimorbus*

6. The plant growth promoting ability of the antagonistic bacterial isolates were evaluated in green house using different bacterization methods such as soil drenching, foliar application in tea and marigold and seed bacterization in chickpea and mungbean. Bacteria were applied either singly or jointly. The growth promotion of different varieties of tea seedling was observed in forms of growth parameters such as increase in shoot length, number of leaves and height. All used tea varieties show growth promotion to single and joint application of bacteria in soil. Foliar spraying with the suspension also led to significantly better growth of the shoot in tea bushes.

7. Green house experiments were conducted to study the effect of *Bacillus pumilus* on Poria root rot caused *Poria hypobrunnea.* Disease development was significantly reduced in PGPR treated plants compared to control plants.

8. The two selected bacteria were characterized *in vitro* for their cultural characteristic as well as for their plant growth promotion activities. Both the bacteria had the property of IAA production, P-solubilization, Siderophores and Volatiles.

9. Active principles were isolated from cell free culture filtrates of the two bacteria which showed antifungal activity in spores germination and radial growth bioassay. Culture filtrates were found heat stable.

10. Bioassay of solvent extracts culture filtrates also showed inhibitory activities in spores germination and radial growth of test pathogens. Diethyl ether fraction was most effectives.

11. Beside plant growth promotion, biochemical changes such as chlorophyll and phenol content and enzyme activities (peroxidase, phenylalanine ammonia lyase, chitinase and β-1, 3 glucanase) were also studied. Enzyme activities as well as phenol accumulation were increased in PGPR treated plants compared to untreated control.

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

13. Polyclonal antibodies (PAbs) were raised against antigens prepared from *Bacillus pumilus* and *Poria hypobrunnea.*

14. Agar gel double diffusion test were performed using crude antibody. Strong precipitin arcs were obtained.

15. The bacterial sustainability in the soil was evaluated by ELISA and Dot- blot.

16. 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. Results revealed that pathogen population had dechned in presence of PGPR.

17. PGPR formulation was developed in talcum powder. The viability of talcum formulation was tested during 60 days and 120 days of storage by ELISA. All treatment with formulation showed enhancement in growth over untreated control.

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