Studies on *Bacillus megaterium* and *Serratia marcescens* as plant growth promoters and biocontrol agents

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by

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DECLARATION

I declare that the thesis entitled "Studies on *Bacillus megaterium* and *Serratia marcescens* as plant growth promoters and biocontrol agents" has been prepared by me under the guidance of Professor Usha Chakraborty and Professor Bishwanath Chakraborty, Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal. No part of this thesis has formed the basis for the award of any degree or fellowship previously.

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CERTIFICATE

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ABSTRACT

The rhizosphere, a zone very rich in nutrients, supports large microbial populations, which exert beneficial, neutral or detrimental effects on plant growth. Plant growth promoting rhizobacteria include those bacteria, which, on inoculation into the soil, colonize the roots of plants and enhance plant growth. With more and more emphasis on organic farming, efforts are on to isolate and identify beneficial microbes, and hence, plant growth promoting rhizobacteria (PGPR) are finding increasing applications today as biofertilizers and bioprotectants.

One of the major plantation crops of India is tea (*Camellia sinensis*), the leaves of which are used in the production of the world's most common hot beverage. The use of chemicals as fertilizers, insecticides and fungicides in tea cultivation has posed serious problems in recent years. Undoubtedly, excess use of chemical fertilizers and fungicides has resulted in several problems including persistence of chemicals in plant products and adverse impact on soil environment which in turn leads to loss in productivity. Hence, there is a pressing need in tea industry for utilizing either biological products completely or reducing the use of chemicals by supplementing with biological products in integrated management practices.

Considering the importance of using such beneficial microbes as plant growth promoters/ biocontrol agents, the study was undertaken with the title "Studies on *Bacillus megaterium* and *Serratia marcescens* as plant growth promoters and biocontrol agents" giving special emphasis on growth promoting traits, potentiality as biocontrol agents and efficacy of bioformulations of two selected PGPR strains- *Bacillus megaterium* (TRS 7) and *Serratia marcescens* (TRS 1), which were originally isolated from the rhizosphere of tea bushes of Nagrakata and Hansqua tea estates.

The main objectives of the present study were to assess the growth promotion efficacy of *Bacillus megaterium* and *Serratia marcescens* in tea in the nursery and field; to determine biocontrol potential of the selected bacterial isolates against sclerotial blight of tea and biochemical changes associated with plant growth promotion and biocontrol; to develop bioformulations of the bacteria and determine the sustainability of applied bacteria in the rhizosphere.

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

Standard methods were used for experimental purpose in order to achieve the above objectives. Different varieties of tea plant, which were maintained in the Germ Plasm bank at Department of Botany, North Bengal University, were used for the experimental purposes.

The two selected bacteria were morphologically, biochemically characterized and finally identified as *Bacillus megaterium* (TRS 7) and *Serratia marcescens* (TRS 1).

The BLAST query of 16S r DNA sequence of the isolates against GenBank database confirmed their identity. The sequences were deposited in NCBI, GenBank database under the accession Nos. JX 312687.1 and JN 020963.1 for *B.megaterium* and *S. marcescens* respectively. The identity of the isolates of *B. megaterium* (JX312687) and *S. marcescens* (JN 020963) were further confirmed by DGGE. Optimal growth conditions of the bacteria were initially determined. Both the bacteria were able to produce IAA, volatiles, siderophores and solubilised phosphates *in vitro* but did not produce HCN and *B. megaterium* was non- chitinase producing strain. *B. megaterium* was resistant to 450µg/ml concentration of rifampicin and ampicillin (A^2, A^{10} and A^{25} mcg/disc) where as *S. marcescens* was found to be resistant to Mt^5, C^{10} and C^{25}.

Both the bacteria showed antagonism to *Sclerotium rolfsii, Rhizoctonia solani, Fusarium graminearum and F. oxysporum*. Effect of culture filtrate of *B. megaterium* and *S. marcescens* in inhibiting one of the root pathogen- *S. rolfsii* in terms of inhibition of germination of sclerotia was noted. Sclerotial germination of *S. rolfsii* with cell free culture filtrates of *B. megaterium* and *S. marcescens* showed about 90-95% inhibition in comparison to control.

Both *B.megaterium* and *S. marcescens* significantly promoted the growth of tea plants in field, pot and tea estates. Percentage increase in height and number of leaves of young saplings of five varieties of tea (TV-18, TV-23, TV-25, TV-26 and T-17) was greater when two bacteria were applied together in comparison to individual application. Similar results were obtained in potted and field grown plants. Bacterial suspensions of *B. megaterium* and *S. marcescens* were also applied to the rhizosphere of 8-yrs old pruned plants of T-17/154 at a regular interval of 15 days in the experimental field of commercial tea garden (Hansqua Tea Garden). A significant increase in no of leaves was observed. Soil P content decreased following application of both bacteria, while root and leaf phosphate contents showed a significant increase both in treated tea plants in potted as well as field conditions in comparison to control. Acid and alkaline phosphatase activities were enhanced following application of

bacteria. Both the bacteria were found to tolerate concentration of insecticides/fungicides at levels much higher than the ones used in the field. Combined application of *B. megaterium* and low dose of acephate (1:2000-0.5mg/ml), commonly used in tea gardens was tried in order to reduce insect attack keeping the growth promotion achieved by *B. megaterium*. Leaves were healthier with a significant reduction in insect attack (% of insect attack reduced to 16-22% after foliar spray of acephate). Significant increase in leaf, branch numbers and reduction in % of insect attack (14-21% from 47-56% after foliar spray of acephate with aqueous suspensions of *S. marcescens*.

In addition to growth promotion, higher activities of CHT, PO, PAL, β-1,3 GLU and accumulation of higher phenolic compound as well as chlorophylls in *S.marcescens* and *B. megaterium* treated tea varieties were observed. No significant changes in either protein content or protein profile was observed by treatments with the two bacteria. In HPLC analysis, new isoforms and increase of isomers were observed in *B. megaterium* treated plants where as few were lost or there was suppression of few isomers by the treatment of *S. marcescens*.

B. megaterium reduced sclerotial blight more significantly in comparison to *S. marcscens*. There was a significant increase in peroxidase and chitinase activities of tea leaves in pathogen inoculated as well as in *B. megaterium*, *S. marcescens* or *B. megaterium+S. marcescens* treated plants challenge inoculated with *S. rolfsii*. In isozyme analysis of peroxidase maximum intensity of bands was noticed in the TV-26 variety after the treatment by *S. rolfsii*. Activities of PAL increased significantly about two times in *B. megaterium+S. marcescens* inoculated plants challenged with pathogen *S. rolfsii*. Maximum phenol content was obtained in the presence of both *B. megaterium+S. marcescens* and *S. rolfsii*. Similar trend was also observed in O-phenol content.

B. megaterium could survive in the range of $6.1\times\log_{10}$ cfu/ml in bioformulations of saw dust, rice husk and $6.98\times\log_{10}$ cfu/ml in tea waste respectively where as *S. marcescens* could survive in the range of 7.12, 7.11 and $7.2\times\log_{10}$ cfu/ml in saw dust, rice husk and tea waste formulations respectively up to nine months. The single and joint effects of *S. marcescens* and *B. megaterium* in three bioformulations were also tested on growth promotion of potted tea plants. All treatments with formulation showed enhancement in growth over untreated controls. Significant increase in activities of PO and CHT was observed. β-1,3-glucanase and phenyl alanine ammonia

lyase activities and phenolic contents increased due to single and joint application of bacteria as formulations of saw dust, rice husk and tea waste.

The bacterial sustainability in the soil was evaluated by ELISA, Western blot and Dot blot using their PAbs. *B. megaterium* and *S. marcescens* could successfully survive and multiply in tea rhizosphere even after three months of application. Populations of *S. rolfsii* were determined in the soil using PAbs raised against *S.rolfsii*. It was observed that the population of the pathogen reduced significantly in *B. megaterium* and *S. marcescens* treated soil, as detected by ELISA and Dot blot. For immunolocalization of chitinase in treated as well as control tea leaves, tea leaves were treated with chitinase antibody and FITC labeled Con A. Strong apple green fluorescence were evident in treated leaves. Expression of chitinase was also observed in appearance of bands in western blotting and in Dot blot.

Results of the present study indicate that two rhizobacteria isolated from tea rhizosphere, *B. megaterium* (TRS-7) and *S. marcescens* (TRS-1) have shown good potential as a plant growth promoter and biocontrol agent. *B. megaterium* (TRS-7) showed better response as a plant growth promoter and biocontrol agent in comparison to *S marcescens* (TRS 1).

PREFACE

Tea plantations of North Bengal region, including Darjeeling hills have been facing problems due to the regular application of fungicides, insecticides and chemical fertilizers. To replace the use of chemicals, an easy and eco-friendly method may be the utilization of biological products completely or reducing the use of chemicals by supplementing with biological products in integrated management practices. Considering the importance of PGPR- beneficial microbes as plant growth promoters/ biocontrol agents, the present study has been undertaken to determine whether rhizobacteria, isolated from tea rhizosphere, could be used for increasing crop productivity and determine their mechanism of action.

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

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INTRODUCTION

Tea (*Camellia sinensis* (L.) O. Kuntze) is one of the important plantation crops of India. The tea plant is an evergreen of the *Camellia* family that is native to China, Tibet and Northern India. There are two main varieties of the tea plant. The small leaf variety, known as *Camellia sinensis*, thrives in the cool, high mountain regions of central China and Japan. The broad leaf variety, known as *Camellia assamica*, grows best in the moist, tropical climates found in Northeast India and the Szechuan and Yunnan provinces of China. The plant produces dark green, shiny leaves and small, white blossoms. Tea plant is a perennial and also grows in several geographical regions of India, including the plains of Assam, North Bengal as well as hills of Darjeeling (Fig.1). In case of tea, the demand of organic tea in the world market is very high and is increasing. Since tea is grown in tropical agro climates, pests, weeds and disease causing organisms are capable of causing serious damage to the crops, for which extensive use of chemicals has been implemented in the past. Pesticide residues and incorporation of byproducts of pesticides into the soil humus have caused serious problems of pollution and loss of land fertility (Bezbaruah 1994). Excessive use of chemicals and the presence of residue in the leaves is a major concern for consumers. The use of beneficial micro-organisms as biofertilizers and biocontrol agents has become more important in recent years not only to improve plant growth and to manage plant diseases but also to avoid environmental pollution. Therefore, there is an urgent need to select natural plant protectors. A safe and easy method to achieve this is to use the naturally occurring rhizobacterial strains which can effectively inhibit growth of pathogens and thereby protect the cultivated plants. Fungal pathogen control through rhizobacterial strains have been reported for several crops. The rhizosphere of tea bushes are expected to be rich source of microorganisms, some of which could be exploited for use as biofertilizer/biocontrol agents capable of improving the growth of the plant, either by suppression of pathogenic soil fungi or by growth promotion through other mechanisms.

Rhizosphere is the habitat in which several biologically important processes and interactions take place. It is the zone of intense activity of various groups of microorganisms. The rhizosphere is the 1mm zone of soil surrounding a plant root where the biology and chemistry of the soil are influenced by the root.

Figure 1: Tea Plantation

The region around the root is relatively rich in nutrients, due to the loss of as much as 40% of plant photosynthates from the roots as root exudates (Lynch and Whipps 1991). Root exudates include amino acids, organic acids, carbohydrates, sugars, vitamins, mucilage and proteins. The exudates act as messengers that stimulate biological and physical interactions between roots and soil organisms. Consequently, the rhizosphere supports large and active microbial populations capable of exerting beneficial, neutral, or detrimental effects on plant growth. The microorganisms grow in close association with the plant and are referred to as rhizobacteria (Bashan 1998). They live at the expense of the plant, feeding on the nutrients released from the plant roots. The beneficial groups of microbes with the capacity to enhance plant growth by increasing seed emergence, plant weight and crop yields are designated as the plant growth promoting rhizobacteria (PGPR). According to their relationship with the plants, PGPR can be divided into two groups: symbiotic bacteria and free-living rhizobacteria (Khan 2005). PGPR can also be divided into two groups according to their residing sites: iPGPR (i.e., symbiotic bacteria), which live inside the plant cells, produce nodules, and are localized inside the specialized structures; and ePGPR (i.e., free-living rhizobacteria), which live outside the plant cells and do not produce nodules, but still promote plant growth (Gray and Smith 2005). The best-known iPGPR are Rhizobia, which produce nodules in leguminous plants (Hayat *et al*. 2010). They facilitate plant growth and development both directly and indirectly (Glick 1995). PGPR are also termed as plant health promoting rhizobacteria (PHPR) or nodule promoting rhizobacteria (NPR) and are associated with the rhizosphere which is an important soil ecological environment for plant–microbe interactions (Burr and Caesar 1984). Currently several genera are designated as PGPR and are known to be associated with several crop plants, viz. *Azotobacter, Azoarcus, Bacillus, Burkholderia, Enterobacter, Erwinia, Gluconacetobacter, Klebsiella, Pseudomonas, Serratia, Rhizobium* etc. The mechanisms by which PGPR can influence plant growth may differ from species to species as well as from strain to strain. Several determinants for mechanisms of growth promotion include bacterial synthesis of plant hormones like Indole-3 acetic acid (IAA), cytokinin, gibberellins, breakdown of plant induced ethylene by bacterial production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase and increase mineral and N- availability in the soil (Kloepper 1992; Glick 1995). Growth promotion mechanism may be direct i.e. production of growth hormones, phosphate

solubilization, nitrogen fixation or indirect viz, suppression of deleterious microorganisms by siderophore production or secretion of antifungal metabolites (Kloepper 1993). Co-inoculation with two or more microorganisms have also shown to yield similar or better results in field studies [Chatterjee *et al.* (2012) reported the effect of co-inoculation of three bacterial isolates- *Bacillus firmus* KUCr1, *Cellulosimicrobium cellulans* KUCr3 and *Pseudomonas aeruginosa* KUCd1 on selected growth parameters of amaranth plants. KUCr1 and KUCr3 were reported to be P-solubilizers and indole acetic acid (IAA) producers, and KUCd1 was a siderophore producer. Co-inoculation of the three isolates gave the best results for overall growth of amaranth plants followed by co-inoculation with KUCr1 and KUCd1, then KUCr1 alone. Among the test isolates, KUCr1 and KUCd1 were found to be better rhizosphere colonizers when co-inoculated. KUCr1 and KUCr3 when co-inoculated produced more IAA in liquid medium. Co-inoculation gave insignificant variation in P-solubilization, but siderophore production by KUCd1 was greatly enhanced when inoculated with other isolates in culture conditions. Co-inoculation of microbes promoted plant growth better than individual isolates. Turan *et al.* (2012) conducted a green house experiments to determine the effects of plant growth-promoting rhizobacteria (PGPR) and boron (B) treatments, applied either alone or in combination, on yield, plant growth, leaf total chlorophyll content, stomatal conductance, membrane leakage, and leaf relative water content of wheat (*Triticum aestivum* L. cv. *Bezostiya*) and barley (*Hordeum vulgare* L. cv. *Tokak*) plants. Results showed that alone or combined B (0, 1, 3, 6, 9 kg ha^{-1}) and PGPR (*Bacillus megaterium* M3, *Bacillus subtilis* OSU142, *Azospirillum brasilense* Sp245, and *Raoultella terrigena*) treatments positively affected dry weight and physiological parameters in both species. Dubey *et al.* (2013) isolated a total of eight motile, aerobic, Gram-positive and straight rod-shaped, endospore forming *Bacillus* spp. from the rhizosphere of chickpea plants collected from different agricultural fields. Phylogeny of the isolates was studied by partial sequencing of 16S rDNA and comparative analysis of the sequence data confirmed that the isolates belong to distinct phylogenetic lineage corresponding to *Bacillus*. Phenotyping clusters correlate with ARDRA pattern and showed resemblance to partial 16S rDNA sequencing. *Bacillus* spp. BSK5 and *Bacillus subtilis* BSK17 were the most potent strains for having plant-growth-promoting attributes. These two strains solubilised inorganic phosphate, produced Indole acetic acid, siderophore, Hydrocyanic acid and

secreted extracellular chitinase and β-1,3-glucanase which antagonised and caused mycelial deformities in two phytopathogens- *Macrophomina phaseolina* and *Fusarium oxysporum* in dual culture and by culture filtrate]. Interest in biological control has increased recently by public concerns. PGPR have been used as good biocontrol agents against soil borne pathogens. Disease suppression by antagonistic bacteria depends on their ability to colonize roots and to produce substances inhibitory to pathogens. Potential biocontrol agents produce antibiotics, siderophores that cause disease suppression and increase yield of plants. Well characterized antibiotics with biocontrol properties include 2,4-DAPG, phenazine, pyrrolnitrin, HCN and lipopeptides. Some biocontrol PGPB strains protect plants by activating gene encoding defense enzymes- chitinase, β-1,3 glucanase, peroxidase, phenylalanine- ammonia-lyase and other enzymes, involved in synthesis of phytoalexin (M Piga *et al.* 1997). According to Van Loon *et al.* (1998) non-pathogenic rhizobacteria can induce a systemic resistance (ISR) in plants that is phenotypically similar to pathogen-induced systemic acquired resistance (SAR). SAR develops when plants successfully trigger their defense mechanism in response to primary infection by a pathogen, notably when the latter induces a hypersensitive reaction through which it becomes limited in a local necrotic lesion of brown, desiccated tissue. Bacterial determinants of ISR include lipopolysaccharides, siderophores, and salicylic acid (SA). Whereas some of the rhizobacteria induce resistance through the SA-dependent SAR pathway, others do not and require jasmonic acid and ethylene perception by the plant for ISR to develop. ISR is effective under field conditions and offers a natural mechanism for biological control of plant disease. PGPR mediated induced systemic resistance (ISR) results in alteration of physiological and biological reactions of plant cells and production of pathogenesis related proteins (PR) and phytoalexins. It has been hypothesised that the inducing rhizobacteria in the plant roots produce signal, which spreads systemically within the plant and increases the defensive competence of the distant tissues from the subsequent infection by the pathogens. The efficacy of the biological control agent would largely depend on the type of formulation and delivery technology (Lumsden *et al* 1995). Experimental formulations of *Bacillus* spp that effectively reduced plant disease included peat and chitin (Sid Ahmed *et al* 2003). The advantages of using *Bacillus* as a biological control agent is its property to form spores resistant to unfavourable natural conditions and its tolerance to antimicrobial substances released

by other microbes in the soil. Treatment with *Ochrobactrum anthropi* decreased brown root rot of tea, caused by *Phellinus noxius* (Chakraborty *et al*. 2009). Multifold increase in activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in tea plants was observed on application of *O. anthropi* to soil followed by inoculation with *Phellinus noxius*. Kim *et al*. (2009) and Fatima *et al*. (2009) both mentioned the role of *Paenibacillus polymyxa* GBR-462 and *Azotobacter* sp. WPR-51 as biocontrol agents in chili pepper and wheat in controlling diseases caused by *Phytopthora capsici* and *Rhizoctonia solani* respectively. Further, Chakraborty *et al*. (2010) also reported that *Serratia marcescens* (TRS 1) showed antagonism to a number of fungal pathogens *in vitro*. It reduced brown root rot of tea caused by *Fomes lamaoensis*. Urrea *et al.* (2011) isolated 120 strains of antagonistic bacteria from the rhizosphere of *Physalis peruviana* and five were selected for their high inhibition of *F. oxysporum* growth, conidia production under *in vitro* conditions. These strains inhibited growth by 41–58% and reduced three- five fold conidia production. In the *in vivo* assays, all the tested isolates significantly reduced fungal pathogenicity in terms of virulence. All isolates were identified as belonging to the genus *Pseudomonas* except for A-19 (*Bacillus* sp.). Simonetti *et al.* (2012) isolated strains of *Pseudomonas fluorescens* and *Bacillus amyloliquefaciens* from soybean rhizosphere, inhibited mycelial growth of *Botrytis cinerea* and *Sclerotinia sclerotiorum in vitro*. Nithya *et al.* (2013) reported that nine bacterial strains (*Bacillus pumilus* SB 21, *Bacillus megaterium* HiB 9, *Bacillus subtilis* BCB 19, *Pseudomonas plecoglossicida* SRI 156, *Brevibacterium antiquum* SRI 158, *B. pumilus* INR 7, *P. fluorescens* UOM SAR 80, *P. fluorescens* UOM SAR 14 and *B. pumilus* SE 34) were tested to induce systemic resistance in sorghum cultivars 296B and Bulk Y against the highly pathogenic grain mould pathogens- *Curvularia lunata* and *Fusarium proliferatum*, respectively. The bacterial isolates were effective in inducing resistance in sorghum. Among the strains tested, SRI 158 was found highly effective in reducing grain mould severity in both the genotypes.

Hence, the time has now come to look for means of reducing chemical outputs in tea as well as other crops. Repeated fungicide applications cause soil contamination, fungicide resistance and harmful effects to non-target organisms. In order to adopt eco-friendly and inexpensive alternate disease management strategies, increasing use of plant growth promoting microbes as biofertilizers and biocontrol

agents provide alternatives to the use of chemicals for disease control. Commercialization of PGPR for bioformulation products will also be effective for the farmers. Considering the importance of using such beneficial microbes as plant growth promoters/ biocontrol agents, the present study was undertaken with the following objectives giving special emphasis on growth promoting traits, potentiality as biocontrol agents and efficacy of bioformulations of two selected PGPR strains- *Bacillus megaterium* (TRS 7) and *Serratia marcescens* (TRS 1), which were originally isolated from the rhizosphere of tea bushes of Nagrakata and Hansqua tea estates (Fig.2).

Objectives of the study:-

1. Assessment of growth promotion efficacy of *Bacillus megaterium* and *Serratia marcescens* in tea in the nursery and field.
2. Determination of biocontrol potential of the selected bacterial isolates against sclerotial blight of tea.
3. Elucidation of bacterial determinants of plant growth promotion and biocontrol.
4. Determination of biochemical changes associated with plant growth promotion and biocontrol.
5. Development of bioformulations of the bacteria.
6. Determination of sustainability of applied bacteria in the rhizosphere.

Figure 2: GIS location of Hansqua Tea Estate (A) and Nagrakata Tea Estate (B).

LITERATURE REVIEW

The thin layer of soil (about 1 to 2mm thick) surrounding crop roots and the volume of soil occupied by roots is known as the rhizosphere. The sheer extent of crop roots in soil dictates that a significant portion of soil is actually within the rhizosphere (about 5 to 40% of soil in the rooting zone depending upon crop root architecture). This zone is where the majority of soil microorganism (bacteria and fungi) reside. Plant growth results from interaction of roots and shoots with the environment. The environment for roots is the soil or planting medium which provide structural support as well as water and nutrients to the plant. Roots also support the growth and functions of a complex of microorganisms that can have a profound effect on the growth and survival of plants. These microorganisms constitute rhizosphere microflora and can be categorized as deleterious, beneficial, or neutral with respect to root/plant health. Beneficial interactions between roots and microbes are common in rhizosphere and can be enhanced. Increased plant growth and crop yield can be obtained upon inoculating seeds or roots with certain specific root-colonizing bacteria- plant growth promoting rhizobacteria.The mechanisms by which PGPR increase crop performance is not well understood. Growth promotion mechanisms may be direct, i.e. production of growth hormones, phosphate solubilization, nitrogen fixation or indirect, such as suppression of deleterious microorganisms by siderophore production or secretion of antifungal metabolites (Kloepper 1993). 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). Inoculant development has been most successful to deliver biological control agents of plant disease, ie. organisms capable of killing other organisms pathogenic or disease causing to crops. Bacteria in the genera *Bacillus*, *Pseudomonas*, *Burkholderia* and *Agrobacterium* are the biological control agents predominantly studied and increasingly marketed. Biofertilizers are also available for increasing crop nutrient uptake of nitrogen from nitrogen fixing bacteria associated with roots (*Azospirillium*), iron uptake from siderophore producing bacteria (*Pseudomonas*), sulfur uptake from sulfur-oxidizing bacteria (*Thiobacillus*), and phosphorus uptake from phosphate-mineral solubilizing bacteria (*Bacillus*,

Pseudomonas). Nitrogen fixing biofertilizers provide only a modest increase in crop nitrogen uptake (at best an increase of 20 lbs N per acre). The popular inoculants presently commercialized for increasing phosphorus uptake through phosphorus solubilization (*Penicillium* and *Aspergillus*) and phosphorus transfer directly to roots (mycorrhizae) are not bacteria but fungi. 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 plant roots for uptake of water and nutrients. These PGPR are referred to as biostimulants and the phytohormones they produce include indole-acetic acid, cytokinins, gibberellins and inhibitors of ethylene production.

Several earlier reviews have focused on the role of PGPR in plant growth promotion (Kloepper, 1993; Zhang *et al*. 1996; Smith *et al*. 1997; Enebak *et al*. 1998; Whipps, 2001; Bai, 2002; Bhattarai and Prasad, 2003; Ryu *et al*. 2003; Khalid *et al*. 2004; Kloepper *et al*. 2005; Lee *et al*. 2005).The review presented below has been compiled mainly on three important aspects i.e. 1.Plant growth promotion; 2. Disease reduction by the use of plant growth promoting rhizobacteria and 3. Development of bioformulations of beneficial bacteria.

Plant growth promotion

Plant growth promoting rhizobacteria (PGPR) are a common group of bacteria that can actively colonize plant roots and increase plant growth (Kloepper and Schroth 1978). These PGPR can prevent the deleterious effects of phytopathogenic organisms on the environment. The mechanisms by which PGPR can influence plant growth may differ from species to species as well as from strain to strain. Growth promotion mechanism may be direct i.e. production of growth hormones, phosphate solubilization, nitrogen fixation or indirect, viz, suppression of deleterious microorganisms by siderophore production or secretion of antifungal metabolites (Kloepper 1993). However, in field conditions, the above traits may not be sufficient to account for the observed growth promotion. The biochemical or physiological changes induced in the host that are activated by the PGPR also lead to plant growth promotion and develop resistance capacity in the host against pathogens.

Krebs *et al*. (1998) isolated several *Bacillus* strains belonging to the *B. subtilis*/*amyloliquefaciens* group from plant-pathogen-infested soil which possessed plant-growth-promoting activity**.** Three out of the four strains investigated were

identified as *B. amyloliquefaciens* and were able to degrade extracellular phytase (myo-inositol hexakisphosphate). Enebak *et al*. (1998) conducted an experiment on effects of PGPR on loblolly and slash pine seedling. Loblolly and slash pine seeds were inoculated at soaking with 1 of 2 different strains of PGPR in the greenhouse, time to germination and seedling densities were determined at 21 days, and seedling biomass was measured at 12 week after sowing. All bacterial strains significantly increased the speed of seedling emergence over non- treated pine seed. By 12th week, however, no differences in stand densities were observed between bacteria-treated and no treated seed for either pine species. Post emergence damping -off was reduced in loblolly pine when seed was treated with 3 of the 12 bacterial strains; however, post emergence damping –off on slash pine seedling was not affected by rhizobacteria. Treatment with either rhizobacteria had a significant positive and negative effect on seedling growth and biomass, which depended on tree species. Loblolly pine shoot and root lengths, as well the above-and belowground biomass were significantly reduced when seeds were treated with strains BSI and BS2. In contrast, loblolly pine shoot and root lengths, as well the above-and belowground biomass were significantly reduced when seeds were treated with strains BS1 BS2. In contrast, loblolly pine seeds treated with strains BS3, PM2, and INR7 significantly retraced shoot length compared with no treated seeds, and biomass were also reduced when treated with strains BS1and BS2. Unlike loblolly pine, no bacterial strain increased slash pine root length or biomass. This study suggests that the effects of rhizobacteria inoculation on seedling emergence and plant growth are independent and that the effects are species specific.

Phosphorus is an essential nutrient for plants and an important component in cell metabolism. The ability of PGPR to convert insoluble phosphorus (P) to an accessible form is an important trait for increasing plant yields. Phosphorus solubilizing activity was determined by the ability of microbes to release metabolites such as organic acids, which through their hydroxyl and carboxyl groups chelate the cation bound to phosphate, the latter being converted to soluble forms (Sagoe *et al.* 1998). Davison (1998) reported that fluorescent pseudomonads enhanced plant growth by improving soil nutrient status, secreting plant growth regulators, and suppressing soil-borne pathogens. In addition to the phosphate-solubilizing capability of many *Pseudomonas* strains, they could promote plant growth by mechanisms such

as the production of plant growth regulators and vitamins, enhancement of plant nutrient uptake and suppression of pathogenic or deleterious organisms Moreover, the tripartite association composed of legume plant, rhizobia and *Pseudomonas spp*. was reported to increase root and shoot weight, plant vigour, nitrogen (N) fixation and grain yield in various legumes (Dashti *et al*. 1998; Sindhu *et al*. 1999). The phosphate solubilising bacteria (PSB) dissolved the soil P through production of low molecular weight organic acids mainly gluconic and keto gluconic acids (Deubel *et al.* 2000), in addition to lowering the pH of rhizosphere. The pH of rhizosphere was lowered through biotical production of proton / bicarbonate release (anion / cation balance) and gaseous (O_2/CO_2) exchanges. *Pseudomonas stutzeri* (Vazquez *et al.* 2000) and *P. putida* (Kumar and Singh 2001) were also reported as phosphate solubilizers. Strains from bacterial genera *Pseudomonas*, *Bacillus*, *Rhizobium* and *Enterobacter* along with *Penicillium* and *Aspergillus* fungi (Whitelaw 2000) and among the soil bacterial communities, ectorhizospheric strains from *Pseudomonas* and *Bacilli*, and endosymbiotic rhizobia were categorised as effective phosphate solubilizers. Phosphorus solubilization ability of PSB had direct correlation with pH of the medium. Ectorhizospheric strains from pseudomonads and bacilli, and endosymbiotic rhizobia were also described as effective phosphate solubilizers (Igual *et al*. 2001). Growth and phosphorus content in two alpine *Carex* species were increased by inoculation with *Pseudomonas fortinii* (Bartholdy *et al.* 2001).

Probanza *et al*. (2001) reported the effect of co-inoculation with *Pisolithus tinctorius* and a PGPR belonging to the genus *Bacillus (Bacillus licheniformis* CECT 5106 and *Bacillus pumilus* CECT 5105) in enhancing growth of *Pinus pinea* plants and the changes that occurred in rhizosphere microbial communities. Both bacterial strains of *Bacillus* promoted the growth of *Pinus pinea* seedlings, but, according to the authors, this biological effect does not imply a synergic effect with mycorrhizal infection. However, the positive response to mycorrhiza in a longer-term experiment it could be expected. The introduction of both inocula causes an alteration in the microbial rhizosphere composition, despite the low levels of inocula that were found at the end of the assay.

According to Bai (2002) *Serratia proteamaculans* 1-102 (1-102) promotes soybean-bradyrhizobia nodulation and growth, but the mechanism is unknown. After adding isoflavonoid inducers to 1-102 culture, an active peak with a retention time of

about 105 min in the HPLC fractionation was isolated using a bioassay based on the stimulation of soybean seed germination. The plant growth-promoting activity of this material was compared with 1-102 culture (cells) and supernatant under greenhouse conditions. The results suggest that this inducible activator might be a lipo-chitooligosaccharide (LCO) analogue. LCOs act as rhizobia-to-legume signals stimulating root nodule formation. The activator could provide additional 'signal', increasing in the signal quality (the signal-to-noise ratio, SNR) of the plant–rhizobia signal exchange process. Several Bacillus strains belonging to the *B. subtilis/amyloliquefaciens* group isolated from plant-pathogen-infested soil possessed plant-growth-promoting activity. Three out of the four strains investigated were identified as *B. amyloliquefaciens* and were able to degrade extracellular phytate (myo-inositol hexakisphosphate). The highest extracellular phytase activity was detected in strain FZB45, and diluted culture filtrates of this strain stimulated growth of maize seedlings under phosphate limitation in the presence of phytate. The amino acid sequence deduced from the phytase phyA gene cloned from FZB45 displayed a high degree of similarity to known Bacillus phytases. Weak similarity between FZB45 phytase and *B. subtilis* alkaline phosphatase IV pointed to a possible common origin of these two enzymes. The recombinant protein expressed by *B. subtilis* MU331 displayed 3(1)-phytase activity yielding D/L-Ins(1,2,4,5,6)P5 as the first product of phytate hydrolysis. A phytase-negative mutant strain, FZB45/M2, whose phyA gene was disrupted, was generated by replacing the entire wild-type gene on the chromosome of FZB45 with a km::phyA fragment and culture filtrates obtained from FZB45/M2 did not stimulate plant growth. In addition, the growth of maize seedlings was promoted in the presence of purified phytase and the absence of culture filtrate. These genetic and biochemical experiments provide strong evidence that phytase activity of *B. amyloliquefaciens* FZB45 is important for plant growth stimulation under phosphate limitation (Idriss *et al*. 2002).

Bacteria were also reported as comparatively more effective in phosphorus solubilization than fungi (Alam *et al.* 2002). Inoculation with PSB increased sugarcane yield by 12.6 percent (Sundara *et al.* 2002). Soil P dynamics was also characterized by physicochemical (sorption-desorption) and biological (immobilization-mineralization) processes. Large amount of P applied as fertilizer enters in to the immobile pools through precipitation reaction with highly reactive

Al^{3+} and Fe^{3+} in acidic, and Ca^{2+} in calcareous or normal soils (Gyaneshwar *et al.* 2002; Hao *et al.*2002).

The inoculation effect of *Bradyrhizobium japonicum* and *Azotobacter chroococum* on soybean (*Glycine max* (L) Merill var. Ransom) was studied by Bhattarai and Prasad (2003). Dual inoculation proved best in all the plant growth parameters. Inoculation with *Azotobacter* alone was also little better than uninoculated control. In order to select potential plant growth promoting rhizobacteria), a selection of strains from the predominant genera in the rhizosphere of four lupine species, based on genetic divergence criteria, was carried out in a study by Gutierrez-Manero *et al.* (2003). This yielded 11 *Aureobacterium* (Aur), four *Cellulomonas* (Cell), two *Arthrobacter* (Arth), two *Pseudomonas* (Ps), and six *Bacillus* (Bc) strains. Cell-free culture filtrates of each bacterium were assayed for effects on germination, growth, and biological nitrogen fixation (BNF) of *Lupinus albus* L. cv. *Multolupa* seeds or seedlings. Four(Aur 6, Aur 9, Aur 11, and Cell 1) of the twenty-five strains assayed promoted germination. *Aureobacterium* 6 and *Aur* 9 also increased root surface, total nitrogen content, and BNF. As a result of the screening, and considering all the variables studied, authors suggested that Aur 6 can be considered a plant growth promoting rhizobacterium suitable for further field trials in other plants and in different production systems. Mamatha *et al*. (2003) performed a greenhouse experiment in which *Bacillus coagulans* and *Pseudomonas fluorescens* were inoculated either singly or dually on growth and nutrition of sandalwood tree. Parameters such as plant height, number of leaves, biomass, P content, alkaline phosphatase & dehydrogenases and microbial population of the root zone soil revealed that all these parameters were higher in plants which were inoculated dually. According to Penrose and Glick (2003) one of the major mechanisms utilized by plant growth-promoting rhizobacteria (PGPR) to facilitate plant growth and development is the lowering of ethylene levels by deamination of 1-aminocyclopropane-1-carboxylic acid (ACC) the immediate precursor of ethylene in plants. The enzyme catalysing this reaction, ACC deaminase, hydrolyses ACC to alpha-ketobutyrate and ammonia. Several bacterial strains that can utilize ACC as a sole source of nitrogen were isolated from rhizosphere soil samples. All of these strains were considered to be PGPR based on the ability to promote canola seedling root elongation under gnotobiotic conditions. The treatment of plant seeds or roots with these bacteria

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

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

yields of tested wheat cv. in pot (up to 14.7 % increase over control) and field experiments (up to 27.5 % increase over control); however, the response varied with cv. and PGPR strains. It was concluded that the strain, which produced the highest amount of auxins in nonsterilized soil, also caused maximum increase in growth and yield of both the wheat cv. Their study suggested that potential for auxin biosynthesis by rhizobacteria could be used as a tool for the screening of effective PGPR strains. Matiru and Dakora (2004) used light, scanning, and transmission electron microscopy to show that roots of sorghum and millet landraces from Africa were easily infected by rhizobial isolates from five unrelated legume genera. With sorghum, in particular, plant growth and phosphorus (P) uptake were significantly increased by rhizobial inoculation, suggesting that field selection of suitable rhizobia/cereal combinations could increase yields and produce fodder for livestock production.

Some bacterial species have mineralization and solubilization potential for organic and inorganic phosphorus, respectively (Khiari and Parent 2005). Inorganic P is solubilized by the action of organic and inorganic acids secreted by PSB in which hydroxyl and carboxyl groups of acids chelate cations (Al, Fe,Ca) and decrease the pH in basic soils (Stevenson 2005). El-Komy HMA (2005) studied the efficacy of strains of *Pseudomonas fluorescens*, *Bacillus megaterium* and *Azospirillum spp*. in *in vitro* solubilization of Ca_3PO_4. *Pseudomonas fluorescens* and *Bacillus megaterium* strains were the most powerful phosphate solubilizers on Pikovskaya (PVK) plates and liquid medium. *Azospirillum lipoferum* strains showed weak zones of solubilisation on the PVK plates. Phosphate solubilization by the tested organisms was accompanied with pH reduction of the culture medium. Maximum pH reduction was 2.8, 1.2 and 0.5 units for *Pseudomonas fluorescens*, *Bacillus megaterium* and *Azospirillum lipoferum* strain 137, respectively. Alginate and agar immobilization of the tested bacteria or coimmobilization of *A. lipoferum* 137 and *B. megaterium* significantly enhanced phosphorus solubilisation for four consecutive 4-day cycles. In a pot experiment, phosphorus mobilization in wheat (*Triticum aestivum* L. cv. Beni Swif 1) inoculated with *B. megaterium* or *A. Lipoferum* 137 as single or mixed inocula (as free or alginate immobilized beads) was studied in presence of Ca_3PO_4.

Wheat inoculated with mixed inocula exhibited high shoot dry weight, total nitrogen (N) yield and the shoot phosphorus content increased by 37 and 53 % compared to the plants inoculated with *A. lipoferum* and uninoculated ones, used as control,

respectively. Maximum nitrogenase activity (measured by acetylene reduction assay) was observed in mixed inoculum treatment, and was increased by 500 and 32 % compared to uninoculated and *A. lipoferum* inoculated plants. Results demonstrate the beneficial influence of coinoculation of *A. lipoferum* and *B. megaterium* for providing balanced N and P nutrition of wheat plants. Combined inoculation of arbuscular mycorrhiza and phosphate solubilising bacteria gave better uptake of both native P from the soil and P coming from the phosphatic rock (Cabello *et al.* 2005).

Kloepper *et al*. (2005) reported that plant growth-promoting rhizobacteria (PGPR) colonize plant roots and exert beneficial effects on plant health and development. They investigated the mechanisms by which PGPR elicit plant growth promotion from the viewpoint of signal transduction pathways within plants. Eight different PGPR strains, including *Bacillus subtilis* GB03, *B. amyloliquefaciens* IN937a, *B. pumilus* SE-34, *B. pumilus* T4, *B. pasteurii* C9, *Paenibacillus polymyxa* E681, *Pseudomonas fluorescens* 89B-61, and *Serratia marcescens* 90-166, were evaluated for elicitation of growth promotion of wildtype and mutant *Arabidopsis in vitro* and *in vivo*. *In vitro* testing on MS medium indicated that all eight PGPR strains increased foliar fresh weight of *Arabidopsis* at distances of 2, 4, and 6 cm from the site of bacterial inoculation. Among the eight strains, IN937a and GB03 inhibited growth of *Arabidopsis* plants when the bacteria were inoculated 2 cm from the plants, while they significantly increased plant growth when inoculated 6 cm from the plants, suggesting that a bacterial metabolite that diffused into the agar accounted for growth promotion with this strain. *In vivo*, eight PGPR strains promoted foliar fresh weight under greenhouse conditions 4 weeks after sowing. To define signal transduction pathways associated with growth promotion elicited by PGPR, various planthormone mutants of *Arabidopsis* were evaluated *in vitro* and *in vivo*. Elicitation of growth promotion by PGPR strains *in vitro* involved signaling of brassinosteroid, IAA, salicylic acid, and gibberellins. *In vivo* testing indicated that ethylene signaling was involved in growth promotion. Results suggest that elicitation of growth promotion by PGPR in *Arabidopsis* is associated with several different signal transduction pathways and that such signaling may be different for plants grown *in vitro* vs. *in vivo*. Timmusk *et al*. (2005) also showed that this bacterium protected *Arabidopsis thaliana* against pathogens and abiotic stress. They studied colonization of plant roots by a natural isolate of *P. polymyxa* which had been tagged with a plasmid-borne *gfp*

gene. Fluorescence microscopy and electron scanning microscopy indicated that the bacteria colonized predominantly the root tip, where they formed biofilms. Accumulation of bacteria was observed in the intercellular spaces outside the vascular cylinder. Systemic spreading did not occur, as indicated by the absence of bacteria in aerial tissues. Studies were performed in both a gnotobiotic system and a soil system. The fact that similar observations were made in both systems suggests that colonization by this bacterium can be studied in a more defined system. They discussed the problems associated with green fluorescent protein tagging of natural isolates and deleterious effects of the plant growth-promoting bacteria. Lee *et al.* (2005) reported that among plant growth promoting rhizobacteria (PGPR) isolated from bean (*Phaseolus vulgaris* L.) nodules. DLA strain was found to increase bean yield and growth when plants were co-inoculated with *Rhizobium leguminosarum* bv. *phaseoli* under nitrogen-free conditions, compared to plant inoculated with *R.leguminosarum* bv. *phaseoli* alone. DLA strain was Gram-positive spore-forming rods. Biolog tests indicated that the DLA strain belonged to the genus *Bacillus*. Phylogenetic analysis of 16S rRNA gene hypervariant region sequences demonstrated DLA strain to be *Bacillus subtilis* strain. Shirkot *et al.* (2005) also observed that treatment of apple seeds with charcoal based inoculants of *Bacillus megaterium* a plant growth promoting rhizobacterium originally isolated from roots of apple seedling significantly increased the various growth attributes of six months old apple seedlings under unsterilized soil conditions. The effect of seed treatment was more pronounced when jaggery was used as adhesive and seed treatment was given after stratification. The percent increase in shoot length, shoot dry weight, root length and root dry weight was ranged between 32.04 to 62.12 per cent over the untreated control. Rhizosphere soil bacterial population and rhizobacterial population of apple seedling were positively and significantly correlated with the above mentioned parameters. Similarly nitrogen, phosphorus and potassium content of whole shoot system was found more in bacterial treated seedling and ranged between 1.15-2.0.13-0. 36 and 1. 21- 1.92, respectively.

Among the whole microbial population in soil, phosphorus solubilising bacteria (PSB) constitute 1 to 50 %, while phosphorus solubilizing fungi (PSF) are only 0.1 to 0.5 % in P solubilization potential (Chen *et al.* 2006). Microorganisms with phosphate solubilizing potential increased the availability of soluble phosphate

and enhanced the plant growth by improving biological nitrogen fixation (Ponmurugan and Gopi 2006). Rhizospheric microorganisms could interact positively in promoting plant growth, as well as N and P uptake. Seed yield of green gram was enhanced by 24 % following triple inoculation of *Bradyrhizobium* + *Glomus fasciculatum* + *Bacillus subtilis* (Zaidi and Khan 2006). Son *et al*. (2006) reported that *Pseudomonas spp*. enhanced the number of nodules, dry weight of nodules, yield components, grain yield, nutrient availability and uptake in soybean crop. In a study by Liu *et al*. (2006) maize and rice seedlings were inoculated with the GFP-labeled *B. megaterium* C4 and then grown in gnotobiotic condition. Observation by confocal laser scanning microscope showed that the GFP-labeled bacterial cells infected the maize roots through the cracks formed at the lateral root junctions and then penetrated into cortex, xylem, and pith, and that the bacteria migrated slowly from roots to stems and leaves. The bacteria were mainly located in the intercellular spaces, although a few bacterial cells were also present within the xylem vessels, root hair cells, epidermis, cortical parenchyma, and pith cells. In addition, microscopic observation also revealed clearly that the root tip in the zone of elongation and differentiation and the junction between the primary and the lateral roots were the two sites for the bacteria entry into rice root. In a study by Chen *et al*. (2006), isolation, screening and characterization of 36 strains of phosphate solubilizing bacteria (PSB) from Central Taiwan were carried out. Mineral phosphate solubilizing (MPS) activities of all isolates were tested on tricalcium phosphate medium by analyzing the soluble-P content after 72 h of incubation at 30°C. Identification and phylogenetic analysis of 36 isolates were carried out by 16S rDNA sequencing. Ten isolates belonged to genus *Bacillus*, nine to genus *Rhodococcus*, seven to genus *Arthrobacter*, six to genus *Serratia* and one each to genera *Chryseobacterium*, *Delftia*, *Gordonia* and *Phyllobacterium*. In addition, four strains namely, *Arthrobacter ureafaciens*, *Phyllobacterium myrsinacearum*, *Rhodococcus erythropolis* and *Delftia* sp. were reported for the first time as phosphate solubilizing bacteria (PSB) after confirming their capacity to solubilize considerable amount of tricalcium phosphate in the medium by secreting organic acids. P-solubilizing activity of these strains was associated with the release of organic acids and a drop in the pH of the medium. HPLC analysis detected eight different kinds of organic acids, namely: citric acid, gluconic acid, lactic acid, succinic acid, propionic acid and three unknown organic acids from the cultures of these isolates. An inverse relationship between pH and P

solubilized was apparent from this study. Identification and characterization of soil PSB for the effective plant growth-promotion broadens the spectrum of phosphate solubilizers available for field application. The effect of different plant-growth promoting rhizobacteria (*Azotobacter chroococcum***,** *Azospirillum brasilense*, *Pseudomonas fluorescens*, *Pseudomonas putida* and *Bacillus cereus*) on pigeonpea (*Cajanus cajan* (L) Milsp.) cv. P-921 inoculated with *Rhizobium* sp. (AR-2–2 k) was assessed.

A glasshouse experiment was carried out by Tilak *et al*. (2006) with a sandy-loam soil in which the seeds were treated with *Rhizobium* alone or in combination with several PGPR isolates. It was monitored on the basis of nodulation, N_2 fixation, shoot biomass, total N content in shoot and legume grain yield. The competitive ability of the introduced *Rhizobium* strain was assessed by calculating nodule occupancy. The PGPR isolates used did not antagonize the introduced *Rhizobium* strain and the dual inoculation with either *Pseudomonas putida*, *P. fluorescens* or *Bacillus cereus* resulted in a significant increase in plant growth, nodulation and enzyme activity over *Rhizobium*-inoculated and uninoculated control plants. The nodule occupancy of the introduced *Rhizobium* strain increased from 50 % (with *Rhizobium* alone) to 85 % in the presence of *Pseudomonas putida*. This study enabled thye authors to select an ideal combination of efficient *Rhizobium* strain and PGPR for pigeonpea grown in the semiarid tropics. Five bacterial strains with phosphate-solubilizing ability and other plant growth promoting traits increased the plant biomass (20–40 %) as tested by paper towel method. Glasshouse and field experiments were conducted using two efficient strains *Serratia marcescens* EB 67 and *Pseudomonas* sp. CDB 35. Increase in plant biomass (dry weight) was 99 % with EB 67 and 94 % with CDB 35 under glasshouse conditions. Increase in plant biomass at 48 and 96 days after sowing was 66 % and 50 % with EB 67 and 51 % and 18 % with CDB 35 under field conditions. Seed treatment with EB 67 and CDB 35 increased the grain yield of field-grown maize by 85 % and 64 % compared to the uninoculated control. Population of EB 67 and CDB 35 were traced back from the rhizosphere of maize on buffered rock phosphate (RP) medium and both the strains survived up to 96 days after sowing (Hameeda *et al.* 2006). Yanni *et al.* (2006) also studied the natural and intimate associations between rhizobia and rice (*Oryza sativa*

L.) and assessed their impact on plant growth in order to exploit those combinations that can enhance grain yield with less dependence on inputs of nitrogen (N) fertilizer.

Erdogan *et al*. (2007) conducted a study with barley in greenhouse conditions in order to investigate seed inoculation with 5 different N_2-fixing (*Bacillus licheniformis* RC02, *Rhodobacter capsulatus* RC04, *Paenibacillus polymyxa* RC05, *Pseudomonas putida* RC06, and *Bacillus* OSU-142) and 2 different phosphate-solubilising (*Bacillus megaterium* RC01 and *Bacillus* M-13) bacteria in comparison to control and mineral fertiliser (N and P) application. Among the strains used in their study, 6 plant growth promoting rhizobacteria (PGPR) stimulated indole acetic acid (IAA) production and 3 of them stimulated phosphate solubilisation; all bacterial strains fixed N_2 and significantly increased the growth of barley. Available phosphate in soil was significantly increased by seed inoculation with *Bacillus* M-13 and *B. megaterium* RC01. Maximum NO_3-N was found in soil after inoculation with N_2-fixing *Bacillus* OSU-142, followed by *P. polymyxa* RC05 and *R. capsulatus* RC04. Total culturable bacteria count increased in all treatments with time, whereas N_2-fixing bacteria decreased with time, except with *B. megaterium* RC01 inoculation. The data suggest that seed inoculation of barley with plant PGPR increased root weight by 17.9%-32.1% as compared to the control, and increased shoot weight by 28.8%-54.2%, depending on the species. N_2-fixing bacterial inoculation significantly increased uptake of N, Fe, Mn, and Zn by barley. The production of hormones is suggested to be one of the mechanisms by which PGPR stimulate barley growth. Effective *Bacillus* species, such as OSU-142, RC07, M-13, *P. polymyxa* RC05, *P. putida* RC06, and *R. capsulatus* RC04, may be used in agriculture. The effect of direct inoculation of seeds with the plant growth promoting rhizobacteria (PGPR) *Azospirillum lipoferum* CRT1 was assessed on maize (*Zea mays*) grown for 35 days after sowing (d.a.s.) in controlled conditions (greenhouse) in a luvisol soil from south-eastern France. WhinRhizo® software was used to describe the following changes in the root system morphology for each plant: distribution and average root diameter, root surface and the number of tips (Czarnes *et al*. 2007). Phosphate solubilizing bacteria enhanced the seedling length of *Cicer arietinum* (Sharma *et al.* 2007), while co-inoculation of PSM and PGPR reduced P application by 50 % without affecting corn yield (Yazdani *et al.* 2009).

Trivedi and Pandey (2008) characterized *Bacillus megaterium* strain B388, isolated from rhizosphere soil of pine belonging to a temperate Himalayan location. The plant growth promotion by the bacterium has been evaluated through petridish and broth based assays. The isolate solubilized tricalcium phosphate under *in vitro* conditions; maximum activity (166 µg/ml) was recorded at 28°C after 15 days of incubation. Production of indole acetic acid demonstrated in broth assays was another important plant growth promoting character. The bacterium produced diffusible and volatile compounds that inhibited the growth of two phytopathogens viz. *Alternaria alternata* and *Fusarium oxysporum.* The carrier based formulations of the bacterium resulted in increased plant growth in bioassays. The rhizosphere colonization and the viability of the cells entrapped in alginate beads were greater in comparison to coal or broth based formulations. The bacterium showed maximum similarity with *Bacillus megaterium* by 16S rRNA analysis. Marulanda *et al.* (2008) investigated how the interaction between three different AMF isolates (*Glomus constrictum* autochthonous, GcA; *G. constrictum* from collection, GcC; and commercial *Glomus intraradices*, Gi) and a *Bacillus megaterium* strain isolated from a Mediterranean calcareous soil affects *Lactuca sativa* L. plant growth. Inoculation with *B. megaterium* increased plant growth when in combination with two of the AMF isolates (GcA and Gi), but decreased it when in combination with GcC. At the same time, plants inoculated with the GcC fungus alone or in combination with *B. megaterium* (*GcC+Bm*) showed leaf symptoms of stress injury by accumulating proline and reducing the amount of photosynthetic pigments, whereas the opposite occurred in plants coinoculated with Gi fungus and *B. megaterium* (*Gi+Bm*). *GcC+Bm* leaves also presented the highest glucose-6- phosphate dehydrogenase (G6PDH) and the lowest glutamine synthetase (GS) enzymatic activities, whereas *Gi+Bm* leaves showed the highest GS activity.

Ochrobactrum anthropi TRS-2, isolated from tea rhizosphere could solubilize phosphate, produce siderophore and IAA *in vitro* and also exhibited antifungal activity against six test pathogens. Application of an aqueous suspension of *O. anthropi* to the rhizosphere of nursery grown tea saplings of five varieties of tea (TV-18, T-17, HV-39, S-449, UP-3 and) led to enhanced growth of the treated plants, as evidenced by increase in height, in the number of shoots and number of leaves per shoot (Chakraborty *et al.* 2009). Screening of rhizobacteria for growth promotion of sorghum (*Sorghum bicolour* (L.) Moench) was conducted under greenhouse

conditions for a total of 78 bacteria isolated from the rhizosphere of sorghum in Ethiopia, and 86 isolates from the rhizosphere and rhizoplane of grasses in South Africa. Three isolates from Ethiopia, all identified as *Bacillus cereus*, enhanced growth promotion by resulting in statistically significant increases in at least five parameters. Of these, *B. cereus* (KBE7-8) resulted in significant increase in shoot and root biomass. Among effective isolates from South Africa, *B. cereus* (NAS4-3) and *Stenotrophomonas maltophilia* (KBS9-B) showed significant increases in all the parameters measured. The isolates which resulted in significant growth promotion colonized the roots effectively with a count up to $>10^8$ cfu/g. In the study conducted to elucidate the possible modes of action by these effective isolates, indole 3-acetic acid-like substances were detected in culture filtrates of the isolates ranging from 4.2 mg/ml by *Serratia marcescens* (KBS9-R) to 22.8 mg/ml by *B. cereus* (KBS5-H) in the presence of 2 mg tryptophan/g nutrient broth solution. Higher rates of solubilization of tricalcium phosphate on Pikovskaya agar medium were shown by *Chryseomonas luteola* (KBS5-F), *S. marcescens* (KBS6-H) and *B. cereus* (KBE9-1). There is very limited knowledge of the use of rhizobacteria in agriculture in Ethiopia and South Africa. The current study therefore generated valuable information towards application of plant growth promoting rhizobacteria as alternatives to chemical fertilizers (Idris *et al*. 2009).

Approximately 30,000 fluorescent bacterial strains isolated from tomato, lettuce, eggplant, Chinese cabbage, and Japanese pepper plants at seven different locations in Hyogo Prefecture, were screened for plant growth- promoting (PGP) activity to induce disease resistance against bacterial wilt in tomato. The 37 strains that had higher PGP activity were subjected to molecular phylogenetic analyses using the sequences of the 16S rRNA, gyrB and rpoD genes. Most of the strains were identified as *Pseudomonas fluorescens* or its close relative, *P. putida*, while a few strains were grouped with more distantly related bacterial species such as *Enterobacter* and *Stenotrophomonas*. The phylogenetic relationships among tomato and lettuce isolates mostly coincided with the source locality and host plants, with a few exceptions. In contrast, isolates from Japanese pepper plants did not form their own cluster but represented several different bacterial species (Kumatani *et al*. 2009). In order to study the effect of phosphate solubilisation microorganisms (PSM) and plant growth promoting rhizobacteria (PGPR) on yield and yield components of corn

Zea mays (L. cv.SC604) an experiment was conducted at research farm of Sari Agricultural Sciences and Natural Resources University, Iran during 2007. Experiment laid out as split plot based on randomized complete block design with three replications. Three levels of manures (consisted of 20 $Mg.ha^{-1}$ farmyard manure, 15 $Mg.^{ha-1}$ green manure and check or without any manures) as main plots and eight levels of biofertilizers (consisted of 1-NPK or conventional fertilizer application; 2-NPK+PSM+PGPR; 3 NP50%K+PSM+PGPR; 4- N50%PK+PSM +PGPR; 5-N50%P50%K+PSM+ PGPR; 6-PK+PGPR; 7- NK+PSM and 8-PSM+PGPR) as sub plots were treatments. Results showed that farmyard manure application increased row number, ear weight, grain number per ear, grain yield, biological yield and harvest index compared to check. Furthermore, using of PSM and PGPR in addition to conventional fertilizer applications (NPK) could improve ear weight, row number and grain number per row and ultimately increased grain yield in green manure and check plots. According to results in all fertilizer treatments application of PSM and PGPR together could reduce P application by 50% without any significant reduction of grain yield. However, this treatment could not compensate 50% reduction of N application (Yazdani *et al*. 2009). Farhat *et al.* (2009) isolated *Serratia marcescens* CTM 50650 from the phosphate mine of Gafsa showed phosphate solubilizing activities. Various insoluble inorganic phosphates, including rock phosphate (RP), calcium phosphate ($CaHPO_4$), tri-calcium phosphate [$Ca_3(PO_4)_2$] and hydroxyapatite were tested as sole sources of phosphate for bacterial growth. Solubilization of these phosphates by *S. marcescens* CTM 50650 was very efficient.

Chakraborty *et al*. (2010) reported that *Serratia marcescens* (TRS-1), as aqueous suspensions, promoted growth in tea saplings as evidenced by increase in height, emergence of new leaves and branches, as well as increase in leaf biomass. Survival of *S. marcescens* in soil after application was determined by ELISA and Dot-Blot using PAb raised against the bacterium. *S. marcescens* solubilized phosphate *in vitro* and *in vivo*. Following application of the bacterium, soil P content decreased, root and leaf phosphate increased, and soil phosphatase activities were also enhanced. Organic acid production and plant growth promotion was also reported as a function of phosphate solubilization by *Acinetobacter rhizosphaerae* strain BIHB 723, isolated from the cold deserts of the trans-Himalayas by Gulati *et al.* (2010).

Hassen and Labuschagne (2010) investigated rhizobacteria isolated from the rhizoplane of grasses growing at the Nylsvlei Nature Reserve in South Africa for growth promotion and root colonization in wheat (*Triticum aestivum* L.) and tomato (*Lycopersicon esculentum* Mill.) under greenhouse and microplot field conditions. The identities of the isolates were determined by means of 16S rRNA gene sequencing as *Bacillus simplex* (KBS1F-3), *Bacillus megaterium* (NAS7-L), *Bacillus cereus* (KFP9-F) and *Paenibacillus alvei* (NAS6G-6). The three *Bacillus* strains were isolated from the perennial grass *Themeda triandra* while the *Paenibacillus* strain was isolated from another perennial grass *Sporobolus fimbriatus*. Enhanced plant shoot and root weight in wheat was achieved by single inoculation with three of the isolates whereas no significant increase was observed in root length. Combined inoculation of *Paenibacillus alvei* (NAS6G-6) and *Bacillus cereus* (KFP9-F) on wheat resulted in significant increase in these parameters. Single inoculations of *Bacillus simplex* (KBS1F-3) and *Bacillus cereus* (KFP9-F) resulted in significant increase in root and shoots fresh weight, root dry weight and total root length in tomatoes. Indoleacetic acid production, phosphate solubilization and siderophore secretion were studied as possible mechanisms by which the bacterial isolates enhanced plant growth. Root colonization was studied by means of spontaneous rifampicin resistant strains of the wild type isolates. Except for *B. megaterium* (NAS7-L), the rest of the isolates colonized the roots efficiently resulting in concentrations of 10^6–10^8 cfu g^{-1} root. The root colonization of *Bacillus simplex* (KBS1F-3) and *Paenibacillus alvei* (NAS6G-6) was visualized by confocal scanning laser microscope (CSLM) after successful transformation of the isolates with the pNF8 plasmid carrying the gene for the green fluorescent protein (gfp). Plant growth promoting activities of 40 different strains of *Pseudomonas fluorescens* and *Pseudomonas putida*, previously isolated from the rhizosphere of wheat (*Triticum aestivum* L.) and canola (*Brassica napus* L.) and maintained in the microbial collection of Soil and Water Research Institute, Tehran, Iran, were evaluated by Miransari *et al.* (2010). The ability of bacteria to produce auxin and siderophores and utilizing P sources with little solubility was determined. Four strains of Wp1 (*P. putida*), Cfp10 (*Pseudomonas sp.*), Wp150 (*P. putida*), and Wp159 (*P. putida*) were able to grow in the DF medium with ACC. Thirty percent of bacterial isolates from canola rhizosphere and 33% of bacterial isolates from wheat rhizosphere were able to produce HCN. The results indicate that most of the bacteria, tested in the experiment, have plant growth-promoting activities. Malleswari and

Bagyanarayana (2010) screened total of 102 bacterial isolates were obtained from the rhizosphere of some medicinal plants viz., *Coleus forskohlii*, *Andrographis paniculata*, *Withania somnifera*, *Ocimum sanctum*, *Aloe vera* and *Tagetes erecta* for their plant growth promoting activities. Only 38 isolates were selected as the best and were further subjected to seed germination tests. In seed germination tests, all these 38 bacterial isolates showed enhancement of growth promotion in sorghum, green gram and maize. Four PGPR isolates- Cf-37, Ap-13, Te-1 and Te-8 were selected to design integrated stable formulations for field applications to promote plant growth and to achieve effective biocontrol of pathogens in commercially grown medicinal plants.

Saharan and Nehra (2011) reported the use of plant growth-promoting rhizobacteria (PGPR) in various crop plants as growth promoters. Inoculation of crop plants with certain strains of PGPR at an early stage of development improved biomass production through direct effects on root and shoot growth. Inoculation of ornamentals, forest trees, vegetables, and agricultural crops with PGPR resulted in multiple effects on early-season plant growth, as seen in the enhancement of seedling germination, plant vigor, plant height, shoot weight, nutrient content of shoot tissues, early bloom, chlorophyll content and increased nodulation in legumes. Effects of plant growth promoting rhizobacteria on growth and yield of Turf (*Eragrostis tef* Zucc. Trotter) under greenhouse condition were also reported by Woyessa and Assefa (2011). Out of 160 isolates, four isolates were found to be potent PGPR viz. AURB15 (*Pseudomonas fluorescens*), AURB34 (*Burkholderia cepacia*), AURB56 (*Bacillus coagulans*) and AURB65 (*Bacillus subtilis*). A Gram positive, rod-shaped potential strain was selected by Dastager *et al.* (2011) from the pool of bacterial isolates obtained from the Western Ghats forest (India) on the basis of zone of Phosphate solubilization activity. Identification based on 16S rRNA gene sequence revealed that the strain -*Bacillus* species, sharing highest sequence similarity to *Bacillus tequilensis* NRRL B-41771^T (99.5%). Strain NII-0943 was able to produce good amount of indole acetic acid (IAA) and was positive for siderophore production. In addition to IAA and siderophore attributes, strain NII-0943 also possessed the characteristics like $Ca_3(PO_4)_2$ solubilization and growth in nitrogen-free medium. Seed inoculation with the strain NII-0943 resulted in significantly higher root initiation in black pepper cuttings grown under pots. The contents of nitrogen and phosphorus in both soil and

plant were also enhanced significantly in treatments inoculated with these bacterial inocula.

Ghosh *et al.* (2012) also reported that six phosphate-solubilizing bacterial strains were isolated from the rhizosphere soils of two seagrasses (*Halophila ovalis* (R. Br.) Hook and *Halodule pinifolia* (Miki) Hartog) in the Vellar estuary. Experimental studies found that the strain PSSG6 was effective in phosphate solubilization with phosphate solubilization efficiency index $E = 375 \pm 8.54$, followed by the strain PSSG5 with phosphate solubilization efficiency index $E = 275 \pm 27.3$. Of the 6 strains isolated, the strains PSSG4 and PSSG5 belonged to the genus *Bacillus*, and PSSG1, PSSG2 and PSSG3 were identified as *Citrobacter* sp., *Shigella* sp., and *Klebsiella* sp., respectively and PSSG6 was identified as *Bacillus circulans*. Bhattacharjee *et al.* (2012) isolated IAA and ACC-deaminase producing rhizobacterium *Rhizobium leguminosarum* bv. *trifolii* SN10, from root nodules of *Trifolium alexandrium* L. *R. leguminosarum* bv. *trifolii* SN10 promoted the growth of four different rice varieties grown in India in terms of biomass, root branching and N content. In addition, using scanning electron microscopy and viable cell counts, it was found that the bacterium successfully colonized the root surface of the rice variety which showed maximum growth promotion upon inoculation. *Pseudomonas fluorescens* 6-8, a rhizosphere isolate was characterized for its ability to produce indole-3-acetic acid and cytokinins production and colonization of canola (*Brassica napus* L.) roots and can promote root elongation under gnotobiotic conditions (Pallai *et al*. 2012). A novel *Enterobacter cancerogenus* MSA2- plant growth promoting gamma-proteobacterium, isolated by Jha *et al.* (2012) from the rhizosphere of *Jatropha cucas* supplemented with 1% carboxymethylcellulose showed overall plant growth promotion effect resulting in enhanced root length (124.14%), fresh root mass (81%), fresh shoot mass (120.02%), dry root mass (124%), dry shoot mass (105.54%), number of leaf (30.72%), chlorophyll content (50.41%), and biomass (87.20%) over control under the days of experimental observation. A field experiment was conducted by Islam *et al*. (2012) to evaluate the suitable combination of plant growth promoting rhizobacteria (*Azospirillum biofertilizer* strain BM9 and BM11) along with different nitrogenous fertilizer levels (0, 20, 40, 60, 80 and 100% N) on rice variety Binadhan 4. Treatment showed a significant increase in growth parameter like plant height, shoot dry weight, root length and dry weights, grain and straw yields, effective

tillers/hill and panicle length, and nitrogen, phosphorus and potassium uptake over uninoculated under most nitrogen levels except 100% N. Mehta *et al.* (2013) isolated two hundred and six phosphate-solubilizing rhizobacteria (PSB) from rhizosphere soil (RS) and root endosphere (ER) of apple trees from different sites of four locations viz., Chamba, Shimla, Kinnaur and Kullu of Himachal Pradesh, Northern India, and PSB were screened for plant growth promoting traits (PGPTs) by using culture dependent procedures. Indole acetic acid (IAA) production was detected in 50 isolates (24.2 %), siderophore synthesis in 53 isolates (25.7 %), hydrocyanic acid (HCN) in 40 isolates (19.4 %) and percentage growth inhibition against *Dematophora necatrix* in 61 isolates (29.6 %). Overall, 54.3 % of PSB isolates from RS and 64.4 % from ER showed none of the PGPTs tested. Among the PSB showing PGPTs, 10.6 % had single trait and 30.6 % had multiple traits showing two (10.7 %), three (14.1 %) and four (5.8 %) types of PGPTs. The Shannon–Weaver diversity index (H′) revealed that PGPT-possessing PSBs in RS were more abundant than ER. Clustering analysis by principal component analysis showed that ER was most important factor influencing the ecological distribution and physiological characterization of PGPT-possessing PSB. Phosphate-solubilizing bacterial strains (PSB) were isolated from maize, onion, jasmine, and tomato rhizosphere soils from four different localities of Salem (Tamil Nadu) - (i) Ammapalayam, (ii) Attayampatty, (iii) Seeragapadi, and (iv) Ariyanoor. Twelve efficient PSB isolates were selected from the colonies based on their ability to form clear zone on Pikovskaya's agar medium. The isolated PSB released high amount of phosphorus from calcium phosphate. The efficient bacterial strains isolated from rhizosphere soils released high amount of phosphorus. Oligonucleotide primers in RAPD characterization showed genetic variation among the bacterial strains (Ranjan *et al.* 2013). Priya *et al.* (2013) also reported that among micro-organisms several bacterial species showed the ability to solubilize tri calcium phosphate. From the rhizosphere soil many species of phosphate solubilizing bacteria like *Bacillus,Pseudomonous* sp , *Xanthomonas* sp. were isolated. The PSB isolates were subjected to production of IAA in pikovskayas broth *in-vitro* condition by adding L-tryptophan as a substrate. Then pH range of *Pseudomonous* sp was initially at 6.8 and finally decreased to 4.63 then produced more Indole acetic acid. So, there is increase evidence that phosphobacteria improve plant growth due to biosynthesis of plant growth substances rather than their action in releasing phosphorous.

Chakraborty *et al*. (2013) isolated a large number of rhizobacteria from the rhizosphere of tea plants, out of which three were selected for various studies. The selected bacteria were *Bacillus amyloliquefaciens*, *Serratia marcescens* and *B. pumilus*. These bacteria showed positive PGPR traits *in vitro*, such as, phosphate solubilization, siderophore production, antagonism to pathogens and IAA production. 16S rDNA sequencing of the bacteria was done and their phylogenetic relationships determined. Under *in vivo* conditions, the PGPR enhanced the seedling growth of tea varieties in the nursery as well as in the field. Plant growth promotion was determined in terms of increase in number of leaves, their biomass and number of shoots. Application of the PGPR led to enhancement in activities of defense related enzymes, such as, phenyl alanine ammonia lyase, peroxidase, chitinase and β1,3-glucanase in tea leaves. Total phenols also increased quantitatively. It is evident from the present study that application of PGPR in the soil lead to biopriming of the plants through induced systemic resistance and other mechanisms. In a study by George *et al.* (2013), two plant growth promoting bacteria designated as KiSII and RNF 267, isolated from the rhizosphere of coconut palms were identified as *Serratia marcescens* and *Enterobacter* sp. based on their phenotypic features, BIOLOG studies and 16S rRNA gene sequence analysis. Both bacteria exhibited phosphate solubilization, ammonification, and production of indole acetic acid, β-1, 3 glucanase activities and 1-aminocyclopropane-1-carboxylate-deaminase activity. They could also tolerate a range of pH conditions, low temperature and salinity (NaCl). In addition, *S. marcescens* KiSII exhibited N- fixation potential, chitinase activity, siderophore production and antibiotics production. Seed bacterization with these bacteria increased the growth parameters of test plants such as paddy and cowpea over uninoculated control in green house assay. In coconut seedlings, significant increase in growth and nutrient uptake accompanied with higher populations of plant beneficial microorganisms in their rhizospheres were recorded on inoculation with both the PGPR. Baniaghil *et al*. (2013) reported the effect of PGPR- *A.lipoferum*, *A.brasilense*, *Azosprillum lipoferum* sp. and two strains of *Pseudomonas-Pseudomonas putida, P.fluorescens* on growth parameters, antioxidant enzymes and microelements of two Canola cultivars- Hyola 401 and RGS 003 under two salinity levels- 80 and 160 mM NaCl. Canola seeds inoculation with PGPR had recovered salinity effects on some growth parameters, and the most effects was related to *Azospirillium* strains specially *A.brasilense*. Out of 51 isolates, *Pseudomonas*

aeruginosa FP6 was identified on the basis of 16S rDNA gene sequence analysis. *P. aeruginosa* FP6 was screened for other plant growth promoting factors like phosphate solubilization, production of IAA, ammonia, siderophore and cell wall degrading enzyme activities- cellulase, chitinase and protease. The isolate was able to solubilize phosphate, produce IAA, siderophore, HCN, ammonia and biosurfactant. Inoculation of cowpea seeds with the *P. aeruginosa* FP6 significantly ($P < 0.05$) enhanced seed germination, seedling vigor index, plant height, and also fresh and dry weight in comparison with the control. Results from this study showed the multifarious plant growth promoting activities of *P. aeruginosa* and suggested its potential use in developing a cost-effective eco-friendly multifunctional biofertilizer (Bhakthavatchalu *et al.* 2013).

Biological Control

Plant-growth promoting rhizobacteria (PGPR) have been used as good biocontrol agents against soil borne pathogens. Potential biocontrol agents should have the following traits- produce antibiotics and siderophores that chelate iron, making it unavailable to pathogens; the ability to synthesize anti-fungal metabolites that cause disease suppression and production of 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 increase yield of plants. The biological control that results from PGPR are reported to be caused by several mechanisms such as competition, antibiosis, and induced resistance (Kloepper *et al*. 1980). PGPR-induced systemic resistance was first observed on carnation where reduced susceptibility to *Fusarium* wilt was observed (Van Peer *et al*. 1991) and on cucumber against *Colletotrichum orbiculare* (Wei *et al*. 1991). In order to determine whether the PGPR could also induce systemic resistance (ISR) in the host, several biochemical analyses known to be associated with ISR were studied. In such cases, similar to SAR, several defense mechanisms were activated. PGPR also produce substances harmful to pathogens, such as HCN and antibiotics that suppress deleterious microorganisms in the soil (Schippers 1992).

PGPR have been applied to a wide range of crops as biological control agents against fungal, bacterial, and viral pathogens (van Loon *et al*. 1998). Plant growth-promoting rhizobacteria (PGPR) strains INR7 (*Bacillus pumilus*), GB03 (*Bacillus subtilis*), and ME1 (*Curtobacterium flaccumfaciens*) were tested singly and in

combinations for biological control against multiple cucumber pathogens. Investigations under greenhouse conditions were conducted with three cucumber pathogens-*Colletotrichum orbiculare* (causing anthracnose), *Pseudomonas syringae* pv. *lachrymans* (causing angular leaf spot), and *Erwinia tracheiphila* (causing cucurbit wilt disease) inoculated singly and in all possible combinations. There was a general trend across all experiments toward greater suppression and enhanced consistency against multiple cucumber pathogens using strain mixtures. The same three PGPR strains were evaluated as seed treatments in two field trials over two seasons, and two strains, IN26 (*Burkholderia gladioli*) and INR7 also were tested as foliar sprays in one of the trials. In the field trials, the efficacy of induced systemic resistance activity was determined against introduced cucumber pathogens naturally spread within plots through placement of infected plants into the field to provide the pathogen inoculum. PGPR-mediated disease suppression was observed against angular leaf spot in 1996 and against a mixed infection of angular leaf spot and anthracnose . The three-way mixture of PGPR strains (INR7 plus ME1 plus GB03) as a seed treatment showed intensive plant growth promotion and disease reduction to a level statistically equivalent to the synthetic elicitor Actigard applied as a spray (Raupach and Kloepper, 1998).According to Van Loon *et al.* (1998) nonpathogenic rhizobacteria can induce a systemic resistance in plants that is phenotypically similar to pathogen-induced systemic acquired resistance (SAR). Bacterial determinants of ISR include lipopolysaccharides, siderophores, and salicylic acid (SA). Whereas some of the rhizobacteria induce resistance through the SA-dependent SAR pathway, others do not and require jasmonic acid and ethylene perception by the plant for ISR to develop. ISR is effective under field conditions and offers a natural mechanism for biological control of plant disease.

Paenibacillus polymyxa is a plant growth promoting rhizobacterium with a broad host range, but so far the use of this organism as a biocontrol agent has not been very efficient. In previous work it was showed that this bacterium protects *Arabidopsis thaliana* against pathogens and abiotic stress (Timmusk and Wagner 1999). Colonization of plant roots by a natural isolate of *P. polymyxa* was studied which had been tagged with a plasmid-borne gfp gene. Fluorescence microscopy and electron scanning microscopy indicated that the bacteria colonized predominantly the root tip where they formed biofilms. Accumulation of bacteria was observed in the

intercellular spaces outside the vascular cylinder. Other studies have reported that soil or seed treatment with *Bacillus subtilis* protected tomato plants from *Fusarium* wilt caused by *Fusarium oxysporum* f.sp.*lycopersici* (Ghonim 1999; Roberti and Selmi 1999) and cucumber and lettuce from rot diseases (Utkhede *et al*. 1999; Amer and Utkhede 2000). The efficacy of various *Pseudomonas fluorescens* isolates was tested for the management of fruit rot of chilli caused by *Colletotrichum capsici*. Among the various isolates tested *P. fluorescens* isolates *viz.* Pf1 and ATR increased the plant growth and produced the maximum amout of indole acetic acid. *P. fluorescens* Pf1 effectively inhibited the mycelial growth of the pathogen under *in vitro* conditions and decreased the fruit rot incidence under greenhouse condition. Seed treatment plus soil application of talc based formulation of *P. fluorescens* isolate Pf1 effectively reduced the disease incidence. Expression of various defense related enzymes and chemicals was found to be 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). The *P. fluorescens* strain Pf1, which performed well against rice sheath blight, sheath rot and blast (Nandakumar *et al*. 2001; Radjacommare *et al*. 2002) and wilt disease of tomato (Ramamoorty *et al*. 2002) , Pythium disease of tomato and hot pepper (Ramamoorty *et al*. 2002). Efficacy of seven strains of *Pseudomonas fluorescens* (*Pfs*17), plant growth-promoting rhizobacteria (PGPR), were tested by Sarma *et al*. (2002) under field conditions for their ability to protect *Cicer arietinum* against *Sclerotium rolfsii* infection. Best protection was observed in strain 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 resistance, qualitative and quantitative alterations of phenolic compounds in different parts of *C.arietinum* were estimated following PGPR application as seed treatment. High performance liquid chromatographic (HPLC) analysis of the leaves, collars and roots of the PGPR-treated and non-treated (control) plants showed the presence of gallic, ferulic, chlorogenic and cinnamic acids with varied amounts in the PGPR treated as well as non-treated (control) plants. Maximum accumulation of cinnamic acid was observed in plants treated with 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. Pfs3 also caused maximum accumulation of total phenolics and gallic acid in all chickpea plant parts as compared to other treatments and untreated

control. A direct relationship between the level of total phenolics and seedling survivability was observed.

Raj *et al.* (2003) demonstrated a potential role for plant growth promoting rhizobacterial formulations in downy mildew management. Five plant growth promoting rhizobacterial formulations, each consisting of two *Bacilli* strains with chitosan as a carrier were tested for their capacity to promote growth and induce resistance against downy mildew in pearl millet under both greenhouse and field conditions. Bhatia *et al.* (2003) observed maximum colony growth inhibition due to *Pseudomonas* PS 2 (74 %) as compared to PS 1 (71 %) on trypticase soy agar (TSM) plates after 5 days of incubation. Light and scanning electron microscope examination showed hyphal coiling, vacuolation and granulation of cytoplasm resulting in lysis of hyphae of *Macrophomina phaseolina* by pseudomonads. Cell free culture filtrates of strains PS1 and PS 2 restricted the growth of mycelium of *M. phaseolina*, PS 1 and PS 2 caused maximum colony growth inhibition by 57 and 61% respectively at 20% conc. of culture filtrate after 4 days of incubation. Volatile substances produced by PS 1 and PS 2 also inhibited the colony growth of *M. phaseolina* by 25 and 32% respectively. Inhibitory effect of volatile substances, however, decreased with advancing in incubation period. Colony growth of *M. phaseolina* was significantly decreased by PS 1 and PS 2 as compared to control both in iron sufficient and iron deficient conditions. PS 2 showed higher antagonistic activity than PS 1, as evidenced by pronounced colony growth inhibition. *Bacillus megaterium* have also been reported to produce antibiotics against several fungal pathogens by Jung and Kim 2003. So, the advantages of using *Bacillus* as a biological control agent is its property to form spores resistant to unfavorable natural conditions and its tolerance to antimicrobial substances released by other microbes in the soil. Murphy *et al.* (2003) also evaluated combinations of two strains of plant growth-promoting rhizobacteria (PGPR) formulated with the carrier chitosan for the ability to induce growth promotion of tomato plants and resistance to infection by cucumber mosaic virus (CMV). Each PGPR combination included GB03 (*Bacillus subtilis*) and one of the following PGPR strains: SE34 (*B. pumilus*), IN937a (*B. amyloliquefaciens*), IN937b (*B. subtilis*), INR7 (*B. pumilus*), or T4 (*B. pumilus*). The PGPR combinations formulated with chitosan are referred to as biopreparations. When plants were challenged with CMV, all plants in the biopreparation treatments and the older control

treatment had significantly greater height, fresh weight, and flower and fruit numbers than that of plants in the CMV-inoculated same age control treatment. CMV disease severity ratings were significantly lower for biopreparation-treated and older control tomato plants than for that of same age control plants at 14 and 28 days postinoculation (dpi). *Pseudomonas fluoresens* strain Pf4 and *P. aeruginosa* strain Pag protected chick pea (*Cicer arietinum*) plants from *Sclerotium rolfsii* infection when applied singly or in combination as seed treatment (Singh *et al*. 2003). Aflatoxin contamination of groundnut, caused by *Aspergillus flavus* (Af) group of fungi, is a major problem in the rain fed agriculture in the seni arid tropics. Biological control could be one of the components of integrated management to reduce pre harvest kernel investment in the field. Thakur *et al.* (2003) evaluated six *Trichoderma* and three *Pseudomonas* strains that were identified as highly antagonistic to Af 11-4 (a highly toxigenic strain) *in vitro*, in field to determine their biocontrol potential. The antagonists were applied as seed dressing and soil application in flowering in Af-sick pots. All the antagonists significantly reduced seed infection in all three field experiments. Two *T. viridae* (Tv 17 and Tv 23), one *T. harzianum* (Th 23) and one *Pseudomonas* (pf 2) isolates provided greater protection to seed infection by Af 11-4 than others. The reduced seed contamination occurred due to significant reduction in Af population in the rhizosphere of groundnut. Biochemical changes in banded leaf and sheath blight affected maize plants caused by *Rhizoctonia solani* f. sp. *sasakii* grown out of seeds treated with *Pseudomonas fluorescens* were studied by Shivakumar and Sharma (2003). There was an increase in phenolic content in maize leaf sheaths inoculated with *R. solani* or in those of maize plants raised from *P. fluorescens* treated seeds. Increase in phenolic content was observed, in leaf sheaths of plants raised from *P. fluorescens* treated seeds when inoculated with *R. solani.* Peroxidase (PO), polyphenol oxidase (PPO) and phenylalanine ammonia lyase (PAL) activities increased when leaf sheaths were inoculated with the pathogen and plants raised from *P. fluorescens* treated seeds showed higher activity. However leaves from *P. fluorescens* treated seeds did not show any further increase in PO and PPO activities after inoculation with *R. solani.* The bacterized seed with *P. fluorescens* led to accumulation of higher phenolic compounds and higher activity of PO, PPO and PAL, that may play a role in defense mechanism in plants against pathogen.

Someya *et al*. (2003) reported that *Serratia marcescens* strain B2 is an antagonistic bacterium that produces the red-pigmented antibiotic prodigiosin and suppresses rice sheath blight caused by *Rhizoctonia solani* AG-1 IA. Rice sheath blight disease was suppressed when plants were inoculated with this bacterium an hour before pathogen inoculation but not when plants were treated 4 weeks before pathogen inoculation. Bacteria isolated from rice plants and rhizosphere mediate the suppression of antibiotic production of biological control agents and that such suppression is common under field conditions. Kloepper *et al*. (2004) studied the effect of plant growth-promoting rhizobacteria on plant growth and systemic protection against blue mold disease of tobacco (*Nicotiana tabacum* L.), caused by *Peronospora tabacina*, using five PGPR strains with known plant growth promotion and induced resistance activities in other crops. PGPR strains were applied as seed treatments alone at planting and in combination with root drenches after planting. When PGPR were applied as seed treatments, PGPR strains 90-166, SE34 and C-9 at 10^9 CFU mL^{-1} increased all or most parameters of plant growth 7 weeks after planting (WAP) ,while 89B-61 and T4 did not enhance any or few parameters. Seed treatments with PGPR strains 90-166 and C-9 at 10^9 CFU mL^{-1} at 13 WAP resulted in significant disease reduction in blue mold severity compared to the nontreated control. When PGPR were applied as seed treatments and root drenches, all PGPR strains at 10^9 CFU mL^{-1} enhanced tobacco growth compared to the nontreated control at 7 WAP. The time interval between the last PGPR treatment and challenge with *P. tabacina* affected systemic disease protection elicited by some PGPR strains. When the time interval was 8 weeks, 3 PGPR strains 90-166, SE34 and T4 at 10^9 CFU mL^{-1} reduced disease severity, while treatments with all tested PGPR strains resulted in significantly lower disease compared to the nontreated control when it was reduced to 6 weeks. Regression analysis demonstrated a significant relationship between plant growth promotion and systemic protection against blue mold elicited by PGPR strain 90-166. Tobacco growth promotion (X) was calculated by percentage of increase in total fresh plant weight relative to the nontreated control. Systemic protection (Y) against blue mold disease was represented by percentage of decrease in disease severity over the nontreated control. This relationship was best described by the model $Y= -4.48 + 0.37 X$ ($r^2 = 0.86$, $P = 0.0001$) when strain 90-166 was applied as seed treatments. In the experiment in which strain 90-166 was applied as seed treatments and root drenches, $Y= 6.60+0.14 X$ ($r^2 = 0.88$, $P < 0.0001$) defined this relationship when the

time interval was 8 weeks. When the time interval was reduced to 6 weeks, Y= 12.30 + 0.28 X (r^2 = 0.80, P = 0.0005) defined the relationship. Endophytic actinobacteria isolated from healthy cereal plants were assessed for their ability to control fungal root pathogens of cereal crops both *in vitro* and in planta. Thirty eight strains belonging to the genera *Streptomyces*, *Microbispora*, *Micromonospora*, and *Nocardioidies* were assayed by Coombs *et al.* (2004) for their ability to produce antifungal compounds *in vitro* against *Gaeumannomyces graminis* var. *tritici* (*Ggt*), the causal agent of take-all disease in wheat, *Rhizoctonia solani* and *Pythium spp*. Salicylic acid (SA)-mediated induction of systemic resistance by *Pseudomonas aeruginosa* strain 7NSK2 and *P. fluorescens* strain CHA0 against soil-borne fungi and viruses have also been reported by Siddiqui and Shaukat (2004). In green house experiments, Guo *et al*. (2004) reported that three strains of PGPRs – *Serratia sp*.J2, fluorescent *pseudomonad* J3 and BB11 – provided disease control in tomato against tomato wilt and increased yield. Jeun *et al.* (2004) cytologically compared the expression of induced resistance between cucumber plants induced with either plant growth-promoting rhizobacteria (PGPR) or chemicals. Inoculation with PGPR strains *Serratia marcescens* (90–166) and *Pseudomonas fluorescens* (89B61) induced systemic protection in the aerial part of cucumber plants against the anthracnose pathogen *Colletotrichum orbiculare*. Disease development was significantly reduced in these plants compared to control plants that were not inoculated with the PGPR strains. Talc-based bioformulations containing cells of *Pseudomonas fluorescens, Bacillus subtilis* and *Saccharomyces cerevisiae* were evaluated for their potential to attack the mango (*Mangifera indica* L.) anthracnose pathogen *Colletotrichum gloeosporioides* Penz. under endemic conditions by Vivekananthan *et al.* (2004). The *Pseudomonas fluorescens* isolate 1 (Pf1) was found to protect the ragi [*Eleusine coracana* (L.) Gaertner] blast fungus, *Pyricularia grisea*. Induction of defense proteins *viz*. chitinase, β-1,3 glucanase, peroxidase (PO) and polyphenol oxidase (PPO) by the Pf1 isolate was studied against *P. grisea*. Chitinase in a resistant, susceptible and commonly used cultivar with and without challenge inoculation of *P. grisea*, revealed changes in the isoform pattern by UV illumination after staining the gel with fluorescent brightner 28. Native PAGE (polyacrylamide gel electrophoresis) of PO showed the single isoform in all the treatments including the control and a significant increase in the intensity of the band in the inoculated control and Pf1

treatment in all the varieties. Isoform analysis of PPO showed the induction of PPO in *P. fluorescens* treated plants challenged with *P. grisea* (Radjacommare 2004).

Saravanakumar *et al*. (2005) evaluated fluorescent pseudomonads based bioformulation for their ability to control *Macrophomina* root rot disease in mungbean (*Vigna mungo*). *P. fluorescens* isolate Pf1 showed the maximum inhibition in mycelial growth of *Macrophomina phaseolina* under in vitro conditions. Bioformulation of Pf1 with chitin was effective in reducing the root rot incidence in green gram both under glasshouse and field conditions. The rhizosphere colonization of *P. fluorescens* was observed appreciable with the green gram plants. However, Pf1 amended with chitin colonized effectively. Furthermore, the induction of defence-related enzymes and chemicals in plants by Pf1 amended with or without chitin and neem were tested. Increased accumulation of defence enzymes viz., phenylalanine ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), chitinase, β-1,3-glucanse and phenolics were observed in Pf1 bioformulation amended with chitin, pre-treated plants challenge inoculated with *M. phaseolina* under glasshouse conditions. The study revealed that in addition to direct antagonism and plant-growth promotion, PGPR strains amended with chitin bioformulation induced defence-related enzymes and pathogenesis related (PR) proteins which collectively enhance the resistance in green gram against the infection of *M. phaseolina*. Bhatia *et al.* (2005) isolated ten isolates of fluorescent *pseudomonads* from rhizosphere of sunflower, potato, maize and groundnut. All the isolates produced fluorescent pigment in succinate broth and displayed siderophore production. Production of hydrocyanic acid (HCN) and indole acetic acid (IAA) by all the isolates was reduced besides phosphate solubilisation. Out of the ten strains, *Pseudomonas* PS I and PS II were found most potential. Bacterisation of sunflower seeds with fluorescent *Pseudomonas* PS I and PS II resulted in increased seed germination, root length, shoot length, fresh and dry weight of roots, shoots and yield of sunflower. Seed bacterisation with strains of fluorescent *Pseudomonas* PS I and PS II reduced incidence of collar rot by 69.8% and 56.9% respectively, in *Sclerotium rolfsii*-infested soil, making the organism a potential biocontrol agent against collar rot of the sunflower. To assess the potential of bacterial antagonists to control *Phytophthora* blight of pepper caused by *P. capsici* using different screening methods was performed by Rajkumar *et al*. (2005).

Among a collection of fluorescent pseudomonads isolated from the rhizosphere of pepper, twelve isolates were initially selected based on dual culture assay on potato dextrose agar and com meal agar. Further, these twelve isolates were screened for the reduction of disease severity caused by *P. capsici* using detached leaves and seedling assay. Most of the antagonists showed varying levels of antagonism against *P. capsici* in both detached leaves and seedlings assay. In addition few isolates increased shoot and root length of pepper in seedling assays. Among them, isolate PS 119 showing highest ability to reduce the disease severity in the *in vitro* seedling assay was found to be the most efficient antagonists against *P. capsici* in the *in vivo* biological control tests. Integration of foliar bacterial biological control agents and plant growth promoting rhizobacteria (PGPR) was investigated by Ji *et al.* (2005) to determine whether biological control of bacterial speck of tomato, caused by *Pseudomonas syringae* pv. *tomato*, and bacterial spot of tomato, caused by *Xanthomonas campestris* pv. *vesicatoria* and *Xanthomonas vesicatoria*, could be improved. Three foliar biological control agents and two selected PGPR strains were employed in pairwise combinations. The foliar biological control agents had previously demonstrated moderate control of bacterial speck or bacterial spot when applied as foliar sprays. The PGPR strains were selected in this study based on their capacity to induce resistance against bacterial speck when applied as seed and soil treatments in the greenhouse. Field trials were conducted in Alabama, Florida, and California for evaluation of the efficacy in control of bacterial speck and in Alabama and Florida for control of bacterial spot. The foliar biological control agent *P. syringae* strain Cit7 was the most effective of the three foliar biological control agents, providing significant suppression of bacterial speck in all field trials and bacterial spot in two out of three field trials. When applied as a seed treatment and soil drench, PGPR strain *Pseudomonas fluorescens* 89B-61 significantly reduced foliar severity of bacterial speck in the field trial in California and in three of six disease ratings in the field trials in Alabama. PGPR strains 89B-61 and *Bacillus pumilus* SE34 both provided significant suppression of bacterial spot in the two field trials conducted in Alabama. Combined use of foliar biological control agent Cit7 and PGPR strain 89B-61 provided significant control of bacterial speck and spot of tomato in each trial. In one field trial, control was enhanced significantly with combined biological control agents compared to single agent inoculations. These results suggested that some PGPR strains may induce plant resistance under field conditions,

providing effective suppression of bacterial speck and spot of tomato, and that there may be some benefit to the integration of rhizosphere-applied PGPR and foliar-applied biological control agents.

One of 500 rhizobacteria isolated from soil, rhizosphere and rhizoplane of healthy tomato plants was selected by Romeiro *et al.* (2005) in laboratory, greenhouse and field tests as a good inducer of systemic resistance. This plant growth-promoting rhizobacterium was identified as *Bacillus cereus* by fatty-acid analysis. *Bacillus cereus* bacterial cells were removed from liquid culture by centrifugation and the supernatant repeatedly dialyzed (cut-off = 12 000 daltons) against distilled water. Dialysates applied to roots protected tomato plants against leaf fungal and bacterial pathogens, evidence that macromolecules synthesized by the PGPR and released into the environment act as elicitors of systemic resistance. Mathivanan *et al*. (2005) reported that the effect of talc formulations of *Pseudomonas fluorescens* Mingula and *Trichoderma viride* Pers. Ex S.F. Gray applications either alone or in combination on crop growth, sheath blight disease and grain yield in rice was investigated in three different field experiments. Application of *P. fluorescens* and *T. viride* resulted in a significant reduction of sheath blight incidence caused by *Rhizoctonia solani* and was comparable to the treatment with a systemic fungicide- Carbendazim. The promotion of plant growth and yield and suppression of sheath blight disease were marginally improved following combined application of *P. fluorescens* and *T. viride*. Although the combined application of both biocontrol agents did not lead to statistically significant additive effects in reducing the sheath blight and increasing grain yield when compared with their individual applications, no negative effect was recorded in this combined application treatment. Hence, the talc formulations of biocontrol agents either alone or in combination can be recommended as one of the crop protection strategies for the management of sheath blight of rice.

The morphological, biochemical, and physiological characteristics of a phosphate solubilising and antagonistic bacterial strain, designated as B0 (*Pseudomonas putida*), isolated from a sub-alpine Himalayan forest site were described by Pandey *et al*. (2006). The isolate also exhibited antifungal activity against phytopathogenic fungi in Petri dish assays and produced chitinase, β-l,3-glucanase, salicylic acid, siderophore, and hydrogen cyanide. The plant growth promotion and antifungal properties were demonstrated through a maize-based

bioassay under greenhouse conditions. Although the bacterial inoculation was found to result in significant increment in plant biomass, it stimulated bacterial and suppressed fungal counts in the rhizosphere.

Inoculation with *Serratia marcescens* (90-166) induced systemic protection in the aerial parts of cucumber plants against anthracnose pathogen- *Colletotrichum orbiculare*. In green house experiments, *Serratia marcescens* NBR11213 was evaluated for plant growth promotion and biological control of foot and root rot of betelvine caused by *Phytophthora nicotianae* (Lavania *et al*. 2006). Bacterization of betelvine (*Piper betle* L.) cuttings with *S. marcescens* NBRI1213 induced phenylalanine ammonia-lyase, peroxidase, and polyphenoloxidase activities in leaf and root. Qualitative and quantitative estimation of phenolic compounds was done through high-performance liquid chromatography (HPLC) in leaf and root of betelvine after treatment with *S. marcescens* NBRI1213 and infection by *P. nicotianae*. Major phenolics detected were gallic, protocatechuic, chlorogenic, caffeic, ferulic, and ellagic acids by comparison of their retention time with standards through HPLC. In all of the treated plant**s**, synthesis of phenolic compounds was enhanced compared with control. Maximum accumulation of phenolics was increased in *S. marcescens* NBRI1213-treated plant**s** infected with *P. nicotianae*. In a greenhouse test, bacterization using *S. marcescens* NBRI1213 decreased the number of diseased plants compared with nonbacterized controls. There were significant growth increases in shoot length, shoot dry weight, root length, and root dry weight, averaging 81 %, 68 %, 152 %, and 290 %, respectively, greater than untreated controls. *Bacillus licheniformis* N1, which has previously exhibited potential as a biological control agent, was investigated to develop a biofungicide to control the gray mold of tomato caused by *Botrytis cinerea*. Various formulations of *B. licheniformis* N1 were developed by Lee *et al.* (2006) using fermentation cultures of the bacteria in Biji medium, and their ability to control gray mold on tomato plants was evaluated.

The secondary metabolites, 2,4-Diacetylphloroglucinol(DAPG), phenazine-1-carboxylic acid (PCA), Pyrrolnitrin (PRN) and hydrogen cyanide (HCN) also play a key role in the suppression of plant pathogens by several strains of *Pseudomonas*. Genes encoding these metabolites were characterized and specific primers designed for their detection (Svercel *et al*. 2007). Kamil *et al*. (2007) also reported that four

hundred bacterial isolates were isolated from rhizosphere of some plants collected from Egypt and screened for production of chitinase enzyme. Only four isolates designated MS1, MS2, MS3 and MS 4 were the most potent chitinolytic bacterial species. SDS-PAGE analysis of vegetative and sporulated cells of the four isolates revealed that the protein profile of the four isolates were different fron each other in their banding pattern and were identified as *Bacillus licheniformis*, *Stenotrophomonas maltophila*, *Bacillus licheniformis* and *B. thuringiensis*. *In vitro* MS 1 and MS 3 were the most active species, so they suppressed the growth of all tested pathogenic fungi (*Rhizoctonia solani*, *Macrophomina phaseolina*, *Fusarium culmorum*, *Pythium sp*, *Alternaria alternata* and *Sclerotium rolfsii*). MS3 also produced the highest level of chitinase enzyme(1.27µ/ml) after 4 days incubation as compared with the other isolates. In green-house experiment, *B. licheniformis* (MS3) significantly reduced the damping off disease caused by *Rhizoctonia solani*, in *Helianthus annus* using the seed coat or soil draing treatments. Principe *et al*. (2007) established a bacterial collection of approximately one thousand native strains, isolated from saline soils of Cordoba province (Argentina). From this collection, a screening to identify those strains showing plant growth promotion and biocontrol activities, as well as salt tolerance, was performed. Strains MEP_2 18 and ARP_2 3 showed antagonistic activity against phytopathogenic fungi belonging to *Sclerotinia* and *Fusarium* genus. Antifungal activity was found in cell-free supernatants, and it was heat and protease resistant. Strains MEP_2 18 and ARP_2 3 were identified as *Bacillus sp*. according to the sequence analysis of 16S rRNA gene. Three formulations of *Trichoderma viride* viz., *Tv* (CPRI), *Tv* (Viricon L, Orgaman) and *Tv* (AAU) were tested as seed treatment against the diseases of potato. All the formulations significantly controlled late blight, black scurf and brown rot diseases apart from providing significantly good germination and tuber yield. *Tv* (AAU) suppressed the occurrence of mild mosaic significantly. Among the three formulations, performance of *Tv* (CPRI) was the best (Gogoi *et al*. 2007). Tiwari and Thrimurthy (2007) isolated *Pseudomonas fluorescens* from the rhizosphere of rice, wheat, maize, soybean and sunflower and also confirmed the *in vitro* evaluation of *P. fluorescens* isolates in the form of their antagonistic ability aginst both *Pyricularia grisea* and *Rhizoctonia solani* in dual culture tests.

Sharma *et al*. (2007) isolated siderophore producing strain of *Pseudomonas aeruginosa* and also showed the antifungal activity of the strain against three

phytopathogenic fungi, viz., *Fusarium moniliformae*, *Alternaria solani* and *Helminthosporium halodes*. Inhibition of these fungal pathogens appeared to be due to production of antifungal secondary metabolites by *P. aeruginosa*. They suggested that *P. aeruginosa* can be developed as an eco- friendly biocontrol agent. Kumar *et al*. (2007) also reported the efficacy of various *Pseudomonas fluorescens* tested for the induction of systemic resistance against dry root rot of chickpea caused by *Macrophomina phaseolina*. Among five isolates of *P. fluorescens*, Pf4-99 was strong siderophore producer and plant growth promoter. This isolate also inhibited the mycelial growth of *M. phaseolina* in *in vitro* and decreased the root rot incidence under polyhouse. In Pf4-99 treated plants, an increase in phenolic content was observed on 5th d while maximum increase in PAL activity was observed on 4th d after challenge inoculation with *M. phaseolina*. A marked increase in chitinase and β-1,3- glucanase activity was observed in response to pathogen inoculation in Pf4-99 treated plants. An increase in PPO activity was observed upon challenge inoculation with pathogen. The level of PO was multifold higher up to 3-day after Pf4-99 treatment. The observations revealed that *P. fluorescens* isolate Pf4-99 systemically induced resistance against dry root rot of chickpea by the accumulation of battery of enzymes in response to pathogen infection. Foliar spray and micro- injection of plant growth promoting rhizobacterial strains viz., *Pseudomonas fluorescens*(Pf4) and *P. aeruginosa*(Pag), on chickpea (*Cicer arietinum* L.) induced synthesis of phenylalanine ammonia lyase (PAL) when challenged against *Sclerotinia sclerotiorum* (Basha and Chatterjee 2007). Ten isolates of *Pseudomonas fluorescens* obtained from rice rhizosphere were tested for antifungal activity against *Magnaporthe grisea*, *Dreschelaria oryzae*, *Rhizoctonia solani* and *Sarocladium oryzae* that are known to attack rice plants (Reddy *et al*. 2008). One isolate, *P. fluorescens* 8 effectively inhibited mycelial growth in all these fungi in dual culture tests (50-85%). All the ten isolates of *P. fluorescens* were further tested for the production of siderophore, hydrogen cyanide and salicylic acid. The isolate *P. fluorescens* 8 showed higher production of siderophore, HCN and salicylic acid. *Lysobacter enzymogenes* C3 was a bacterial biological control agent that exhibited antagonism against multiple fungal pathogens and its antifungal activity was attributed in part to production of lytic enzymes (Li *et al*. 2008). Domenech *et al*. (2007) evaluated the ability of six putative plant growth promoting rhizobacteria, isolated from the rhizosphere of *Nicotiana glauca* L., to stimulate growth and induce

systemic resistance against *Xanthomonas campestris* CECT 95 in *Arabidopsis thaliana* L. Col-0. The six bacterial strains significantly reduced the disease symptoms caused by the pathogen compared to the controls, with the best results obtained with the *Bacillus* strain N11.37 and the *Stenotrophomonas* strain N6.8. These two strains were tested on *A. thaliana* NahG plants and jar1- 1 and etr1-1 mutants, to elucidate whether the salicylic acid (SA)-dependent or SA-independent pathway was involved in the induction of systemic resistance. Results indicated that N6.8 induces the SA-dependent pathway. For N11.37 it is as yet not clear as in the etr1-1 mutants and NahG plants ISR is not expressed, while in jar1- 1 it is. In addition, levels of SA were measured in Col-0 plants treated with N6.8 and N11.37 to confirm whether or not the two strains produced an increased level of SA. N6.8- and N11.37-induced plants showed higher levels of SA than the controls. Authors concluded that N6.8 induces a SA-dependent pathway while N11.37 induces a pathway that is both ethylene (ET)- and SA-dependent.

Pseudomonas corrugata, a soil bacterium originally isolated from a temperate site of Indian Himalayan Region (IHR) was examined by Trivedi *et al.* (2008) for its antagonistic activities against two phytopathogenic fungi, *Alternaria alternata* and *Fusarium oxysporum*. Although the bacterium did not show inhibition zones due to production of diffusible antifungal metabolites, a reduction in growth between 58 % and 49 % in both test fungi, *A. alternata* and *F. oxysporum*, was observed in sealed petri plates after 120 h of incubation due to production of volatile antifungal metabolites. Reduction in biomass of *A. alternata* (93.8) and *F. oxysporum* (76.9) in Kings B broth was recorded after 48 h of incubation in dual culture. The antagonism was observed to be affected by growth medium, pH and temperature. The reduction in fungal biomass due to antagonism of bacteria was recorded maximum in the middle of the stationary phase after 21 h of inoculation. The production of siderophore, ammonia, lipase and chitinase in growth medium by *P. corrugata* were considered contributing to the antagonistic activities of the bacterium. Saravanakumar *et al.* (2008) reported that plant growth-promoting rhizobacterial strains were isolated from different agroecosystems of Tamil Nadu, India, and were tested for their efficacy against the sheath rot pathogen *Sarocladium oryzae* under in vitro, glasshouse and field conditions. Vigour and a relative performance index (RPI) were used to assay the growth promotion and antagonistic activity of *Pseudomonas* strains against *S.*

oryzae under *in vitro* conditions. Results revealed significant performance by strains Pf1, TDK1 and PY15 compared to other strains. Further, the combination of *Pseudomona*s strains Pf1, TDK1 and PY15 was more effective in reducing sheath rot disease in rice plants compared to individual strains under glasshouse and field conditions. Quantitative and native polyacrylamide gel electrophoresis (PAGE) analysis of peroxidase (PO), polyphenol oxidase (PPO) and chitinase activity in rice plants showed an increased accumulation of defence enzymes in the treatment with a combination of Pf1, TDK1 and PY15 compared to the treatment with individual strains and untreated controls. The present study revealed the probable influence of antagonism, plant growth promotion and induced systemic resistance (ISR) by the mixture of *Pseudomonas* bioformulations in enhancing the disease resistance in rice plants against sheath rot disease. Nandeeshkumar *et al*. (2008) reported that induction of resistance to downy mildew caused by *Plasmopara halstedii* in sunflower was studied after treatment with PGPR strain INR7 (*Bacillus spp*). Treatment of sunflower seeds with 1×10^8 cfu/ml of PGPR strain INR7 resulted in decreased disease severity and offered 51 and 54% protection under green house and field conditions, respectively. The induction of resistance to *P. halstedii* by PGPR strain INR7 was accompanied by the accumulation of various host defense-related enzymes in susceptible sunflower seedlings. Enhanced activation of catalase (CAT), phenylalanine ammonia-lyase (PAL), peroxidase (POX), polyphenol oxidase (PPO) and chitinase (CHT) was evident at 6,9 and 12 h post inoculation, respectively, in sunflower seedlings raised from seeds treated with PGPR strain INR7. This enhanced and early activation of defense-related responses in the susceptible cultivar after treatment with PGPR strain INR7 was comparable to that in the resistant cultivar. The results indicate that PGPR strain INR7 induced resistance against *P. halstedii* in sunflower is mediated through enhanced expression of defense mechanism.

Survival of *Pseudomonas sp*. SF4c and *Pseudomonas sp*. SF10b (two plant-growth-promoting bacteria isolated from wheat rhizosphere) was investigated in microcosms. Spontaneous rifampicin resistant mutants derived from these strains (showing both growth rate and viability comparable to the wild-strains) were used to monitor the strains in bulk soil and wheat rhizosphere. Studies were carried out for 60 days in pots containing non-sterile fertilized or non-fertilized soil. The number of viable cells of both mutant strains declined during the first days but then became

established in the wheat rhizosphere at an appropriate cell density in both kinds of soil. Survival of the strains was better in the rhizosphere than in the bulk soil. Finally, the antagonism of *Pseudomonas spp*. against phytopatogenic fungi was evaluated in vitro. Both strains inhibited the mycelial growth (or the resistance structures) of some of the phytopathogenic fungi tested, though variation in this antagonism was observed in different media. This inhibition could be due to the production of extracellular enzymes, hydrogen cyanide or siderophores, signifying that these microorganisms might be applied in agriculture to minimize the utilization of chemical pesticides and fertilizers (Fischer *et al*. 2010). In another study, treatment with *Ochrobactrum anthropi* decreased brown root rot of tea, caused by *Phellinus noxius* (Chakraborty *et al*. 2009). Multifold increase in activities of chitinase, β-1,3-glucanase, peroxidase and phenylalanine ammonia lyase in tea plants was observed on application of *O. anthropi* to soil followed by inoculation with *Phellinus noxius*. A concomitant increase in accumulation of phenolics was also obtained. Further, Chakraborty *et al*. (2010) also reported that *Serratia marcescens* (TRS 1) showed antagonism to a number of fungal pathogens *in vitro*. It also reduced brown root rot of tea caused by *Fomes lamaoensis*. Significant increase in phenolics, as well as peroxidise, chitinase, β-1,3-glucanase and phenylalanine ammonia-lyase, were observed in tea plants on application of *S. marcescens* alone or followed by *F. lamaoensis*.

Gopalakrishnan *et al.* (2011) isolated about 360 bacteria from the rhizosphere of a system of rice intensification (SRI) fields. Those bacteria were also characterized for the production of siderophore, fluorescence, indole acetic acid (IAA), hydrocyanic acid (HCN) and solubilization of phosphorus. Of them, seven most promising isolates (SRI-156, 158, 178, 211, 229, 305 and 360) were screened for their antagonistic potential against *Macrophomina phaseolina* (causes charcoal rot in *Sorghum*) by dual culture assay, blotter paper assay and in greenhouse. All the seven isolates inhibited *M. phaseolina* in dual culture assay. The sequences of 16S rDNA gene of the isolates SRI-156, 158, 178, 211, 229, 305 and 360 were matched with *Pseudomonas plecoglossicida*, *Brevibacterium antiquum*, *Bacillus altitudinis*, *Enterobacter ludwigii*, *E. ludwigii*, *Acinetobacter tandoii* and *P. monteilii*, respectively in BLAST analysis. Antibiotic-producing *Pseudomonas chlororaphis* strains DF190 and PA23, *Bacillus cereus* strain DFE4 and *Bacillus amyloliquefaciens* strain DFE16 were tested for elicitation of induced systemic resistance (ISR) and direct antibiosis in control of

blackleg in canola caused by the fungal pathogen *Leptosphaeria maculans*. Bacteria controlled the blackleg disease in canola (Ramarathnam *et al.* 2011). Patil *et al.* (2011) mentioned that prodigiosin produced by *Serratia marcescens* NMCC46 could be used as a mosquito larvicidal agent against *Aedes aegypti* and *Anopheles stephensi*. Aballay *et al.* (2011) found that *Bacillus megaterium*, *B. brevis*, *Pseudomonas corrugata*, *P. savastanoi*, *Stenotrophomonas maltophilia* and *Serratia plymuthica* reduced root damage and suppressed populations, meanwhile strains of *B. brevis* and *Comamonas acidovorans* increased plant growth but did not control nematodes.

Rhizobacteria isolated from the rhizosphere soil were evaluated for their ability to control rhizome rot in turmeric (*Curcuma longa* L). These isolates were characterised as *Pseudomonas fluorescens* and *Bacillus subtilis*. Under *in vitro* condition, two isolates, namely *P. chlororaphis* (PcPA23) and *B. subtilis* (BsCBE4), showed maximum inhibition of mycelial growth of *Pythium aphanidermatum*, were found effective in reducing rhizome rot of turmeric both under greenhouse and field conditions and increased the plant growth and rhizome yield. Both the isolates were further tested for its ability to induce production of defense-related enzymes and chemicals in plants. Increased activities of phenylalanine ammonia lyase, peroxidase, polyphenol oxidase, chitinase and b-1,3-glucanase were observed in PcPA23 and BsCBE4 pre-treated turmeric plants challenged with *P. aphanidermatum*. Moreover, higher accumulation of phenolics was noticed in plants pre-treated with PcPA23 and BsCBE4 challenged with *P. aphanidermatum*. Thus, the study showed that in addition to direct antagonism and plant growth promotion, induction of defense related enzymes involved in the phenyl propanoid pathway collectively contributed to enhance resistance against invasion of *Pythium* in turmeric (Kavitha *et al.* 2012).

A total of 51 bacterial isolates from the rhizosphere soil samples were isolated and screened for their antagonistic activity against wide range of phytopathogens. Bacterial antagonist showing highest percent and broad spectrum antagonism against fungal phytopathogens was selected and further identified as *Pseudomonas aeruginosa* FP6 on the basis of 16S rDNA gene sequence analysis. Study on effects of volatile and non-volatile antibiotic compounds on fungal phytopathogens inhibition showed volatile and diffusible metabolites as the major mechanism (Bhakthavatchalu *et al.* 2013). Kumar *et al.* (2013) isolated plant growth promoting rhizobacteria (PGPR) strains, such as *Bacillus* and *Pseudomonas*, by using Nutreint Dextrose Agar

medium or Potato Dextrose Agar medium. The selection of PGPR strains were done by duel culture methods against the potato pathogens. The interaction of PGPR (*Bacillus*) with potato seeds or vegetative parts showed promising antagonism by virtue of producing siderophore and antibiotics against black scurf and stem canker diseases of potato caused by *Rhizoctonia solani*, thereby resulting in increase of potato yield. The effectiveness of PGPR strain (*Bacillus* spp.) in improving the yield of potato in greenhouse conditions and in the field was observed. 21 isolates of *Pseudomonas fluorescens* were isolated and confirmed through various biochemical tests, of which five were tested positive for 2,4-DAPG production with specific primers. Biocontrol potential of these isolates on groundnut stem rot pathogen (*Sclerotium rolfsii*) was determined through *in vitro* dual culture assays. The eight isolates were found effective against *S. rolfsii* (up to 75% inhibition) in dual culture method. All the five 2,4-DAPG-producing plant growth-promoting rhizobacteria isolates were highly antagonistic to *S. rolfsii* (Asadhi *et al.* 2013).

Bioformulations

Isolation of microorganisms, screening for desirable characters, selection of efficient strains and production of inocula are important steps for making use of this microbe-based technology. One of the common means of application of bacterial inoculants to soil is in the form of bioformulations. Use of plant growth promoting microbes as biocontrol agents and biofertilizers give eco-friendly and inexpensive alternatives to the use of chemicals. However, for easy handling of such bacteria, it is necessary to pack such bacteria in inert materials which can also be packaged and stored. Initially, it is essential to determine whether the bacteria can survive in the bioformulations for a reasonable period of time and whether they can induce similar effects to those observed by live bacterial cells. For commercialization, viability of bioinoculant in a prescribed formulation for a certain period with preservation of strain characteristics is also desirable (Fages 1992; Smith 1992). Krishnamurthy and Gnanamanickam (1998) developed talc based formulation of *P. fluorescens* for the management of rice blast caused by *Pyricularia grisea*, in which methyl cellulose and talc was mixed at 1: 4 ratio and blended with equal volume of bacterial suspension at a concentration of 10^{10}cfu/ml. Treatment of cucumber seeds with strain mixtures comprising of *Bacillus pumilus* - INR7, *B. subtilis* – GB03 and *Curtobacterium flaccumfaciens* – ME1 with a mean bacterial density of $5x10^9$cfu/seed reduced intensity of angular leaf spot and

anthracnose equivalent to the synthetic elicitor Actigard and better than seed treatment with individual strains (Raupach and Kloepper 1998). For field applications, the inoculum is required in an appropriate formulation. Viability of inoculum in an appropriate formulation for a certain length of time is important for commercialization of the technology (Bashan 1998). PGPR can also survive in alginate beads for longer periods of time (Bashan and Gonzalez 1999). Press mud is a byproduct of sugar industries. It was composted using vermin-composting technique and later used as a carrier for *Azospirillum* spp. This carrier maximizes the survival of *Azospirillum* spp than lignite, which is predominantly used as a carrier material in India (Muthukumarasamy *et al*. 1999). Formulation development must consider factors such as shelf life, compatibility with current application practices, cost, and ease of application. Health and safety testing may be required to address such issues as non-target effects on other organisms including toxigenicity, allergenicity and pathogenicity, persistence in the environment, and potential for horizontal gene transfer. According to Jeyarajan and Nakkeeran (2000), an ideal formulation should have some characteristics:- 1. Should have increased shelf life. 2. Should not be phytotoxic to the crop plants.3. Should dissolve well in water and should release the bacteria. 4. Should tolerate adverse environmental conditions. 5. Should be cost effective and should give reliable control of plant diseases. 6. Should be compatible with other agrochemicals. 7. Carriers must be cheap and readily available for formulation development .

There are mainly two categories of bioinoculants as presented below:

Liquid Based	**Carrier based**
Easy for application as a foliar spray	Time consuming
Dosage per acre 250 ml	Dosage per acre 5 kg
Contamination is Nil	Contamination chances are high
Very good shelf life 12 months from the manufacture	Poor shelf life 3- 6 months
Cell count 1 x 10^9cfu/ml	Cell count 1 x 10^7cfu/ml
Adequate moisture	Moisture loss may occur
Cells protected with additives from biotic stress	Cells are not protected

For the development of successful formulations of PGPR, rhizobacteria should also possess following characteristics: 1. High rhizosphere competence. 2. High competitive saprophytic ability. 3. Enhanced plant growth. 4. Ease for mass multiplication. 5. Broad spectrum of action. 6. Excellent and reliable control. 7. Safe

to environment. 8. Compatible with other rhizobacteria. 9. Should tolerate desiccation, heat, oxidizing agents and UV radiations (Jeyarajan and Nakkeeran 2000).

Seed treatment of lettuce with either vermiculite or kaolin based carrier of *B. subtilis* (BACT-0) significantly reduced root rot caused by *P. aphanideramtum* and it also increased the fresh weight of lettuce under greenhouse conditions. Treatment of tomato seeds with powder formulation of PGPR (*B. subtilis, B. pumilus*) reduced symptom severity of ToMoV and increased the fruit yield (Murphy *et al*. 2000). Seed treatment with vermiculite based *P. putida* also reduced fusarium root rot of cucumber and increased the yield and growth of cucumber (Amer and Utkhede 2000). Viveganandan and Jauhri (2000) also reported the superiority of alginate-based formulations over charcoal-based ones in maintaining the population of two phosphate solubilizing bacteria during storage at different temperatures and moisture content. Due to the limitations of direct inoculation and the use of various solid-phase bacterial inoculants, several polymer- based formulations, such as alginate beads, wet and dry alginate microbeads and gum-arabic preparations of bacterial species like *Azospirillum brasilense Cd*, *Pseudomonas fluorescens*, and *Rhizobium s*p. have been evaluated (Forestier *et al*. 2001; Bashan *et al.* 2002). Alginate beads have also been reported to preserve the beneficial properties of PGPRs under storage (Russo *et al*. 2001). Soaking of rice seeds in water containing 10g of talc based formulation of *P. fluorescens* consisting mixture of PF1 and PF2 (10^8cfu/g) for 24h controlled rice sheath blight under field condition (Nandakumar *et al*. 2001). Experimental formulations of *Bacillus spp* that have effectively reduced plant diseases have included ca- alginate, alginate manucol (Schmidt *et al*. 2001); peat and chitin (Manjula and Podile 2001; Sid Ahmed *et al*. 2003).

Shelf life of formulation is an important factor which also differs according to the use of different bacterial inoculants as well as carrier materials. Different rhizobacterial formulations and their shelf life are given below.

Yuen *et al*. (2001) also found that incorporation of chitin in the medium increased bacterial population when compared to the non-chitin amended medium and improved the efficiency of PGPR strains in reducing the severity of rust disease in bean plants. Three plant growth promoting rhizobacterial (PGPR) strains of *Pseudomonas spp*, PF1, FP7 and PB2, were also tested alone and in combinations for

suppression of rice sheath blight disease and promotion of plant growth under glasshouse and field conditions. The mixture of PGPR strains significantly reduced the sheath blight incidence when applied as either bacterial suspension through seed, root, foliar and soil application in glasshouse conditions, or as talc-based formulation under field conditions, compared to the respective individual strains (Nandakumar *et al*. 2001)**.** Chitin amendment of soil may have effects in the rhizosphere, such as the stimulation of growth of chitinolytic microorganisms (Ahmed *et al.* 2003), their increased biocontrol activity and elicitation of plant defense proteins (Bharathi *et al.* 2004). All these effects may culminate in enhancing plant protection. Five plant growth promoting rhizobacterial formulations, each consisting of two bacilli strains with chitosan as a carrier were tested for their capacity to promote growth and induce resistance against downy mildew in pearl millet under both greenhouse and field conditions. Three modes of applications were tested: seed treatment, soil amendment, and seed treatment+soil amendment. In general, irrespective of application method, most of the formulations, in comparison with the control, increased plant growth and vigor as measured by seed germination, seedling vigour, plant height, fresh and dry weight, leaf area, tillering capacity, number of earheads, length and girth of earhead, 1000 seed weight and yield. The time of flowering was also advanced by 4-5 days over the control. Likewise all the formulations significantly reduced downy mildew incidence relative to the nontreated control. However, the rate of growth enhancement and disease suppression varied considerably with the formulations. Formulations LS256 and LS257 besides being the best growth promoters were also the most efficient resistance inducers. None of the formulations matched the level of the fungicide metalaxyl in offering protection against downy mildew. Among the application methods tested, soil amendment was found to be the most suitable and desirable way of delivering the formulations. Combination of seed treatment and soil amendment produced the same effect that was produced by soil amendment alone. This study by Raj *et al.* (2003) demonstrated a potential role for plant growth promoting rhizobacterial formulations in downy mildew management. Application of PGPR strains GB03 (*Bacillus subtilis*) and IN937a (*Bacillus amyloliquefaciens*) with the carrier chitosan to the tomato led to protection against cucumber mosaic virus (Murphy *et al.* 2003). Though there are several reports of PGPR strains amended with chitin against plant pathogens, there is only little information available about the induction of defence enzymes against virus diseases.

Further, the development of commercial formulations require newer molecules in order to enhance the survival and efficacy of the plant growth-promoting rhizobacterial (PGPR) strains. Sharathchandra *et al.* (2004) also reported that a bioformulation of *Bacillus* was able to induce plant growth promotion and induce resistance in pearl millet. Endospores of *B. megaterium* were formulated in granule formulations with sodium alginate, lactose and poly vinyl pyrrolidone (PVP K-30) by the wet granulation technique. The granule formulation exhibited good physical characteristics, such as high-water solubility and optimal viscosity that would be suitable for spray application. The bacteria remained viable in the dry granule formulation at 10^9 c.f.u./g after 24 months storage at room temperature. Under laboratory conditions, aqueous solutions of the formulation showed high activity against mycelial growth of *R. solani* (99.64 ± 0.14% mycelial inhibition). High viability of the bacterial antagonist on leaf sheath and leaf blade at day 7 after spraying with the formulation was observed (approximately 10^6 c.f.u./g of plant). Application of an equivalent number of un-formulated endospores resulted in much loss of the bacterial endospores even 1 day after application.

A commercially developed aqueous Chitosan formulation Elexa was also used in different concentrations viz,1:5,1:10,1:15,1:19 and 1:25 as seed soaking treatment to pearl millet for 3,6 and 9 h duration to test for its effect o germination and vigor index. Among the treatments used 1: 19 for 6 h soaking recorded maximum germination and seedling vigor. Seed treatment, foliar spray and combination of seed treatment and foliar spray were tested for control of downy mildew diseases caused by *Scleropora garminicola* in pearl millet under greenhouse and field conditions.

Shelf life of different formulations in different carrier materials

Carriers	**Bacteria**	**Shelf life**	**References**
Talc	Rhizobacteria	2 months	Kloepper and Schroth(1981)
Talc	*P. fluorescens* (P7NF, TL3)	12 months (8.4 Logcfu/g)	Caesar and Burr(1991)
Talc	*P. fluorescens*(Pf1)	8 months ($1.3 x 10^7$cfu/g)	Vidhyasekaran and Muthamilan (1995)
Talc	*B. subtilis*	45 days (1.0×10^6 cfu/g)	Amer andUtkhede(2000)
Talc	*P. putida*	45 days (1.0×10^3 cfu/g)	Amer and Utkhede(2000)
Talc	*P. putida* strain30 and 180	6 months (>1×10^8cfu/g)	Bora *et al*. (2004)
Lignite	*P. fluorescens* (Pf1)	4 months (2.8×10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)

Carriers	Bacteria	Shelf life	References
Peat	*P. fluorescens* (Pf1)	8 months (7.0 x10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)
Peat supplemented with chitin	*B. subtilis*	6 months (>1 x 10^9cfu/g)	Manjula and Podile(2001)
Peat	*P. chlororaphis* (PA23) and *B. subtilis* (CBE4)	6 months (>1 x 10^8cfu/g)	Nakkeeran *et al*.(2004)
Vermiculite	*P. fluorescens* (Pf1)	8 months (1.0 x10^6cfu/g)	Vidhyasekaran and Muthyamilan (1995)
Vermiculite	*B. subtilis*	45days (>1.0 x 10^6 cfu/g)	Amer and Utkhede(2000)
Vermiculite	*P. putida*	45days (>1.0 x 10^3 cfu/g)	Amer and Utkhede(2000)
Farm yard manure	*P. fluorescens* (Pf1)	8 months (1.0 x10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)
Kaolinite	*P. fluorescens* (Pf1)	4 months (2.8 x10^6cfu/g)	Vidhyasekaran and Muthamilan (1995)

Metalaxyl at the rate of 2.1% a.i. in the form of Apron 35SD seed treatment was used as cheek. Under greenhouse conditions seed treatment offered 48% protection. Foliar spray was carried out to two, seven and 14-dy-old seedling and there was marked reduction in downy mildew severity to 42.5% and recorded 38% protection, whereas foliar spray to 7day-old seedling gave 67% protection and reduced severity to 25% and combination of seed treatment by 23%. The nature of disease control mechanisms has been investigated and the results indicated that it is due to induction of systemic resistance. The indication of resistance was observed as early as 24 h time gap between the inducer treatment and pathogen inoculation and the maximum resistance developed at 24-48 h time gap and maintained thereafter. Elexa treated to pearl millet seeds offered growth promoting effect under greenhouse conditions and recorded increase in plant, ear head length and seed weight. Hence, authors inferred that Elexa is a good downy mildew management commercial formulation and also exhibits growth-promoting effect in pearl millet.

The plant growth-promoting rhizobacteria *Pseudomonas fluorescens* (FP7) amended with chitin sprayed at fortnightly intervals gave the maximum induction of flowering, a yield attribute in the preharvest stage, consequently reduced latent symptoms were recorded at the postharvest stage. An enormous induction of the defence-mediating lytic enzymes chitinase and β-1,3-glucanase was recorded in colorimetric assay and the expression of discrete bands in native PAGE analysis after FP7 + chitin treatment. The enhanced expression of defence-mediating enzymes may

collectively contribute to suppress the anthracnose pathogen, leading to improved yield attributes. In China, for instance, a liquid formulation has been extensively tested to control sheath blight disease in farm ricefield conditions (Mew *et al.* 2004). Although this type of formulation can be produced in large quantity using a simple fermentation and formulation processes, it may be difficult to store and may have a relatively short shelf life. Talc-based bioformulations containing cells of *Pseudomonas fluorescens, Bacillus subtilis* and *Saccharomyces cerevisiae* were also evaluated for their potential to attack the mango (*Mangifera indica* L.) anthracnose pathogen *Colletotrichum gloeosporioides* Penz. under endemic conditions by Vivekananthan *et al.* (2004). Talc and peat based formulations of *P. chlororaphis* and *B. subtilis* were prepared and used for the management of turmeric rhizome rot (Nakkeeran *et al*. 2004). *Pseudomonas putida* strain 30 and 180 also survived up to 6 months in talc based formulations. The population load at the end of 6th month was 10^8 cfu/g of the product (Bora *et al*. 2004).

Carrier-based preparations of two plant growth-promoting rhizobacteria (PGPR) viz. *Bacillus subtilis* and *Pseudomonas corrugata*, developed in five formulations were also evaluated for their growth promotion, rhizosphere colonization, and viability under storage. The effect of these formulations as fresh preparations, and after 6 months of storage at 4ºC and room temperature, was also determined. The bacterial inoculants in all the formulations were found to enhance the growth parameters of the test plant species; best results were obtained in case of alginate-based formulations. Maximum numbers of inoculated bacteria were recovered from the rhizosphere of alginate-based formulation-treated plants after 6 weeks of growth. Viability of bacterial inoculants was maximal in alginate beads, and alginate beads supplemented with skim milk formulations, after 180 days of storage at 4ºC (Trivedi *et al.* 2005). The bacterial populations that were recorded, initially lower in the case of alginate-based formulations, increased with time. In contrast to this, the maximum number of bacteria in the case of charcoal- and broth-based formulations was recovered after 7 days of inoculation declined after 14 and 21 days in case of both bacteria. Maximum colonization of rhizosphere (5.819 and 5.431 log10 c.f.u. g^{-1} of soil) was recorded in alginate-coated seeds by *B. subtilis* and in the alginate bead formulation by *P. corrugata*, respectively. Minimum colonization was recorded in case of the broth based formulation (3.125 and 3.410 log10 c.f.u. $g)^{-1}$ of soil) by *B. subtilis* and *P. corrugata*, respectively. The formulations of strain of B*acillus subtilis*

AUBS-1 inhibitory to the growth of damping-off pathogen, *Pythium aphanidermatum*, for seed treatment was provided by Jayraj *et al*. (2005). The formulation included the talc-based powder, lignite based powder, lignite and fiyash-based powder, wettable-powder, bentonite-paste, polyethylene glycol (PEG) paste and water dispersible tablet. Formulations were stored at room temperature for 2 years and frequently sampled to test their shelf life. Populations of bacteria in the formulations were stable for up to 2 years storage at room temperature 28°C. Viability of propagules was significantly reduced in talc, wettable powder, and PEG paste and tablet formulations beyond 1 year of storage. Seed treatment of tomato with these formulations resulted in effective control of damping-off caused by *P. aphanidermatum* and also enhanced plant biomass under glasshouse and field conditions. Active rhizosphere colonization by the bacterium was observed on tomato plants grown from the seeds treated with above formulations. Fluorescent pseudomonads based bioformulation was evaluated for their ability to control *Macrophomina* root rot disease in mungbean (*Vigna mungo*). *P. fluorescens* isolate Pf1 showed the maximum inhibition in mycelial growth of *Macrophomina phaseolina* under in vitro conditions. Bioformulation of *Pf1* with chitin was effective in reducing the root rot incidence in green gram both under glasshouse and field conditions. The rhizosphere colonization of *P. fluorescens* was observed appreciable with the green gram plants. However, *Pf1* amended with chitin colonized effectively. Furthermore, the induction of defence-related enzymes and chemicals in plants by Pf1 amended with or without chitin and neem were tested. Increased accumulation of defence enzymes viz., phenyl alanine ammonia lyase (PAL), peroxidase (PO), polyphenol oxidase (PPO), chitinase, β-1,3-glucanse and phenolics were observed in *Pf1* bioformulation amended with chitin, pre-treated plants challenge inoculated with *M. phaseolina* under glasshouse conditions. The present study revealed that in addition to direct antagonism and plant-growth promotion, PGPR strains amended with chitin bioformulation induced defence-related enzymes and pathogenesis related (PR) proteins which collectively enhance the resistance in green gram against the infection of *M. phaseolina* (Saravanakumar *et al*. 2005). There are also several previous reports on application of PGPRs as formulations (Mathivanan *et al*. 2005; Trivedi *et al*. 2005; Hassan-El and Gowen 2006 and Tilak and Reddy 2006). Different formulations of *Bacillus licheniformis* were evaluated on their own and in combination with prochloraz and stroburilin for their ability to reduce mango post-harvest fruit diseases

[anthracnose and stem-end rot (SR)] when applied as a dip treatment in a mango pack house. Untreated fruit and fruit treated with either prochloraz or stroburilin alone served as controls. In these trials treatments integrating chemical pesticides with *B. licheniformis* controlled anthracnose and SR as effectively as the chemical control. The antagonist was more effective especially in the control of post-harvest diseases when fruit were kept in cold storage to simulate export conditions. In two of the three trials, results obtained when fruit was treated with the antagonist in combination with the commercial chemical were comparable to that obtained with the commercial chemical control. In a study by Govender and Korsten (2006), it was found that the antagonist when used in mango pack house treatments could provide an effective alternative to fungicides. Furthermore, the powder formulation of the antagonist can be successfully incorporated into the existing pack line. Previous reports are also available where *Bacillus* bioformulations could survive upto one year or more in several bioformulations (El-Hassan and Gowen 2006). Plant growth-promoting rhizobacteria (PGPR) bioformulations (*Pseudomonas* and *Bacillus)* were also tested for their efficacy against blister blight (*Exobasidium vexans*) disease in tea (*Camellia sinensis*) under field conditions for two seasons. Among the bioformulations tested, foliar application of *Pseudomonas fluorescens Pf1* at 7-d intervals consistently reduced the disease incidence of blister blight for two seasons, almost comparable with that of chemical fungicide. In addition to disease control, it also increased tea yield significantly compared to the untreated control. Induction of defense enzymes such as peroxidase, polyphenol oxidase, phenylalanine ammonia lyase, chitinase, *β*-1,3-glucanase and phenolics were studied. The enzyme accumulation was greater in *P. fluorescens Pf1*-treated plants compared to control. The study revealed the probable influence of plant growth promotion and induced systemic resistance (ISR) in enhancing the disease resistance in tea plants against blister disease by PGPR bioformulations (Saravanakumar *et al.* 2006).

Pseudomonas fluorescens strains CHA0 and Pf1 were investigated for their biocontrol efficacy against Banana bunchy top virus (BBTV) in banana (*Musa spp*.) alone and in combination with chitin under glasshouse and field conditions. Bioformulation of *P. fluorescens* strain CHA0 with chitin was effective in reducing the banana bunchy top disease (BBTD) incidence in banana under glasshouse and field conditions. In addition to disease control, the bioformulation increased the economic yield significantly compared to the untreated control. Increased

accumulation of oxidative enzymes, peroxidase (PO), polyphenol oxidase (PPO), phenylalanine ammonia lyase (PAL), pathogenesis-related (PR) proteins, chitinase, β-1,3-glucanase and phenolics were observed in CHA0 bioformulation amended with chitin-treated plants challenged with BBTV under glasshouse conditions. Indirect ELISA indicated the reduction in viral antigen concentration in *P. fluorescens* strain CHA0 with chitin-treated banana plants corresponding to reduced disease ratings. Induction of defence enzymes by *P. fluorescens* with chitin amendment reduced the BBTD incidence and increased bunch yield in banana (Kavino *et al.* 2008). According to Kim *et al*. (2008), *Phytophthora* blight of pepper caused by *Phytophthora capsici* has devastating consequences when combined with other pathogens, including *Rhizoctonia solani*, *Fusarium oxysporum*, and *Fusarium solani*. In order to develop a field-effective biocontrol strategy against *Phytophthora* blight of pepper, three chitinolytic bacteria, *Serratia plymuthica* strain C-1, strongly antagonistic to *P. capsici*, *Chromobacterium sp*. strain C-61, strongly antagonistic to *R. solani*, and *Lysobacter enzymogenes* strain C-3, antagonistic to *R.solani* and *Fusarium spp.*, were selected. In pot studies, application of cultures combining the three bacterial strains effectively suppressed *Phytophthora* blight more than application of any single bacterial strain. Bioformulations developed from growth of the strains in a simple medium containing chitin under large batch conditions resulted in effective control in field applications. Efficacy of the bioformulated product depended on both the dose and timing of application.In a small pilot field study, an aqueous solution of the formulation (3%w/v) applied by spraying at days 1, 5 and 10 after pathogen inoculation of the rice plants was more effective in suppressing rice sheath blight disease than one application of a fungicide (Iprodione) at day 1. Additionally, rice plants sprayed with the aqueous solution of the granule formulation had higher panicle and whole kernel weights than those of fungicide-treated and control (untreated) plants (Chumthong *et al.* 2008). *Pseudomonas fluorescens Pf1* along with chitin amendment was effective for survival and colonization of bacteria under field conditions. The efficacy of the talc-based bioformulation of *Beauveria* (B2) strain was tested as seed treatment + seedling dip + soil application + foliar spray against rice leaffolder under *in vitro* and greenhouse conditions. The percentage damage was significantly less (5.5) in B2 as compared to untreated healthy control (25.8). In addition, the same treatment increased the activities of defenserelated enzymes, namely peroxidase, polyphenol oxidase, phenylalanine ammonia-lyase, chitinase, and

phenolics in rice (Sivasundaram *et al.* 2008). Two plant growth-promoting bacteria, *Bacillus subtilis* and *Pseudomonas corrugata*, immobilized in a sodium alginate based formulation were evaluated for their survival, viability and plant growth-promoting ability after 3 years of storage at 4 °C. Populations of both of the bacterial isolates recovered from the immobilized sodium alginate beads were in the order of 10^8 cfu g^{-1}. The plant-based bioassay indicated that the plant growth promotion ability of both of the bacterial isolates was equal to those of fresh broth-based formulations. After 42 days, cfu g-1dry weight of root was 5.60 and 5.10 for alginate beads as compared to 3.24 and 3.31 for fresh broth formulations of *B. subtilis* and *P. corrugata*, respectively. The bacterial isolates retained the root colonization, and antifungal and enzyme activities in the alginate-based formulation during storage (Trivedi and Pandey 2008).

In a study by Chakraborty *et al*. (2009) talc based formulation of *Ochrobactrum anthropi* was prepared and its survival determined every month up to a period of 12 months. *O. anthropi* could survive in the formulation up to a period of 9 months with a concentration of 7.0 $\log_{10}$ CFU g^{-1}, after which there was a decline. Talc formulation was as effective as aqueous suspensions in both plant growth promotion and disease suppression. Chakraborty *et al*. (2010) also reported that *Serratia marcescens* (TRS 1), either as aqueous suspensions or in bioformulations of saw dust, rice husk and tea waste, promoted growth in tea saplings as evidenced by increase in height, emergence of new leaves and branches, as well as increase in leaf biomass. *Bacillus megaterium* (TRS 4) and *Serratia marcescens* (TRS 1) could survive in bioformulations of saw dust, rice husk and tea waste in the range of 6.1-8.0×$\log_{10}$ cfu/ml for more than 9 months *in vitro*.Saw dust, rice husk and tea waste bioformulations of these bacteria were found to enhance growth of tea varieties- TV-18, TV-23, TV-25, TV-26 and T-17 (Chakraborty *et al.* 2012). A greenhouse experiment by Antieno *et al.* (2012) was set to assess the formulation effect of one strain i.e. *Bradyrhizobium japonicum*, 532c (granules, liquid and broth) and also to determine the efficiency of coinoculation of Bacillus with two commercial strains of *B. japonicum* (532c and RCR 3407) on 2 soybean (*Glycine max* L.) varieties. PCR–RFLP analysis was used to determine the nodule occupancy in each treatment. Most of the inoculants showed increased nodulation and biomass yields (by approximately 2–5 and 4–10 g $plant^{-1}$ respectively) as compared to the uninoculated controls. TGx1740-2F showed no significant differences in nodule fresh weights for the

formulation effect while the co-inoculants increased the nodule fresh weights by up to 4 g plant^{-1}. The liquid and granule-based inoculants induced higher biomass yields (4–8 g plant^{-1}) suggesting a possible impact of formulation on the effectiveness of the inoculants. The coinoculants also gave higher yields but showing no significant differences to the rhizobial inoculants. Nodule occupancy was 100 % for the rhizobial inoculants as well as the co-inoculants emphasizing the infectivity and high competitiveness of 532c and RCR 3407 strains despite the high population of indigenous rhizobia. In a study by Chakraborty *et al.* (2013), Bioformulations of *Bacillus amyloliquefaciens*, *Serratia marcescens* and *B. pumilus* in talc powder, saw dust and rice husk were prepared and their viability tested. The bacteria showed good survivability even up to 9 months of storage.

Three main aspects i.e. role of plant growth promoting rhizobacteria (PGPR) in crop improvement, how the bacteria help the plants in their defense against pathogen attack and application of these bacteria into the soil, taking into account suitable carriers, shelf life and sustainability of the rhizobacteria in field have been focused in this review. It is more or less clear that many potent rhizobacterial strains viz. *Bacillus*, *Serratia*, *Pseudomonas*, *Rhizobium*, *Azotobacter*, *Burkholderia*, *Enterobacter* etc are used in agriculture to improve the plant yield. Out of these strains some are free living and some prefer symbiotic association. Direct or indirect mechanisms involved in plant growth promotion and induced systemic resistance i.e. biological nitrogen fixation, solubilization of insoluble phosphates, production of phytohormones, increased expression of defense enzymes, activation of genes for PR proteins, increased uptake of nutrients of roots by the PGPR strains have been highlighted. Regarding the mechanism of action of the bacteria it seems probable that these bacteria act through a combination of methods. It is difficult to predict the actual happening in the soil environment but probably the PGPR secrete metabolites into the soil which in turn elicit responses in the host.

It is quite evident that initial information regarding their suitability, ability to survive for sufficiently long periods in the bioformulations as well as sustainability of the applied bacteria in soil has to be generated. Once this is done, commercial mix of PGPR in suitable formulations for various aims such as in improvement of crop yield or suppression of pests and disease may be used as an alternative where use of biological products to replace or supplement chemical use is the need of the hour.

MATERIALS AND METHODS

3.1. Plant materials

3.1.1. Tea

Different varieties of tea plant (*Camellia sinensis* (L) O. Kuntze), which were maintained in the Germ Plasm bank at Department of Botany, North Bengal University, were used for the experimental purposes.

3.1.1.1. Selection

For clonal propagation of tea plants, initially nodal cuttings of 15 varieties (TV- 23, TV- 26, TV-18, TV-29, TV-25, TV-30, TV-22, T-17, BSS-2, UP -9, UP -2, P-1258, K-1/1, AV-2 and S-449) were made and these were planted in sleeves. The plant varieties were originally collected from Tea Experimental Station, Tocklai (TV-23, TV- 26, TV-18, TV-29, TV-25, TV-30, TV-22 and T-17), Darjeeling Tea Research Centre, Darjeeling (K1/1, P-1258, S 449 and AV-2) and United Planters Association of South India, Tamil Nadu (UP-9 and UP-2). Finally out of 15 varieties, five varieties (TV-18, TV-23, TV-25, TV-26 and T-17) were selected for determination of plant growth promotion and disease assessment followed by bacterial application.

3.1.1.2. Propagation by cuttings

Tea plants were propagated by cutting. Soil preparation is one of the most important steps in propagation technique. Sandy soil (75 % sand and 25 % soil) with a pH 5.6-5.8 was used. Soil pH was adjusted by applying 2 % aluminium sulphate solution followed by leaching with water to remove excess aluminium sulphate.

Polyethylene sleeves (8"x6") were filled up with the prepared soil and stacked in rows in bed and watered thoroughly. Cuttings were made from the tips of the shoots and these were placed in individual sleeves and allowed for rooting after dipping them in rooting hormone along with the maintenance of pH(5.6-5.8) of soil in sleeves. The cuttings were covered with plastic cloche to maintain humidity and watered on alternate days. At the end of 2-3 months, once shoots had appeared, the plastic cloche was removed and watering was done regularly. The whole setup was kept under a green agro house. (Fig. 3)

Figure 3: A-D: Stages of propagation of tea cuttings of different varieties in mother bed as well as in sleeves.

3.1.1.3. Experimental set ups

3.1.1.3.1. Potted plants

Eighteen month old plants of the selected five varieties of tea plants- TV-18, TV-23, TV-25, TV-26 and T-17 were transplanted to 12" earthenware pots and allowed to get acclimatized for further experimental purposes. For each treatment, 10 pots were taken as replicate.

3.1.1.3.2. Experimental field

In the experimental field, eighteen month old plants of TV-18, TV-23, TV-25, TV-26 and T-17 were planted in randomized block design. Three replicate plots were designed and in each plot four treatments were given- control, *B.megaterium, S.marcescens* and *B.megaterium+S.marcescens*. For each treatment, each of the 5 varieties had 10 replicates each. Thus, each variety had 30 replicate plants for each treatment. Before the transplantation, pH of soil of individual plotted area was measured and pH ranged in between (5.6-6.0). During transplantation, proper management practices were adopted and soils were mixed with rock phosphate(30kg), super phosphate(30kg) and 2.5 g phorate [O,O-diethyl S-(ethylthiomethyl) phosphor dithioate] and were placed in pits of 1ft. deep. Spacings in between rows was maintained at 65 cm and in between replicates within each row, there was a space of 1m.Tea plants were watered regularly for proper maintenance. (Figs. 4 C&D; 5 A-H)

3.1.1.3.3. Tea Estate

In the experimental plot of commercial tea garden, 8 yr old pruned plants of T-17/1054 were selected for experimental purposes. The plot was divided into a 2-replicated randomized block design. Two replicate plots were designed and in each plot 4 treatments were given- Control, T1 (*B.megaterium*), T2 (*S.marcescens)* and T3 (*B.megaterium+S.marcescens)*. For each treatment, a total no of 100 pruned plants were selected (Total- 100×4=400). Height of all pruned plants were 18". Standard chemical fertilization practices of the tea garden were not applied in this experimental plot. In order to avoid any biasness in data recording, sample no of plants (10 or 9 plants out of 25 in each block for each treatment) was tagged on the basis of almost same branch numbers. (Fig. 4 A&B)

Figure 4: A& B: General field of Hansqua Tea Estate; C&D: Experimental plot for application of biofertilizers on tea, maintained at NBU.

Figure 5: A-H: Experimental field maintained at NBU in randomized block design showing plants at various stages of growth; after 18 months (A&B), 24 months (C-F) and 36 months (G&H).

3.1.1.4. Maintenance of mature plants

Mature tea plants were also properly maintained by irrigation. 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.

3.2. Bacterial cultures

3.2.1. Source of cultures

Initially several microorganisms were isolated from different rhizospheric soil of tea gardens and screened for *in vitro* PGPR activities. Two bacterial strains showed positive responses in *in vitro* PGPR tests. Two bacterial isolates were isolated from the rhizosphere soil of tea bushes from Nagrakata Tea estate and Hansqua Tea Estate, respectively. These cultures were maintained in PDA and NA by regular sub-culturing. They were also preliminarily identified on the basis of morphological, microscopic and biochemical characterization, and finally identity of these two strains were confirmed from the Plant Diagnostic and Identification Services, UK and also by 16S rDNA sequencing.

3.2.2. Assessment of growth in liquid medium

B. megaterium and *S.marcescens* were cultured separately in Nutrient Broth medium (Himedia, M002-100G, ingredients- peptic digest of animal tissue- 5.00g/litre, sodium chloride- 5.00g/l, beef extract- 1.50 g/l, yeast extract- 1.50 g/l, final pH 25°C -7.4±0.2) and were allowed to grow properly, with shaking at 37°C at 120 r.p.m for 24, 48, 72, 96 and 120 h. At the interval of 24 hr, optical density of both bacterial suspensions were measured by colorimeter and those O.D. values were converted to log cfu/ml broth. Then the standard curves for optimization of growth of *B. megaterium* and *S.marcescens* were also prepared and for field application of bacterial aqueous suspensions log phase (bacterial cultures at 48 h) was regarded as best growth phase. At the end of the log phase, bacterial culture was centrifuged at 10,000 r.p.m for 15 mins and the supernatant was discarded, selecting the bacterial pellet. Pellet was scraped into sterile distilled water. The aqueous suspensions were diluted as necessary to maintain the bacterial concentration at10^9 c.f.u/ml.

3.3. *In vitro* characterization of plant growth promoting activities

3.3.1. IAA production

For detection and quantification of IAA, the selected bacterial cells were grown for 24 h to 48 h in high C/N ratio medium. Tryptophane (0.1 mM) was added in order

to enhance acetic acid (IAA) production by the bacteria (Prinsen *et al*. 1993). Production of IAA in culture supernatant was assayed by Pillet-Chollet method as described by Dobbelaere *et al*. (1999). For the reaction, 1 ml of reagent, consisting of 12 g $FeCl_3$ per litre in 7.9 M H_2SO_4 was added to 1 ml of sample supernatant, mixed well, and kept in the dark for 30 min at room temperature. Absorbance was measured at 530 nm.

3.3.2. Phosphate solubilisation

Primary phosphate solubilizing activities of *B. megaterium* and *S. marcescens* were carried out by allowing the bacteria to grow in selective medium i.e., Pikovskaya's agar (Himedia- M520; ingredients- yeast extract-0.50 g/l, dextrose-10.00 g/l, calcium phosphate- 5.00 g/l, ammonium sulphate- 0.50 g/l, potassium chloride- 0.20 g/l, magnesium sulphate- 0.10 g/l, manganese sulphate- 0.0001 g/l, ferrous sulphate- 0.0001 g/l and agar- 15.00 g/l) for 7 to 10 days at 37°C (Pikovskaya, 1948). The appearance of transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacteria.

3.3.3. Siderophore production

The bacterial isolates were characterized for siderophore production following the method of Schwyn and Neiland (1987) using blue indicator dye, chrome azurol S (CAS). For preparing CAS agar, 60.5 mg CAS was dissolved in 50 ml water and mixed with 10 ml iron (III) solution (1 mM $FeCl_3.6H_2O$ in 10 mM HCl) and volume made up to 1L. With constant stirring this solution was added to 72.9 mg hexa-decytrimethyl ammonium bromide (HDTMA), dissolved in 40 ml water. The resultant dark blue liquid was autoclaved. The dye solution was mixed into the medium along the glass wall with enough agitation to achieve mixing without the generation of foam, and poured into sterile petriplates (20 ml per plate). The plates were inoculated with the bacteria and incubated for 10-15 days till any change in the color of the medium was observed.

3.3.4. HCN production

Production of hydrocyanic acid was determined using the procedure described by Reddy *et al.* (1991) with slight modification. The selected bacterial isolates were grown at room temperature (37ºC) on a rotary shaker in nutrient broth(NB) media. Filter paper (Whatman no.1) was cut into uniform strips of 10 cm long and 0.5 cm wide saturated with alkaline picrate solution and placed in side the conical flasks in a hanging position. After incubation at 37ºC for 48 hr, the sodium picrate present in the

filter paper was reduced to reddish compound in proportion to the amount of hydrocyanic acid evolved. The color was eluted by placing the filter paper in a clean test tube containing 10 ml distilled water and the absorbance was measured at 625 nm.

3.3.5. Chitinase production

For detecting the chitinolytic behavior of the bacteria chitinase detection agar (CDA) plates were prepared by mixing 1.0% (w/v) colloidal chitin with 15 g of agar in a medium consisted of (Na_2HPO_4 6.0 g, KH_2PO_4 3.0 g, NaCl 0.5 g, NH_4Cl 1.0 g, yeast extract 0.05g and distilled water 1 L; pH 6.5).

The CDA plate was spot inoculated with organism followed by incubation at 30°C for 7-10 days. Colonies showing zones of clearance against the creamy background were regarded as chitinase producing strains (Kamil *et al*. 2007).

The colloidal chitin was prepared by following the method described by Roberts and Selitrennikoff (1988). 5 g of chitin powder was slowly added to 60 ml of concentrated HCl and left at 4°C overnight with vigorous stirring. The mixture was added to 2 L of ice cold 95 % ethanol with rapid stirring and kept overnight at 25°C. The precipitation formed was collected by centrifugation at 7000 rpm for 20 min at 4°C and washed with sterile distilled water until the colloidal solution became neutral (pH 7). The prepared colloidal chitin solution (5 %) was stored at 4°C until further use.

3.3.6. Lipase production

Lipolytic activities of *B. megaterium* and *S. marcescens* were performed by allowing them to grow on spirit blue agar media. Lipase production by bacteria was assessed by the method of Marshall (1992).

3.3.7. Protease production

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

3.4. Extraction of DNA

The broth cultures of bacterial isolates were centrifuged at 10,000 rpm at 28ºC for 5 mins and the pellets were collected by discarding the supernatant. The pellets were washed thrice with distilled water and resuspended in 0.5 ml of lysis solution(100mM Tris Hcl, pH-7.5, 20mM EDTA, 250mM NaCl, 2% SDS, 1mg/ml lysozyme). To it 5µl of RNase(50mg/ml) was added and incubated at 37ºC for 3 hr.

Then 10µl proteinase K solution(20mg/ml) was added and incubated at 65ºC for 3 min. The lysate was extracted with equal volume of water saturated phenol: chloroform: isoamylalcohol (25:24:1) and then centrifuged at 10,000 rpm for 5 min. The aqueous phase was collected in clean tube and 2 volume of chilled absolute ethanol was added to this aqueous phase. The mixture was centrifuged at 10,000 rpm for 5 min at 4ºC, the pellet was air dried and finally dissolved in 40µl TE buffer and stored at 4ºC.

3.4.1. Quantification

The genomic DNA, isolated from *B. megaterium* and *S. marcescens* were checked for their purities by A260/A280 ratio. The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample. The reading at 280 nm gives the amount of protein in the sample. Pure preparations of DNA and RNA have OD_{260}/OD_{280} values of 1.8 to 2.0, respectively. If there is contamination with protein or phenol, this ratio will be significantly less than the values given above, and accurate quantitation of the amount of nucleic acid will not be possible.

3.4.2. Agarose gel electrophoresis

The quality of the genomic DNAs were also checked on 0.8% agarose gel electrophoresis. The DNA from bacterial isolates produced clear sharp bands, indicating good quality of DNA.

3.4.3. Amplification of 16 S rDNA by PCR

For PCR amplification, DNA was amplified by mixing the template DNA (50 ng), with the polymerase reaction buffer, dNTP mix, primers and Taq polymerase. Polymerase chain reaction was performed in a total volume of 100 µl, containing 78 µl deionized water, 10 µl 10 X Taq polymerase buffer, 1 µl of 1U Taq polymerase enzyme, 6 µl 2 mM dNTPs, 1.5 µl of 100 mM forward (5’-AGAGTRTGATCMTYGCTWAC-3’) and reverse (5’-CGYTAMCTTWTTACG RCT-3’) primers and 3.5 µl of 50 ng template DNA. PCR was programmed with an initial denaturing at 94^0C for 5 min followed by 30 cycles of denaturation at 94^0C for 60 s, annealing at 59^0C for 60 s and extension at 70^0C for 2 min and the final extension at 72^0C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 µl) was mixed with loading buffer (8µl) containing 0.25 % bromophenol blue, 40 % w/v sucrose in water, and then loaded in 2 % Agarose gel with 0.1 % ethidium bromide for examination with horizontal electrophoresis. The PCR product was sent for sequencing to Chromous Biotech, Bangalore, India.

3.4.4. 16 S rDNA sequencing and phylogenetic analyses

The 16SrDNA sequences obtained from PCR products were subjected to BLAST analyses and aligned with extype isolate sequences from NCBI GenBank for confirmation of identification. The DNA sequences were deposited to NCBI GenBank through BankIt procedure and approved as the sequence after complete annotation and given accession numbers.

The evolutionary history was inferred using the UPGMA method (Sneath and Sokal, 1973). The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al*. 2004) and these are represented in the units of number of base substitutions per site. Codon positions included were 1st+2nd+3rd+noncoding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). Phylogenetic analysis was done in Mega4 software (Tamura *et al*. 2007). 16S rDNA of two bacterial isolates were aligned to study the range of homology present in the conserved regions following the ClustalW algorithm (Thompson *et al*. 1994) using the Bioinformatic tool BioEdit. Combinations and percentage of occurrence of different nucleotide in the entire sequence of *B. megaterium* and *S. marcescens* were calculated using the bioinformatics algorithm from the website: http://www.ualberta.ca/~stothard/javascript/dna_stats.html.

3.4.5. Denaturing Gradient Gel Electrophoresis (DGGE)

Materials:

40% Acrylamide:bisacryl-amide (37.5:1)

50 x DGGE/TAE buffer solution

Trizma-Base:	484.4 g
Sodium-Acetate:	272.0 g
trisodium EDTA	37.2 g
H_20	2 l

pH 7.40 adjusted with about 230 ml of glacial acetic acid.

Preparation of Denaturants

100% Denaturant:

Urea	42.0 g
38.5% Acrylamide (makes a 6.5% gel)	16.9 ml
50x DGGE/TAE	2.0 ml
Formamide	40.0 ml

Filled up to 100 ml with distilled H_2O.

0% Denaturant:

38.5% Acrylamide	16.9 ml
50x DGGE/TAE	2.0 ml
Ammonium Persulphate	10% (w/v)
TEMED	20µl

Methods:

Creating the gel sandwich (DCode System BioRad)

Large glass-plates were cleaned with soap and a soft sponge and rinsed with tap water. After drying, they were cleaned again with 96% ethanol. Both 1mm spacers were also cleaned with 96% ethanol and placed on the large glass plates. The clamps were screwed to the sides of the sandwich, in order to be sure that the spacers, 2 glass-plates and especially the glass plates were aligned at the bottom side of the sandwich and placed in the holder. The clamps were unscrewed and the alignment card slid between the glass plates to align the spacers. The clamps were screwed and the alignment of the glass-plates was checked. Then the sandwich was placed on top of the rubber gasket and the handles pressed down.

Preparing the gel

One tube of APS 10% per gel was prepared. The tubing needle was replaced with a new one, the screw between the compartments was opened and the compartments rinsed with water using pump at a uniform speed. The system was completely drained and flushed with compressed air. The gel solutions were prepared as required. Stacking gel was also prepared according to following table.

UF solution	[UF](%)	[Acrylamid/Bis] (%)	Volume UF solution (ml)	Volume APS 10% (µl)	Volume TEMED (µl)
Low	30-45	6	13	78	6
High	60	6	13	78	6

APS and TEMED was added to the low and high solutions according to table, stirred gently by hand and proceeded immediately for pouring the high concentration solution in the compartment closest to the outlet of the gradient mixer and the low

concentration solution in the other compartment by the delivery system. The whole system was kept for polymerization.

Running the gel

Fresh 0.5x TAE buffer was added to the buffer tank to the mark "Fill". The DCode™ Universal Mutation Detection System (Bio-Rad) was switched on at least 60 minutes before electrophoresis, so that the buffer can heat up to 60°C. After 2-3 hours of polymerization, the comb was removed carefully and the bottom of the sandwich was rinsed with tap water to remove non-polymerized gel .The sandwich was set in the sandwich-holder. A dummy sandwich was also set at the other side to get a closed upper buffer compartment. (A dummy consists of a large and small glass plate stuck together with no spacers in between). The DCode™ was then switched off and the lid taken off after 1 minute. The sandwich holder was slided into the buffer tank, with the red dot of the cathode at the right side. The DCode™ pump and the stirrer underneath the tank were switched on (300 rpm) until samples were loaded.

Staining of gels and photography

Before taking DGGE units out from the tank, the run - evaporated H_2O was replaced up to the marked level. Carefully the DGGE unit was dismantled. The ethidium bromide stain was added into a tray with 50XTAE buffer and the gel was stained for 5 minutes and distained with running buffer. The gel was photograph under UV transilluminator.

3.5. Application of bacteria

The bacteria were grown in NB for 48 h at 37°C and centrifuged at 15000 rpm for 15 min. The pellet obtained was resuspended in sterile distilled water. The optical density of the suspension was adjusted using a UV-VIS spectrophotometer following method to obtain a final density of 2.8×10^9 cfu ml^{-1}.

3.5.1. In sleeves

In the nursery, aqueous suspensions of *B. megaterium* and *S. marscescens*, prepared from 48 hr old log phase were applied to the cuttings in sleeves of the selected five varieties @ 25 ml/sleeve. In this case, there were 4 treatments- control, *B. megaterium*, *S. marcescens* and *B. megaterium*+ *S. marcescens*. Each treatment had 10 replicates for each of the variety.

3.5.2. Rhizosphere of potted plants

Aqueous suspensions of the isolates were diluted as necessary to achieve bacterial concentration at 10^9 c.f.u/ ml. The aqueous suspensions were then applied to the pots @ 100 ml/ pot as a soil drench.

3.5.3. Rhizosphere of 2 yrs old plants

The prepared aqueous bacterial suspensions were also applied as a soil drench as well as a foliar spray (bacterial suspensions along with a few drops of Tween-20), @ 100 ml/ plant to the rhizosphere of 2 yr old tea plants. Application was done at an interval of one month and three applications were done. Since insect attack was a problem in the field, in one of the three plots, an integrated approach was tried. In this case , a low dose 1:2000 (0.5 mg/ml) of a commonly used insecticide- acephate was sprayed at an interval of 14 days along with application of bacterial suspension.

3.5.4. Rhizosphere of full grown bushes

B. megaterium and *S. marcecsens*, singly or jointly were applied as a soil drench, as well as foliar spray @ 100 ml/ plant to the rhizosphere of 8 yr old pruned tea plants of T-17/1054. Application was done at an interval of two month and two applications were done. Observations were recorded after 2 and 4 months of 1st application.

3.6. *In vivo* determination of plant growth promoting activities

Growth promotion was studied in potted plants in terms of increase in height, number of leaves, and leaf dry mass in comparison to control. Each treatment was carried out under same physical and environmental condition(temperature 30-34°C; R.H. 60-80 %; 16 h photoperiod). Observations were recorded after 2 months of application.

The growth promotion by individual bacteria was assessed in the experimental field in terms of % increase in height, no of branches, increase in number of leaves, number of shoots and leaf dry mass in comparison to control plot. Each treatment was carried out under same physical and environmental condition. Observations were recorded after 6 and 12 months of 1st application.

Growth promotion was also studied in terms of increase in number of leaves in comparison to control plot in the experimental field of commercial tea garden. Each treatment was carried out under experimental design as indicated earlier. Observations were recorded after 2 and 4 months of 1st application.

3.7. Determination of phosphate contents

3.7.1. Soil phosphate

Soil sample (1g) was air dried and suspended in 25 ml of the extracting solution (0.025N H_2SO_4, 0.05N HCl) to which activated charcoal (0.01g) was also added, shaken well for 30 min on a rotary shaker and filtered through Whatman No. 2 filter paper (Mehlich, 1984). Quantitative estimation of phosphate was done following ammonium molybdate-ascorbic acid method as described by Knudsen and Beegle (1988). For estimation, 2 ml aliquot of the soil extract was transferred to test tube along with the transfer of 2 ml aliquots of each of the working standards. Then 8 ml of the colorimetric working solution (25 ml conc. ammonium paramolybdate solution, 10 ml ascorbic acid soln., final volume- 1000ml) was added to each test tube and mixed thoroughly. They were allowed to wait for 20 mins for colour development. Finally, % transmittance of all standards and samples on a colorimeter with wavelength set at 882 nm was recorded.

3.7.2. Leaf and root phosphate

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

3.8. Extraction and assay of phosphatase activities

About 2 g portions of soil sample and plant material were used for enzyme extraction and assays. The activities of enzymes were expressed according to method of Tominaga and Takeshi (1974) with modifications.

For acid phosphatase (EC 3.1.3.2) assay, samples were extracted in 5 ml of 50 mM sodium acetate buffer (pH5.0) using a chilled mortar and pestle which was then transferred into a tube and solution was shaken well. 1 ml of 5 mM p- nitrophenyl phosphate solution was added to the tube. All the tubes along with control were allowed to incubate at $37^{\circ}C$ for 1 hour. After incubation, 2 ml of solution was transferred into centrifuge tubes. Centrifugation was done at 10,000 r.p.m for 2 min at $4^{\circ}C$. Finally the supernatant was transferred into clean spectrophotometer cuvettes and the reaction was terminated by addition of 4.0 ml of 100 mM NaOH. The amount of p-nitrophenol liberated was determined from the absorbance at 400 nm. Enzyme activity was expressed as μmol p- nitrophenol liberated/sec/g of soil.

The procedure for the assay of alkaline phosphatase (EC 3.1.3.1) was similar to acid phosphatase except that for enzyme extraction and incubation 100 mM sodium bicarbonate buffer (pH 10.0) was used.

3.9. Extraction and estimation of phenols from leaves

3.9.1. Extraction

Phenol was extracted from the fresh young leaves following the method of Mahadevan and Sridhar (1982). One g of leaf tissues were cut into pieces and immediately immersed in 10 ml of boiling alcohol. After 15 minutes of boiling it was cooled and crushed in mortar using pestle thoroughly at room temperature. The extract was filtered through Whatmann No. 1 filter paper. Final volume was adjusted with 80 % ethanol. The whole extraction of phenol was done in dark to prevent light induced degradation of phenol.

3.9.2. Estimation

3.9.2.1. Total phenol

Total phenol content was estimated by Folin Ciocalteau's reagent, following the method of Mahadevan and Sridhar (1982). To 1 ml of the alcoholic extract, 1 ml of 1 N Folin Ciocalteau's reagent followed by 2 ml of 20 % sodium carbonate solution was added in a test tube. The test tube was shaken and heated on a boiling water bath for 1 minute. After cooling, the volume of the reaction mixture was raised to 25 ml. Absorbance of the blue colored solution was measured at 650 nm. Quantity of total phenol was estimated using caffeic acid as standard.

3.9.2.2. O-phenol

O-dihydroxy phenol was also estimated following the method of Mahadevan and Sridhar (1982). 1 ml of alcoholic extract was mixed with 2 ml of 0.05 N HCl, 1 ml of Arnow's reagent ($NaNO_2$ - 10 g, Na_2MoO_4 - 10 g, distilled water - 100 ml) and 2 ml of 1 N NaOH and mixed thoroughly at room temperature following which the volume of the reaction mixture was raised to 10 ml. Absorbance of the colored solution was recorded at 515 nm. Quantity of the O- dihydroxy phenol was estimated using caffeic acid as standard.

3.10. Extraction of enzymes from leaves

3.10.1. β-1, 3-glucanase (EC 3.2.1.38)

β-1,3-glucanase was extracted from tea leaf samples following the method of Pan *et al.* (1991). Leaf sample (1g) was crushed in liquid nitrogen and extracted using

5 ml of chilled 0.05 M sodium acetate buffer (pH 5.0). The extract was then centrifuged at 10000 rpm for 15 min at 4°C and the supernatant was used as crude enzyme extract.

3.10.2. Chitinase (EC. 3.2.1.39)

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

3.10.3. Phenylalanine ammonia lyase (EC.4.3.1.5)

Enzyme was extracted by the method described by Chakraborty *et al.* (1993). Leaf samples were crushed in liquid nitrogen and extracted using 5 ml of sodium borate buffer (pH8.8) containing 2 mM β mercaptoethanol in ice followed by centrifugation at 15000 rpm for 20 min at 4°C. The supernatant was collected and after recording its volume, used immediately for assay or stored -20°C.

3.10.4. Peroxidase (EC.1.11.1.7)

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

3.11. Assay of enzyme activities

3.11.1. β-1, 3-glucanase

Estimation of β-1,3-glucanase activity was done by following the laminarin dinitrosalicylate method described by Pan *et al.* (1991). The crude enzyme extract of 62.5 µl was added to 62.5 µl of laminarin (4 %) and then incubated at 40°C for 10 min. The reaction was stopped by adding 375 µl of dinitrosalicylic reagent and heating for 5 min on a boiling water bath. The resulting colored solution was diluted with 4.5 ml of water, vortexed and absorbance was recorded at 500 nm. The blank was the crude enzyme preparation mixed with laminarin with zero time incubation. The enzyme activity was expressed as µg glucose released min^{-1} g^{-1} fresh wt. tissue.

3.11.2. Chitinase

Chitinase activity was measured according to the method described by Boller and Mauch (1988). Assay mixture consisted of 10µl of 1M Na-acetate buffer (pH4), 0.4 ml enzyme solution and 0.1 ml colloidal chitin. Colloidal chitin was prepared as per the method of Roberts and Selitrennikoff, (1988). Incubation was done for 2 hrs at 37°C and centrifuged at 10,000 r.p.m for 3 min. 0.3 ml supernatant, 30µl of 1M K-PO_4 buffer (pH7.1) and 20µl Helicase (3%) were mixed and allowed to incubate for 1 h at 37°C. 70µl of 1M Na-borate buffer (pH9.8) was added to the reaction mixture. The mixture was again incubated in a boiling water bath for 3 min and rapidly cooled in ice water bath. 2 ml DMAB (2% di methyl amino benzaldehyde in 20% HCl) was finally added and incubated for 20 min at 37°C.The amount of GlcNAc released was measured spectrophotometrically at 585 nm using a standard curve and activity expressed as µg GlcNAc released $min^{-1}g^{-1}$ fresh wt. tissue.

3.11.3. Phenylalanine ammonia lyase

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

3.11.4. Peroxidase

The reaction mixture contained 1 ml of 0.2M Na-phosphate buffer (pH5.4), 1.7 ml dH_2O, 100µl crude enzyme, 100 µl O-dianisidine (5mg/ml methanol) and 0.1 ml of 4mM H_2O_2. O-dianisidine was used as substrate and activity was assayed spectrophotometrically at 465 nm by monitoring the oxidation of O-dianisidine in presence of H_2O_2 (Chakraborty *et al.* 1993). Specific activity expressed as the increase in Δ A 465 min^{-1} g^{-1} fresh wt. tissue.

3.12. Isozyme analysis of peroxidise

Polyacrylamide gel electrophoresis (PAGE) was performed for isozyme analysis of peroxidase. Extract for isozyme analysis was prepared by crushing 1 g of leaf tissue in a mortar and pestle in 2 M sodium phosphate buffer (pH 7.0) in ice cold

condition as described by Davis (1964) and used immediately for the isozyme analysis.

(1) Preparation of the stock solution

Solution A: Acrylamide stock solution (Resolving gel)

For the preparation of acrylamide stock solution for resolving gel 28 g of acrylamide and 0.74 g of N' N' methylene bisacrylamide was dissolved in 100 ml of distilled water. The stock solution was filtered with Whatman No. 1 filter paper and stored at 4°C in dark bottle.

Solution B: Acrylamide stock solution (stacking gel)

For the preparation of acrylamide stock solution for stacking gel 10 g of acrylamide and 2.5 g of N' N' bisacrylamide was dissolved in 100 ml of distilled water. The stock solution was filtered and stored at 4°C in dark bottle.

Solution C: Tris- HCl (Resolving gel)

36.6 g of Tris base was mixed with distilled water and 0.25 ml of N, N, N', N'- tetramethyl ethylene diamine (TEMED) was added. The pH was adjusted to 8.9 with concentrated HCL. The volume of the solution was made up to 100 ml with distilled water. The solution was then stored at 4°C for further use.

Solution D: Tris- HCl (Stacking gel)

5.98 g of Tris base was mixed with distilled water and 0.46 ml of TEMED and the pH was adjusted to 6.7 with concentrated HCl. The volume of the solution was made up to 100 ml with distilled water. The solution was stored at 4°C for further use.

Solution E: Ammonium persulphate solution (APS)

Fresh solution of APS was prepared by dissolving 0.15 g of APS in 10 ml of distilled water.

Solution F: Riboflavin solution

Fresh solution of Riboflavin was prepared by dissolving 0.4 mg of riboflavin in 10 ml distilled water. The solution was kept in dark bottle to protect from light.

Solution G: Electrode buffer

Electrode buffer was prepared freshly by dissolving 0.6 g of Tris base and 2.9 g glycine in 1 L of distilled water.

(2) Preparation of gel

For the polyacrylamide gel electrophoresis of peroxidase isozymes mini slab gel was prepared. For slab gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any trace of grease and then dried. 1.5 mm thick

spacers were placed between the glass plates on three sides and these were sealed with high vacuum grease and clipped thoroughly to prevent any leakage of the gel solution during pouring. 7.5 % resolving gel was prepared by mixing solution A: C: E: distilled water in the ratio of 1: 1: 4: 1 by pipette leaving sufficient space for (comb + 1 cm) the stacking gel.

This resolving gel was immediately over layered with water and kept for polymerization for 2 hours. After polymerization of the resolving gel was complete, over layer was poured off and washed with water to remove any unpolymerized acrylamide. The stacking gel solution was prepared by mixing solutions B: D: F: distilled water in the ratio of 2: 1: 1: 4.

Stacking gel solution was poured over the resolving gel and comb was inserted immediately and over layered with water. Finally the gel was kept for polymerization for 30- 45 minutes in strong sunlight. After polymerization of the stacking gel the comb was removed and washed thoroughly. The gel was now finally mounted in the electrophoretic apparatus. Tris- Glycine running buffer was added sufficiently in both upper and lower reservoir. Any bubble, trapped at the bottom of the gel, was removed very carefully with a bent syringe.

(3) Sample Preparation

Sample (32 μl) was prepared by mixing the sample enzyme (20 μl) with gel loading dye (40 % sucrose and 1 % bromophenol blue in distilled water) in cyclomixture in ice cold condition. All the solutions for electrophoresis were cooled. The samples were immediately loaded in a predetermined order into the bottom of the wells with a microlitre syringe.

(4) Electrophoresis

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

(5) Fixing and Staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally stained. Staining of the gel was performed following the method of Reddy and Gasber (1973).

The gel was incubated in the aqueous (80 ml) solution of Benzidine (2.08 g), Acetic acid (18 ml), 3 % H_2O_2 (100 ml) for 5 minutes. The reaction was stopped with

7 % Acetic acid. After the appearance of clear blue colored bands, analysis of isozyme was done immediately.

3.13. Extraction of soluble proteins

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

3.13.1. Estimation of protein

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

3.13.2. SDS-PAGE analysis of protein

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

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

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

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

(2) Sodium Dodecyl Sulphate (SDS)

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

(3) Tris Buffer

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

(ii) 1.0 M Tris buffer was prepared for use in the stacking and loading buffer. The pH of this buffer was adjusted to 6.8 with conc. HCl and stored at 4ºC for use.

(4) Ammonium Persulphate (APS)

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

(5) Tris –Glycine electrophoresis buffer

Tris running buffer consists of 25 mM Tris base, 250 mM Glycine (pH 8.3) and 0.1 % SDS. A 1X solution was made by dissolving 3.02 g Tris base, 18.8 g Glycine and 10 ml of 10 % SDS in 1 L of distilled water.

(6) SDS gel loading buffer

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

(7) Preparation of gel

Mini slab gel (plate size 8 cm x10 cm) was prepared for the analysis of protein patterns by SDS-PAGE. For gel preparation, two glass plates were thoroughly cleaned with dehydrated alcohol to remove any traces of grease and then dried. Then 1.5 mm thick spacers were placed between the glass plates at three sides and sealed with high vacuum grease and clipped tightly to prevent any leakage of the gel solution during pouring. Resolving and stacking gels were prepared by mixing compounds in the following order and poured by pipette leaving sufficient space for comb in the stacking gel (comb + 1cm).

After pouring the resolving gel solution, it was immediately overlayered with isobutanol and kept for polymerization for 2 hours. After polymerization of the resolving gel was complete, overlay was poured off and washed with water to remove any unpolymerized acrylamide. Stacking gel solution was poured over the resolving gel and the comb was inserted immediately and overlayered with water. Finally the gel was kept for polymerization for 30 - 45 minutes. After polymerization of the stacking gel the comb was removed and the wells were washed thoroughly. The gel was then finally mounted in the electrophoresis apparatus. Tris - Glycine buffer was added sufficiently in both upper and lower reservoir. Any bubble trapped at the bottom of the gel, was removed carefully with a bent syringe.

Name of the compound	10 % Resolving gel (ml)	5% Stacking gel (ml)
Distilled water	2.85	2.10
30 % acrylamide	2.55	0.50
Tris*	1.95	0.38
10 % SDS	0.075	0.030
10 % APS	0.075	0.030
TEMED**	0.003	0.003

*For 1.5 M Tris pH 8.8 in resolving gel and for 1 M Tris pH 6.8 in stacking gel

** N, N, N', N'- Tetramethyl ethylene diamine

(8) Sample preparation

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

(9) Electrophoresis

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

(10) Fixing and staining

After electrophoresis the gel was removed carefully from the glass plates and then the stacking gel was cut off from the resolving gel and finally fixed in glacial acetic acid: methanol: water (10:20:70) for overnight. The staining solution was prepared by dissolving 250 mg of Coomassie brilliant blue (Sigma R 250) in 45 ml of methanol. After the stain was completely dissolved, 45 ml of water and 10 ml of glacial acetic acid were added. The prepared stain was filtered through Whatman No. 1 filter paper.

The gel was removed from the fixer and stained in this stain solution for 4 hours at 37°C with constant shaking at low speed. After staining the gel was finally destained with destaining solution containing methanol, water and acetic acid (4.5: 4.5: 1) at 40°C with constant shaking until the background became clear.

3.14. Extraction and quantification of chlorophyll

3.14.1. Extraction

The chlorophyll was extracted from leaves of the treated and control plants following the method of Harborne (1973) with modifications. Crushing of 1 g leaf tissue was done in a mortar and pestle using 80% acetone in the dark to prevent the photo oxidation of chlorophyll. The extract was filtered through Whatmann No. 1 filter paper, adding sufficient amount of acetone and final volume was made up 10 ml.

3.14.2. Estimation

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

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

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

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

3.15. Analysis of catechins

3.15.1. Extraction from leaves

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

3.15.2. HPLC analysis of catechins

Catechin analysis of the extract was carried out on HPLC (Shimadzu Advanced VP Binary Gradient) using C-18 hypersil column with linear gradient elution system as follows- mobile phase A 100 % acetonitrile; mobile phase B 2 % acetic acid in water. Elution: 88 % B for 6 min then linear gradient to 75 % B over 5

min. The elution was complete after 25 min. Flow rate was fixed as 1 ml min^{-1} with sensitivity of 0.5 aufs. Injection volume was 20 µl and monitored at 278 nm.

3.16. Preparation of pathogen inoculum and inoculation techniques

Cultures of *Sclerotium rolfsii* were grown in sand- maize meal medium (maize meal: sand: water- 1:9:1.5 w:w:v); (Biswas and Sen 2000) in autoclavable plastic bags (sterilized at 20 lbs. pressure for 20 min) for a period of three weeks at 28°C until the mycelia completely covered the substrate. Selected five varieties of tea plants were then inoculated by adding 100g of prepared inoculum of *S. rolfsii* to the rhizosphere of each plant. Inoculation was done 3 days after final application of bacteria.

3.17. Assessment of sclerotial blight disease

To determine biocontrol potential of the two bacterial species against sclerotial blight of tea, initially, sick plot was prepared. For this, roots of infected plants were chopped and mixed with the soil, along with inocula of *S.rolfsii*. Separate plots were earmarked for the different treatments. Once the sick plot was ready, bacteria were applied to the field followed by transplantation of tea saplings. For control set, a different plot was used.

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

3.18. Preparation of bioformulations

3.18.1. Talc-based

For development of bio-formulation 10 g of carboxy methyl cellulose (CMC) was mixed with 1 kg of talcum powder and pH was adjusted to 7.0 by adding calcium carbonate. It was then sterilized for 30 min in two consecutive days. To this sterilized

talcum powder 400 ml of bacterial inocula containing $3x10^9$ cfu ml^{-1} were 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.

3.18.2. Saw dust

2.5g of carboxymethyl cellulose was added to 250 g of saw dust and pH was adjusted to 7 by adding calcium carbonate. They were sterilized for 30 min in two consecutive days. 100 ml of bacterial suspensions (for *Bacillus megaterium*- 2.8×10^{10} c.f.u/ml and for *Serratia marcescens*- $1x10^{10}$ c.f.u/ ml) were added to the mixtures under sterile condition. The mixture was dried under shade to bring the moisture to less than 20%. Formulation was packed in milky white color polythene bags, sealed & stored at room temperature.

3.18.3. Rice husk

For development of rice husk formulation, the same procedure was followed as described in preparation of saw dust formulations.

3.18.4. Tea waste

Tea waste bioformulations of *B. megaterium* and *S. marcescens* were also prepared by the same procedure that was followed in saw dust as well as rice husk preparations. However, tea waste was soaked in water overnight to reduce the phenolic components.

3.19. Determination of *in vitro* survivability

Viability of bacteria in the formulations were checked by dilution-plate technique using Nutrient Agar medium. The plates were inocubated at 37°C and survivability was expressed in the form of colony forming unit (cfu). The viability tests were carried out with the fresh as well as stored preparations. Viability was checked at an interval of one month till nine months. The data was expressed in log form for analysis.

3.20. Application of bioformulations in rhizosphere

Five different varieties of tea (TV-18, TV-23, TV-25, TV-26 and T-17) were maintained in 12″ earthenware pots in randomized block design. Tea plants were watered regularly for proper maintenance. 100 ml of aqueous bacterial suspensions at 10^9 cfu/ml were added to sterilized 250g of talc, saw dust, rice husk or tea waste along with 2.5 g of carboxy methyl cellulose as a carrier and calcium carbonate under sterile

condition. To test *in vivo* efficacy of the four bioformulations for plant growth promotion, 2-mo-old as well as fresh formulations were applied in potted soil at the rate of 100g/pot.

For determining growth promotion of tea plants followed by treatments, increase in height, number of leaves were noted in potted plants. Plants were grown under natural conditions of light and temperature (30±2°C). Observations were recorded after 2 months of application. In each plot, for each treatment 10 replicates were taken and average of the 10 replicate plants were analysed.

3.21. Preparation of antigens

3.21.1. Bacterial antigens

Soluble protein was obtained by strong sonication of bacterial cells in 0.05 M sodium phosphate buffer (pH 7.2) and centrifuged at 4ºC for 20 minutes at 10,000 rpm. The supernatant was used as antigen for the preparation of antibodies against it.

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

3.21.2. Soil antigens

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

3.21.3. Fungal antigens

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

3.22. Raising polyclonal antibody against different antigens

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

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

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

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

3.23. Purification of IgG

(1) Precipitation

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

(2) Column preparation

8 gram of DEAE cellulose (Sigma Co. USA) was suspended in distilled water for overnight. The water was poured off and the DEAE cellulose was suspended in 0.005 M phosphate buffer (pH 8.0) and the washing was repeated for 5 times. The gel was then suspended in 0.02 M phosphate buffer, (pH 8.0) and was transferred to a column (2.6 cm in diameter and 30 cm height) and allowed to settle for 2 h. After the column material had settled, 25 ml of buffer (0.02 M sodium phosphate, pH 8.0) washing was given to the column material.

(3) Fraction collection

At the top of the column, 2 ml of ammonium sulphate precipitatewas applied and the elution was performed at a constant pH and a molarity continuously changing from 0.02 M to 0.03 M. The initial elution buffer (1) was 0.02 M sodium phosphate buffer (pH 8.0). The buffer was applied in the flask on which rubber connection from its bottom was supplying the column. Another connection above the surface of buffer (1) was connected to another flask with buffer (2). The buffer (2) had also connection to the open air. During the draining of buffer (1) to column, buffer (2) was mixed into buffer (1) thereby producing a continuous raise in molarity. Ultimately, 40 fractions each of 5 ml were collected and the optical density values were recorded at 280 nm using UV-Vis spectrophotometer (SYSTRONICS).

(4) Estimation of IgG concentration

IgG concentration was also estimated as described by Jayaraman (1981). Absorbance was taken for selected fractions at 280 nm and 260 nm and then concentration of IgG was calculated by the following formula: protein concentration (mg/ml).=1.55×A280- 0.76×A260.

3.24. Immunodiffusion

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

(1) Diffusion

Agar gel double diffusion tests were carried out using antigen and antisera following the method of Ouchterlony (1967). The antigen and undiluted antisera (50 μl well^{-1}) were pipetted directly into the appropriate well in a laminar chamber. The diffusion was allowed to continue in a moist chamber for 72 h at 25°C. Precipitation

reaction was observed in the agar gel only in cases where common antigen was present.

(2) Washing, staining and drying of slides

After immunodiffusion, the slides were initially washed with sterile distilled water and then with aqueous NaCl solution (0.9% NaCl and 1% NaN_3) for 72 h with 6 hourly changes to remove unreacted antigens and antisera widely dispersed in the agarose gel. Then slides were stained with Coomassie brilliant blue (R250, Sigma; 0.25 g Coomassie blue, 45 ml methanol, 45 ml distilled water and 10 ml glacial acetic acid) for 10 minutes at room temperature. After staining, the slides were washed in destaining solution (methanol: distilled water: acetic acid in 45:45:10 ratio) with changes until the background become clear. Finally slides were washed with distilled water and dried in hot air oven for 3 h at 50°C.

3.25. Determination bacterial sustainability in soil

3.25.1. Immunotechnique

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

3.25.1.1. Dot-blot

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

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

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

(c) Blocking solution 10 % (w/v) skim milk powder (casein hydrolysate, SLR) in (0.05 M Tris-HCl, 0.5 M NaCl) 5 % (v/v) Tween 20, pH 10.3)

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

Nitrocellulose membrane (Millipore, 7 cm x 10 cm, pore size 0.45µm, Millipore corporation, Bedford) was cut carefully into the required size and placed inside the template. 4 µl of coating buffer was loaded into each well of the template over the NCM and was kept for 30 min to dry. Following this 5 µl of test samples (antigen) was loaded into the template wells over the NCM and was kept for 1 h at room temperature. Template was removed and blocking of NCM was done with 10 % non fat dry milk (casein hydrolysate, SRL) prepared in TBST for 30 - 60 min on a shaker, respective polyclonal antibody (IgG 1:500) prepared against that antigen was added

directly in the blocking solution and further incubated at 4ºC for overnight. The membrane was then washed gently with running tap for 3 minutes, following three times 5 minutes washes in TBST (pH 7.4) (Wakeham and White, 1996). The membrane was then incubated in alkaline phosphatase conjugated goat antirabbit IgG (diluted 1: 10,000 in alkaline phosphatase) for 2 h at 37ºC. 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.

3.25.1.2. ELISA

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

3. 25.1.3. Bacterial colony transfer

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

3.25.2. Antibiotic resistance tests

The resistance of *B. megaterium* and *S. marcescens* against different antibiotics were tested *in vitro*. This was felt necessary because it would help in

determining the sustainability of the applied bacteria in the field. To determine the antibiotic resistance of the two isolates, they were tested by agar cup bioassay, against the following antibiotics: Benzyl Penicillin, Streptomycin, Rifampicin, Tetracycline, Ampicillin, Carbenicillin, Metronidazole, Kanamycin and Chloramphenicol. Different concentrations were prepared from crude capsule of Rifampicin (450 mg), Tetracycline (500 mg). Different disc concentrations of Ampicillin (A^2, A^{10}, A^{25}- 2,10,25 mcg/disc), Chloramphenicol (C^{10}, C^{25}- 10,25 mcg/disc), Kanamycin(K^{30}- 30 mcg/disc) and Metronidazole (Mt^5- 5 mcg/disc) were also used for these bioassays.

3.26. Determination of pathogens in soil

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

3.26.1. Dot blot

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

3.26.2. ELISA

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

3.27. Localization of pathogens in root tissues by light microscopy

Cross sections of infected (tea plants infected with *S. rolfsii*) tea roots were cut and stained with cotton blue and lactophenol. Then the samples were placed under microscope and photographs were taken in order to observe the invasion of mycelia of *S. rolfsii* in infected root tissues.

3. 28. Immunodetection of chitinase in tea plants

3.28.1. Localization of chitinase by immunofluorescence

Indirect fluorescence staining of cross-section of tea roots was done using FITC labelled goat antirabbit IgG following the method of Chakraborty and Saha (1994). Initially, cross sections of healthy and infected tea roots were cut and

immersed in phosphate buffer saline (PBS), pH 7.2. These sections were treated with normal serum or antiserum diluted (1:50) in PBS and incubated for 1h. at room temperature. After incubation, sections were washed thrice with PBS-Tween (pH 7.2) for 15 min and transferred to 40µl of diluted (1:40) goat antirabbit IgG conjugated with fluorescein isothiocyanate (FITC). The sections were incubated for 30 min in dark. After that sections were washed thrice with PBS-Tween as mentioned above and then mounted on a grease free slide with 10% glycerol. Fluorescence of the root section were observed using Leica Leitz biomed Microscope with fluorescence optics equipped with UV-filter set-I-3 and photograph was taken.

3.28.2. Western blot

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

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

(ii) Tranfer buffer (Towbin buffer):
(25mM Tris, 192mM glycine 20% reagent grade Methanol, pH 8.3).
Tris- 3.03g; Glycine- 14.4g; 200 ml Methanol (adjusted to 1lit. with dist. Water).

(iii) Phosphate buffer Saline, PBS, (0.15M, pH 7.2)
Stocks
A. Sodium dihydrogen phosphate- 23.40g in 1000ml dist. water
B. Disodium hydrogen phosphate- 21.294g in 1000ml dist. water
280 ml of stock A was mixed with 720 ml of stock B and the pH was adjusted to 7.2. Then
0.8% NaCl and 0.02% KCl was added to the solution.

(iv) Blocking solution
5% non fat dried milk + 0.02% sodium azide in PBS with 0.02% Tween 20.

(v) Washing buffers:
(a) Washing buffer-1: PBS
(b) Washing buffer-2: (50mM Tris-HCl, 150 mM NaCl, pH 7.5).
Tris- 6.07 g; NaCl- 8.78g; made up to 1lit. with distilled water.

(vi) Alkaline phosphatase buffer:
(100mM NaCl, 5mM $MgCl_2$, Tris- HCl, pH 9.5).

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

(vii) Substrate

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

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

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

phosphatase buffer.

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

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

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

Extraction and estimation of protein

Protein extraction and estimation was done as described earlier.

3.28.2.1. SDS PAGE of protein

SDS-PAGE was carried out as mentioned earlier.

3.28.2.2 Blot transfer process

Following the SDS-PAGE, the gel was transferred in prechilled transfer (Towbin) buffer for 1h. The nitrocellulose membrane (BIO-RAD, 0.45µm) and the filter paper (BIO-RAD, 2mm thickness) were cut to gel size, wearing gloves and soaked in Towbin buffer for 15 min. The transfer process was done in Trans- Blot SD Semi-Dry Transfer cell (BIO-RAD) through BIO-RAD power pack. The presoaked filter paper was placed on the platinum anode of the semi-dry cell. A pipette glass (or glass rod) was rolled over the surface of the filter paper to exclude all air bubbles. The prewetted membrane was placed on top of the filter paper and air bubbles were rolled out. The equilibrated gel was carefully placed on the membrane and air bubbles were rolled out. Finally another presoaked filter paper was placed on the top of gel and air bubbles were removed. The cathode was carefully placed on the sandwich and pressed to engage the latches with the guide posts without disturbing the filter paper stack. The blot unit was run for 45 min at a constant volt (15V). After the run the membrane was removed and dried on a clean piece of 3mm filter paper for 1h. and proceeded for immunological probing.

3.28.2.3. Immunoprobing

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

4.1. Characteristics of *B. megaterium* and *S. marcescens*

4.1.1. Microscopic observation

Bacillus megaterium and *Serratia marcescens* were studied under both light and scanning electron microscope.

4.1.1.1. Light microscopy

Morphological observation of *B. megaterium* and *S. marcescens* showed that *B. megaterium* was rod shaped, Gram +(ve), with wavy cell margin, rough surface and opaque nature in density. *B. megaterium* also produced endospores. *S. marcescens* was small rod shaped, Gram–(ve) with smooth cell margin, smooth surface, opaque nature in density. *S. marcescens* also produced red pigment when maintained in culture at 30ºC for 5-6 days (Figs. 6 & 7, A-D).

4.1.1.2. Scanning electron microscopy

Scanning electron micrographs also confirmed the structure of bacteria- i.e. *B. megaterium* larger rod shaped (size-2µm and width-8.5mm) where as *S. marcescens* was much smaller rod shaped bacterium (size-1µm & width-8.7mm) (Figs.6&7,E-H).

4.1.2. Cultural characteristics

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

4.1.2.1. Incubation period

The growth of *B. megaterium* and *S. marcescens* was found to be best at 48 h. After that growth of both bacteria declined. At 48 h (log phase) log cfu values of *B. megaterium* and *S. marcescens* were found to be highest- 17.2 log cfu/ml broth and 16.42 cfu/ml broth respectively (Fig. 8A).

4.1.2.2. pH

B. megaterium and *S. marcescens* grew best at pH 6.0 with log cfu values of 22.8 log cfu/ml and 23.07 log cfu/ml (Fig. 8B).

4.1.2.3. Temperature

The growth of these bacteria was observed at different temperatures ranging from 20 to 50ºC. Both the bacteria grew best at 35ºC with 18.81 log cfu/ml and 16.69 log cfu/ml in case of *B. megaterium* and *S. marcescens* respectively (Fig.8C).

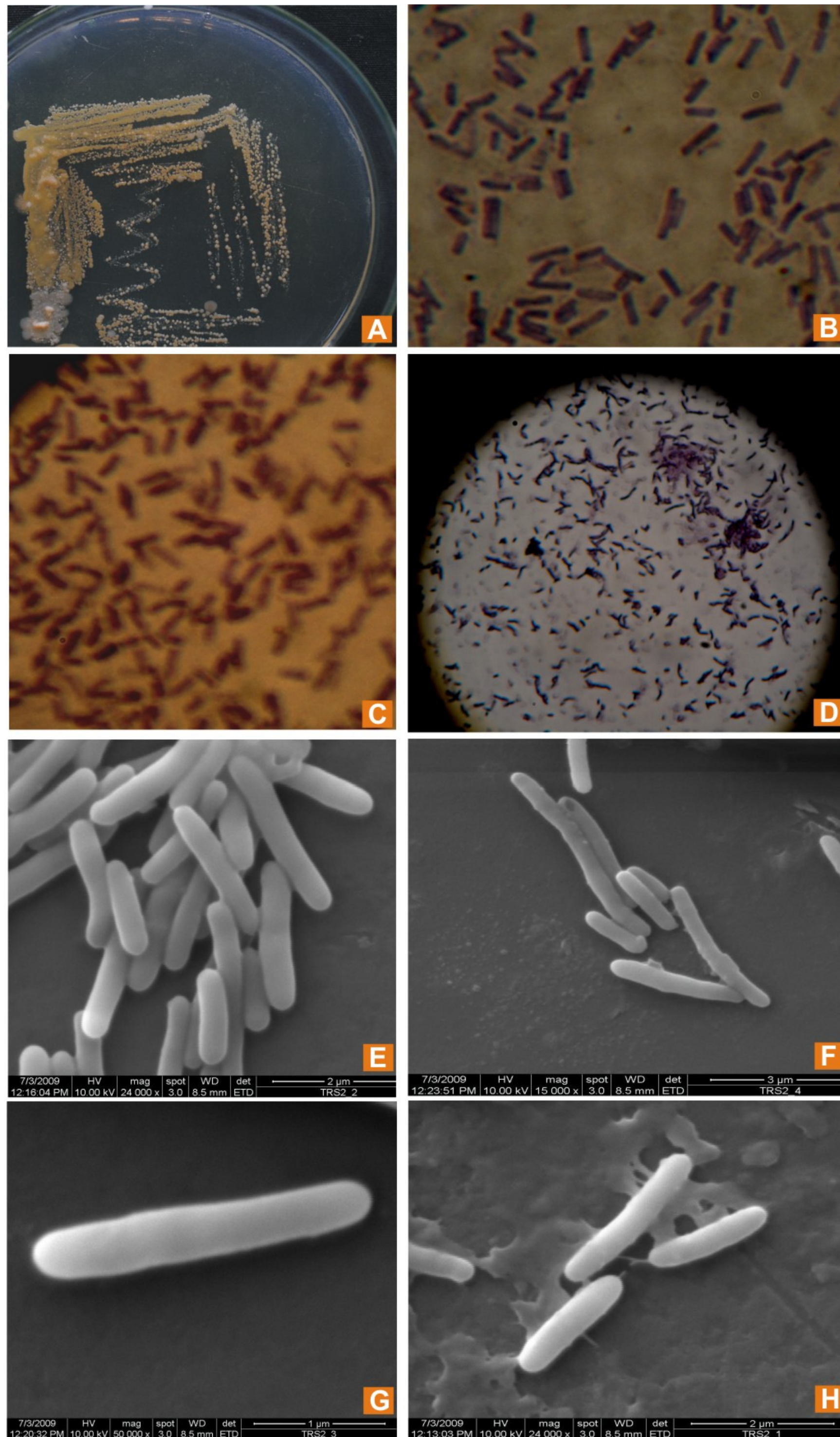


Figure 6: A-H: *Bacillus megaterium*. A- streak, B&C-microscopic view in100X in oil immersion, D-40X and E-H- scanning electron micrographs.

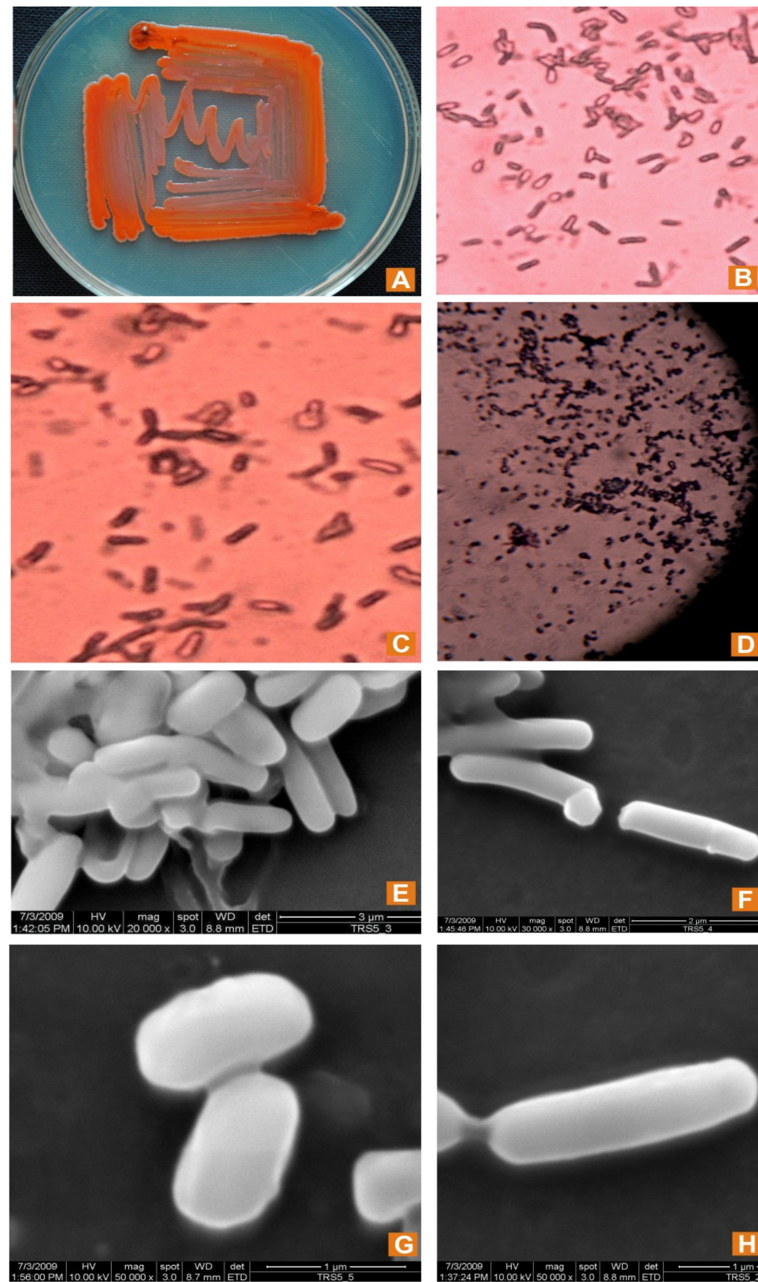


Figure 7: A-H: *Serratia marcescens*. A- streak, B&C- microscopic view in 100X in oil immersion, D- 40X and E-H- scanning electron micrographs.

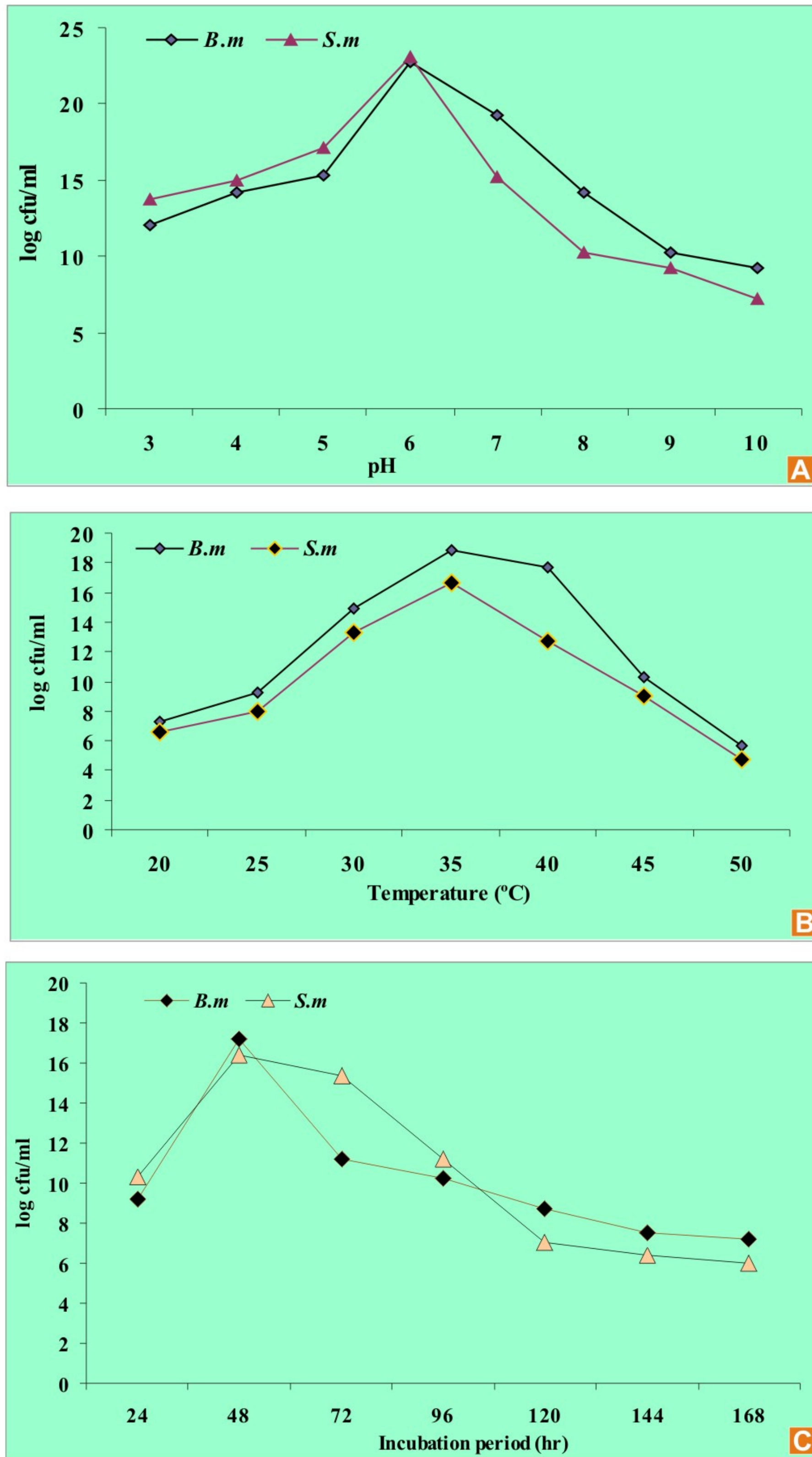


Figure 8: Effect of pH (A), temperature (B) and incubation period (C) on growth of *B. megaterium* and *S. marcescens*.

4.1.3. Molecular analyses

4.1.3.1. Protein pattern

Soluble proteins were extracted from cells of *B. megaterium* and *S. marcescens*, arrested at 48 h in the log phase. In case of *B. megaterium*, whole cell protein (sonicated), extracellular protein (supernatant after sonication) and also pellet dissolved in 0.05(M) sodium-phosphate buffer, pH 7.2 after sonication were loaded on SDS gel as described in Materials and Methods. Protein band patterns in all lanes were similar except the differences in band intensities (Fig. 9, A&B). The molecular weight of protein bands visualized after staining with coomassie blue were determined from the known molecular weight marker. Whole cell protein of *B. megaterium* exhibited 15 bands in SDS-PAGE ranging in molecular weight (12,13,14.3,20,22,26,29,38,40,41,42,43,66,68,95,97kDa) and bands were of varying intensities and more proteins of lower molecular were present (Plate VII, A). Similarly, 42µg of whole cell proteins of different isolates of *S. marcescens* (MTCC 86, 97, 7298, 2645, 4822, 7103, 4301 and 3124) along with original isolated *S. marcescens* (TRS 1) were loaded in lanes. SDS gel of different isolates of *S. marcescens* showed that there were slight differences in band patterns and intensites among them (Fig. 10, A&B). Whole cell proteins of different isolates of *S. marcescens* exhibited 6-9 bands with molecular weights of 13,14.3,19,29,38,43,68,95 KDa (*S. marcescens* TRS 1 and MTCC 86 isolate), 13,14.3,19,29,38,43 KDa(MTCC 97, 4822 and 7103 isolates), 13,14.3,19,20,29,38,68,95 KDa (MTCC 7298), 13,14.3,19,29,38,95 KDa (MTCC 2645), 14.3,20,29,42,43,67,68,95 KDa (MTCC 4301) and 14.3,29,41,42,43,67,68,95,97 KDa (MTCC 3124).

4.1.3.2. 16 S rDNA sequencing and diversity analysis

The BLAST query of 16S r DNA sequence of the isolates against GenBank database confirmed their identity. The sequences have been deposited in NCBI, GenBank database under the accession Nos. JX 312687.1 and JN 020963.1 for *B.megaterium* and *S. marcescens* respectively. The sequenced PCR product was aligned with extype isolate sequences from NCBI GenBank for identification as well as for studying phylogenetic relationship with other ex-type sequences. The phylogenetic analyses conducted using the UPGMA method among the isolates of *S. marcescens* with other ex-type strains obtained from NCBI GeneBank database by MEGA4.1 software. The evolutionary history was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 2.19563315 is shown. The percentage of

replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura *et al.* 2004) and are in the units of the number of base substitutions per site. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 56 positions in the final dataset (Figs. 11 & 12). The evolutionary history was also inferred using the UPGMA method among the isolates of *Bacillus megaterium.* The optimal tree with the sum of branch length = 0.02325875 is shown. A partial chromatogram of the 16S rDNA gene sequenced. These sequences are presented in Figs. (13 & 14). 16S rDNA sequence alignments of *S. marcescens* with extypes isolates and *B. megaterium* with extypes isolates were also shown. Multiple sequence alignment revealed that there were regions in the sequences which were not similar and, hence, gaps were introduced in these regions. Presence of regions with similar sequences indicated relationships among the isolates of *B. megaterium* and the isolates of *S. marcescens* separately. The conserved regions of the gene were demonstrated in different colour (Fig.15 A&B, Tables 1&2).

4.1.3.3. DGGE

Genetic diversity among the isolates of *B. megaterium* (**JX 312687**, MTCC 428, MTCC 1684 and MTCC 2949) and *S. marcescens* (**JN 020963**, MTCC 86, MTCC 97, MTCC 2645, MTCC 3124, MTCC 4301, MTCC 4822 and MTCC 7298) obtained from tea rhizosphere were analyzed on the basis of DGGE analysis of 16 S rDNA sequences. The 16S rDNA regions were flanked by T397F-5'GATGAAGAAGGCAGCGAAATGCGATA-3' and reverse primer sequence T397F-5' CGTATTACCGCGGCTGCTGG-3'. DGGE gels were run at 110 V for 06 hours in 1X TAE buffer (pH 8.0) at 60°C and stained with ethidium bromide. DNA bands on the DGGE gels were excised under UV trans-illumination. The gel photographs were taken and analysed. In this uniform gradient gel of 0% to 100%, the banding pattern among the four isolates of *B. megaterium* and the eight isolates of *S. marcescens* were identical to that of the reference isolates used for conformation (Fig. 15 C). The isolates of *B. megaterium* (JX312687) and *S. marcescens* (JN 020963) were further confirmed by DGGE.

Table 1: Identified *Serratia marcescens* and comparison with referred NCBI GenBank

Strain No	GenBank accession no	Identified as	Country of Origin	Identity (%)
H3010	EF194094.1	*Serratia marcescens*	China	99%
NIOB 798	EU816970.1	*Serratia marcescens*	India	99%
818A	DQ904615.1	*Serratia marcescens*	USA	99%
HPC1387	DQ059503.1	*Serratia marcescens*	India	99%
Clone:7568	AB498898.1	*Serratia marcescens*	Japan	99%
Clone: 7534	AB498897.1	*Serratia marcescens*	Japan	99%
BC16	FM163485.2	*Serratia marcescens*	Vietnam	99%
TRS-1	**JN020963.1**	***Serratia marcescens***	**India**	**99%**
KR-4	DQ866838.1	*Serratia marcescens*	India	99%
L1	EF208031.1	*Serratia marcescens*	China	99%
CC-SB818H2a	AY337583.1	*Serratia marcescens*	Taiwan	99%
PCPTE1	AF536218.1	*Serratia marcescens*	India	99%
clone HQ260324.1	FR865468.1	*Serratia marcescens*	India	99%

Table 2: Identified *Bacillus megaterium* and comparison with referred NCBI GenBank

Strain No	GenBank accession no	Identified as	Country of Origin	Identity (%)
MSBN04	HQ874436.1	*Bacillus megaterium*	India	99%
Z8B-36	HQ238702.1	*Bacillus megaterium*	China	99%
S433Ba-73	HQ238636.1	*Bacillus megaterium*	China	99%
Z4B-24	HQ238516.1	*Bacillus megaterium*	China	99%
tu35	FJ544404.1	*Bacillus megaterium*	China	99%
tu12	FJ544394.1	*Bacillus megaterium*	China	99%
st15	FJ544368.1	*Bacillus megaterium*	China	99%
821	FJ544337.1	*Bacillus megaterium*	China	99%
811	FJ544328.1	*Bacillus megaterium*	China	99%
TRS 7	**JX312687.1**	***Bacillus megaterium***	**India**	**94%**
YJ6	JQ790514.1	*Bacillus megaterium*	India	99%
GZC-1	JQ917435.1	*Bacillus megaterium*	China	99%
108	AB334764.1	*Bacillus megaterium*	Japan	99%
EN2	JN642548.1	*Bacillus megaterium*	India	99%
Pb-WC11121	JX913831.1	*Bacillus megaterium*	China	99%
CC-YY12	AY961982.1	*Bacillus megaterium*	Taiwan	99%

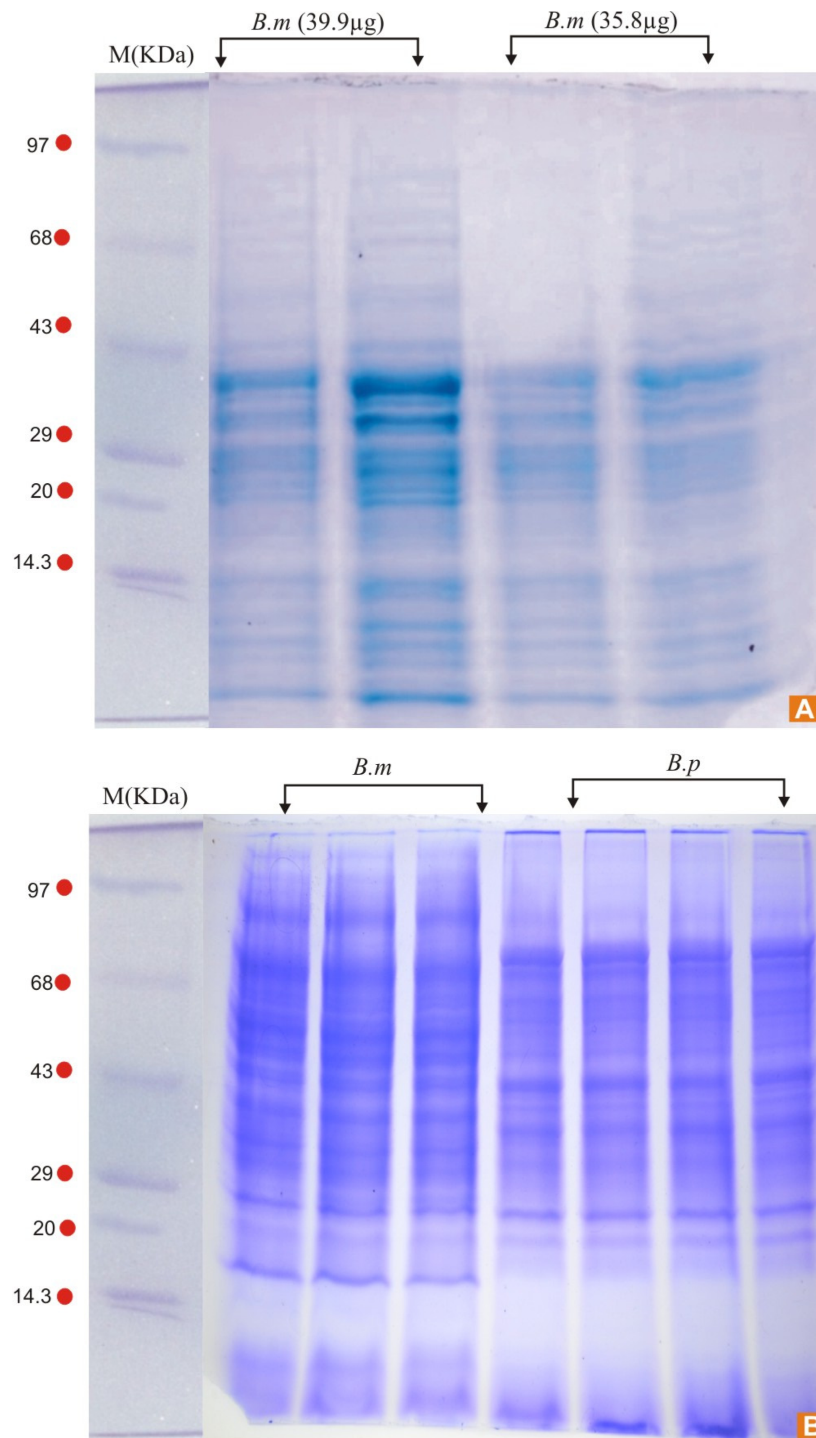


Figure 9: A&B: SDS-PAGE analysis of proteins of *B. megaterium.* A:different protein concentrations; B: comparison with extracellular proteins of *B. pumilus.*

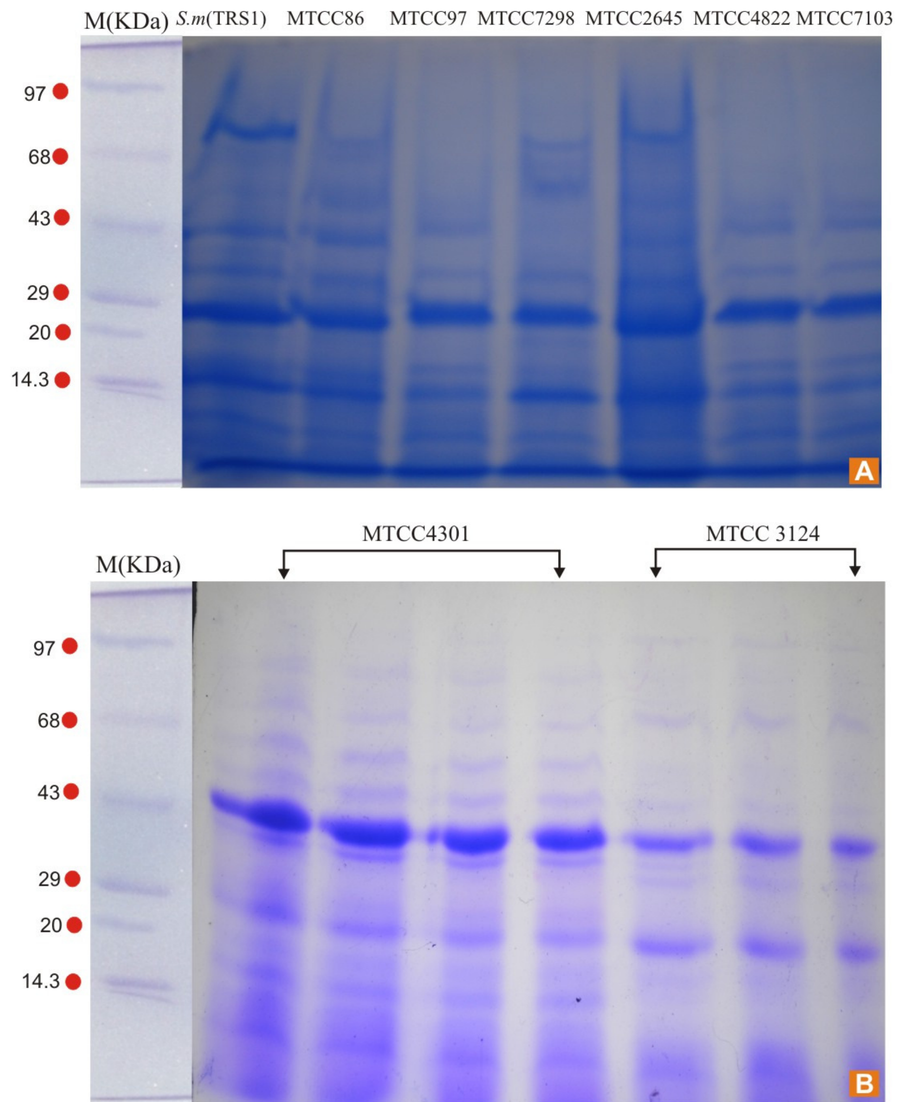


Figure 10: A&B: SDS-PAGE analysis of proteins of various isolates of *S.marcescens.*

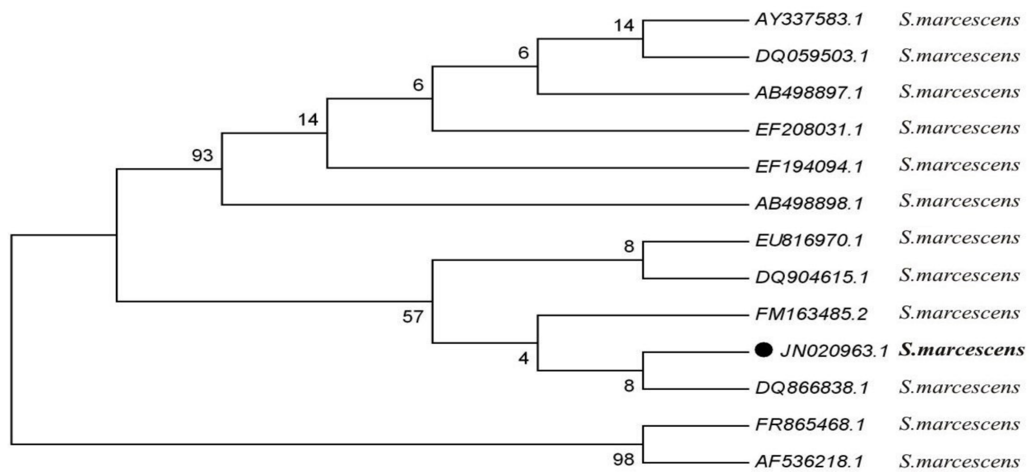


Figure 11: Phylogenetic analysis of 16S rDNA sequences of *Serratia marcescens* (JN 020963) with other ex-type strains obtained from NCBI GeneBank database by MEGA4.1 software.

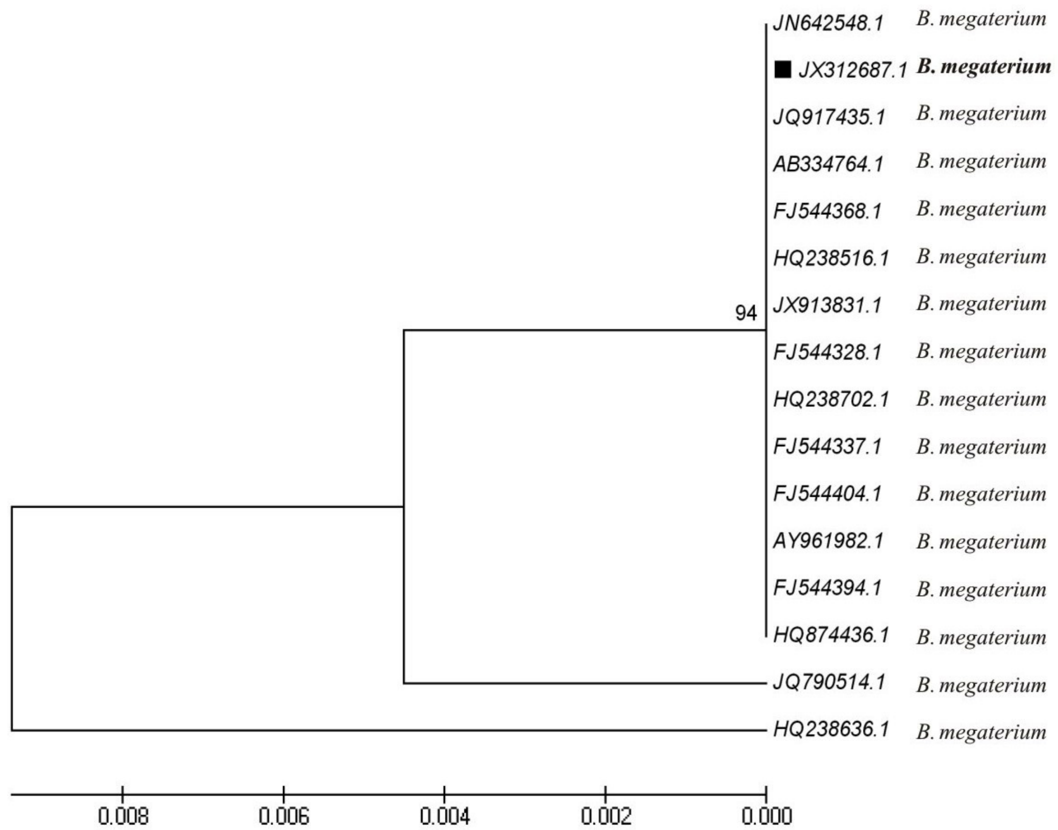
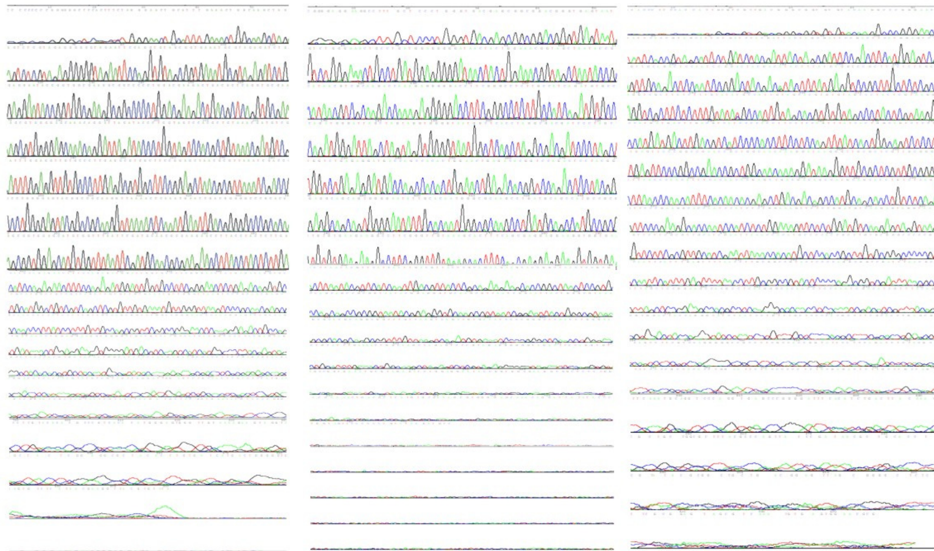


Figure 12: Phylogenetic analysis of 16S rDNA sequences of *Bacillus megaterium* (JX 312687) with other ex-type strains obtained from NCBI GeneBank database by MEGA4.1 software.

Chromatogram



Partial sequence of 16S rDNA region

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GCCCAGATGGGATTAGCTAGTAGGTGGGGTAATGGCTCACCTAGGCGACGATCCCTAGCTGGTCTGAGAGGATGACCAGCCA
CACTGGAACTGAGACACGGTCCAGACTCCTACG GGAGGCAGCAGTGGGGAATATTGCACAATGGGCGCAAGCCTGATGCAG
CCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAACTTAATACGTTCATC
AATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCG
GAATTACTGGGCGTAAAGCGCACGCAGGCGGTTTGTTAAGTCAGA TGTGAAATCCCCGGGCTCAACCTGGGAACTGCATTTG
AAAACTGGCAAGCTAGAGTCTCGTAGAGGGGGGTAGAATTCCAGGTGTAGCCGGTGAAATGCGTAGAGATCTGGAGGAATA
CCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTG
GTAGTCCACGCTGTAAACGATGTCGATTTGGAGGTTGTGCCCTTGAGGCGTGGCTTC CGGAGCTAACGCGTTAAATCGACCG
CCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTC
GATGCAACGCGAAGAACCTTACCTACTCTTGACATCCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGAGA
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTTGTGAAATGTTGGGTTAAGTCCCGCAACGAGCGCAAC CCTTATCCTTTGTTG
CCAGCGGTTCGGCCGGGAACTCAAAGGAGACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGC
CCTTACGAGTAGGGCTACACACGTGCTACAATGGCATATACAAAGAGAAGCGACCTCGCGAGAGCAAGCGGACCTCATAAA
GTATGTCGTAGTCCGGATTGGAGTCTGCAACTCGACTCCATGAAGTCGGAATCGCTAGTAATCGTAGATCAGAATGCTA CGG
TGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCAACGACGCAATGGTGGAAGGGTGT

Sequence Deposited: NCBI Title: *Serratia marcescens* strain TRS-1 16S ribosomal RNA gene, partial sequence.

ACCESSION: **JN020963**
VERSION: JN020963.1
GI: 339521472 ORIGIN

1 cggggaggaa ggcctttgct ccctgggtga cgagcggcgg acgggtgagt aatgtctggg
61 aaactgcctg atggaggggg ataactactg gaaacggtag ctaataccgc ataacgtcgc
121 aagaccaaag agggggacct tcgggcctct tgccatcaga tgtgcccaga tgggattagc
181 tagtaggtgg ggtaatggct cacctaggcg acgatcccta gctggtctga gaggatgacc
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421 tcgcagaaga agcaccggct aactccgtgc cagcagccgc ggtaatacgg agggtgcaag
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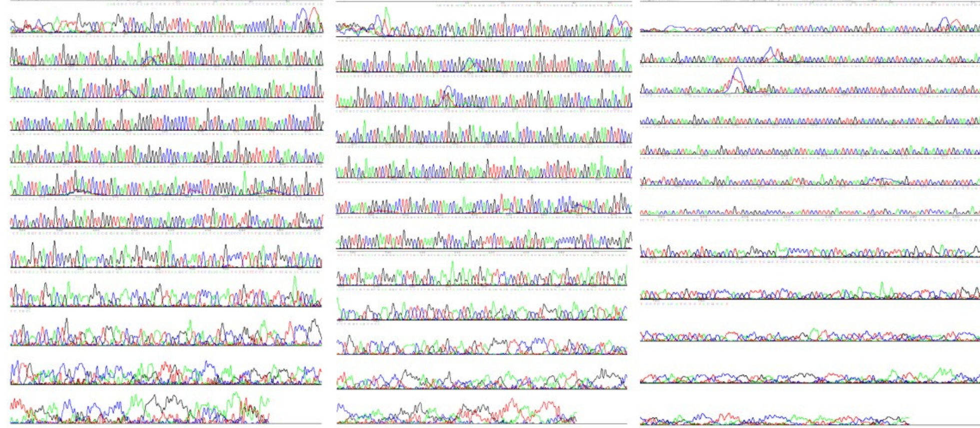
1081 tcaaaggaga ctgccagtga taaactggag gaaggtgggg atgacgtcaa gtcatcatgg
1141 cccttacgag tagggctaca cacgtgctac aatggcatat acaaagagaa gcgacctcgc
1201 gagagcaagc ggacctcata aagtatgtcg tagtccggat tggagtctgc aactcgactc
1261 catgaagtcg gaatcgctag taatcgtaga tcagaatgct acggtgaata cgttcccggg
1321 ccttgtacac accgcccgtc aacgacgcaa tggtggaagg gtgt

Figure 13: Chromatogram and sequence deposition of 16S rDNA region of *Serratia marcescens* (TRS 1)

Chromatogram



Partial sequence of 16S rDNA region

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ggttcggctacacttacaatggcccgcgggcattagctagttggtgaggtaacggctcaccaaggcacgatgcgtagccgacctgagagggtgatcggccacac
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gtaacacccgaagtcggtgg

Sequence Deposited: NCBI Title: *Bacillus megaterium* strain TRS-7 16S ribosomal RNA gene, partial sequence.

ACCESSION: **JX312687**
VERSION: JN312687.1
GI: 402693811 ORIGIN

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1141 gttcggatga gctgcaactc gctctgaagc tggaatcgct agtaatcgcg gatcagcatg
1201 ccgcggtgaa tacgttcccg ggccttgtac acaccgcccg tcacaccacg agagtttgta
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Figure 14: Chromatogram and sequence deposition of 16S rDNA region of *Bacillus megaterium* (TRS 7)

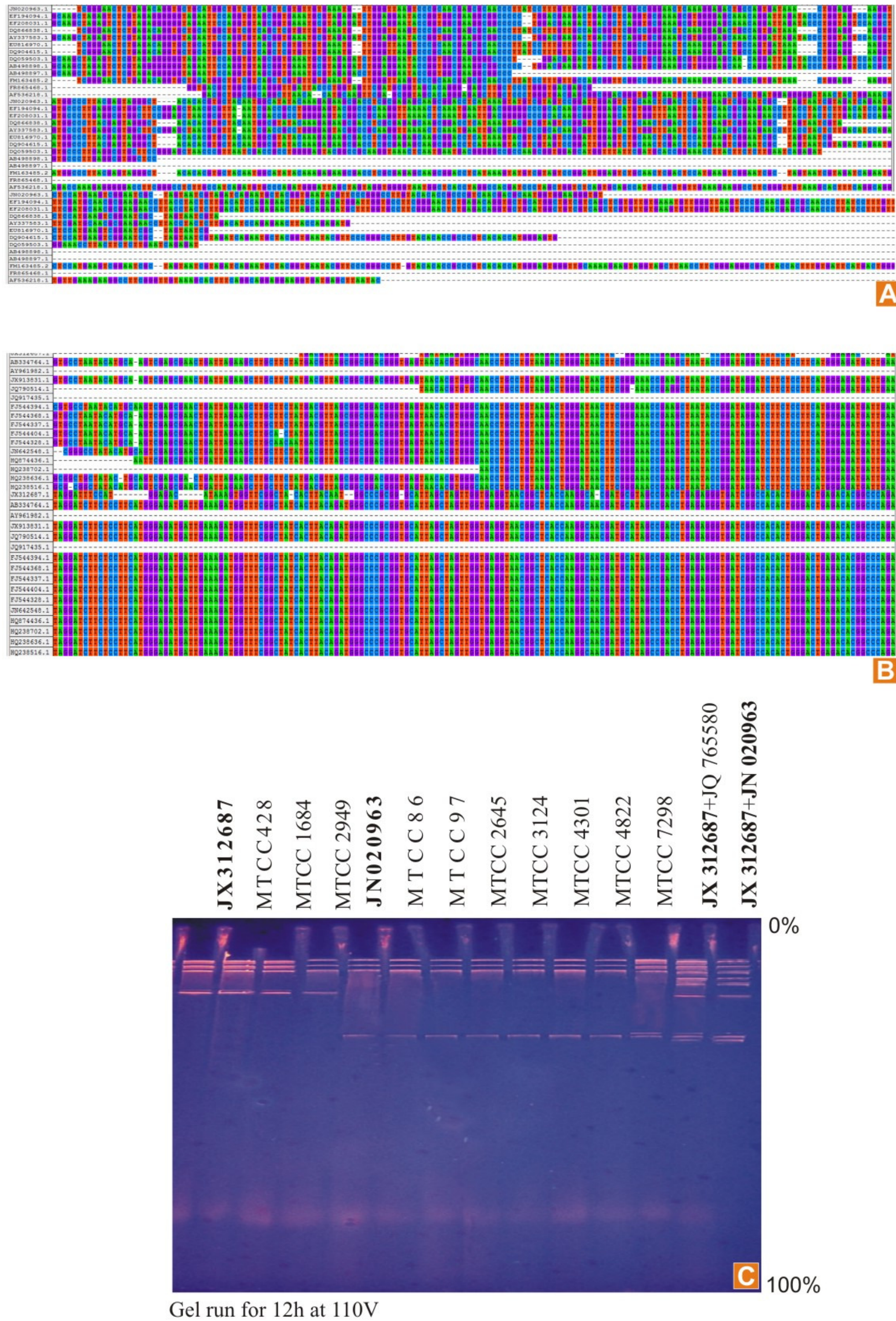


Figure 15: A-C: 16S rDNA sequence alignments of *Serratia marcescens* (JN020963) with extypes isolates. The conserved regions of the gene are demonstrated in different colour (A), 16S rDNA sequence alignments of *Bacillus megaterium* (JX312687) with extypes isolates. The conserved regions of the gene are demonstrated in different colour (B), Genetic diversity among the four isolates of *B. megaterium* (**JX 312687**, MTCC 428, MTCC 1684 and MTCC 2949) and eight isolates of *S. marcescens* (**JN 020963**, MTCC 86, MTCC 97, MTCC 2645, MTCC 3124, MTCC 4301, MTCC 4822 and MTCC 7298) on the basis of DGGE analysis of 16S rDNA sequences (C) .

4.2. *In vitro* PGPR activities of the two bacteria

In order to determine whether the bacteria possess plant growth promoting activities, initially several *in vitro* tests were conducted and results are presented below (Table 3 and Fig. 16, A-F).

4.2.1. Siderophore production

Siderophore production by bacterial strains was detected by growing the bacteria individually in chrome azurol S agar plate. The plates were observed for 10-15 days after inoculation with bacteria. The appearance of yellow halo region was observed around both *B. megaterium* and *S. marcescens* which indicated that both the bacterial isolates were able to chelate Fe^{3+} from chrome azurol S agar. The diameters of halo region were 1.8 cm and 2.0 cm for *B. megaterium* and *S. marcescens* respectively after 12 days of incubation (Table 3; Fig.16, A&B).

4.2.2. Phosphate solubilisation

Formation of clear zone around the colony grown in Pikovskaya's medium is an indication of phosphate solubilisation by rhizobacteria. In Pikovskaya's medium *B. megaterium* and *S. marcescens* produced clear zones of 1.7cm and 2.1cm diameter after 5-7 days of incubation, indicating that both the isolates could solubilise insoluble phosphate (Table 3; Fig. 16, C&D).

4.2.3. Protease production

The bacteria were spot inoculated in Skim milk agar medium and incubated at 30ºC for 5-7 days. The appearance of clear region was observed around both *B. megaterium* and *S. marcescens* which indicated that both the bacterial isolates were able to produce protease. The diameters of clear zone were 2.8 cm and 3.9 cm for *B. megaterium* and *S. marcescens* respectively (Table 3; Fig.16, E&F).

4.2.4. IAA production

Both the bacterial strains were assessed for their ability to produce indole acetic acid by growing them in Nutrient Broth/ Luria Bertani Broth supplemented with tryptophane (0.1 mM). For quantification, HPLC analysis of IAA from *B. megaterium* and *S. marcescens* was done by injecting 10µl of the filtered extracts onto a (C18,5µm 25×0.46 cm) in a chromatograph equipped with a differential ultraviolet detector absorbing at 280 nm. Mobile phase was methanol: H_2O- 80:20

(vol:vol), flow rate- 1.5ml/min. Retention times for peaks were compared to IAA standard (peak at retention time of 2.5 min for IAA standard) and quantified. *B. megaterium* recorded IAA production of 0.05 mg/ml. *S. marcescens* was found to produce 0.03 mg/ml (Fig. 17 A&B).

4.2.5. HCN production

To determine the ability of *B. megaterium* and *S. marcescens* to produce Hydrocyanic acid (HCN) the bacteria were grown in medium amended with glycine. Results were observed after 4-7 days. Both *B. megaterium* and *S. marcescens* were found to be non-cyanogenic in nature (Table 3).

4.2.6. Chitinase production

The bacteria were spot inoculated in the 5 % colloidal chitin amended minimal medium and incubated at 30ºC for 7-10 days. It was observed that no extracellular chitinase was secreted by *B. megaterium* even when grown on chitin amended medium. But *S. marcescens* secreted extracellular chitinase indicating a clear zone around the colony of the bacterium on CDA plate (Table 3).

4.2.7. Lipase production

B. megaterium and *S. marcescens* showed negative responses for lipolytic activities (Table 3).

Table 3: *In vitro* PGPR characteristics of *B. megaterium* and *S. marcescens*

Characteristics	***B. megaterium***	***S.marcescens***
Phosphate solubilisation	+	+
Siderophore production	+	+
Protease production	+	+
Chitinase production	-	+
HCN production	-	-
Volatile production	+	+
IAA production	+	+

+= activity present; - = activity absent

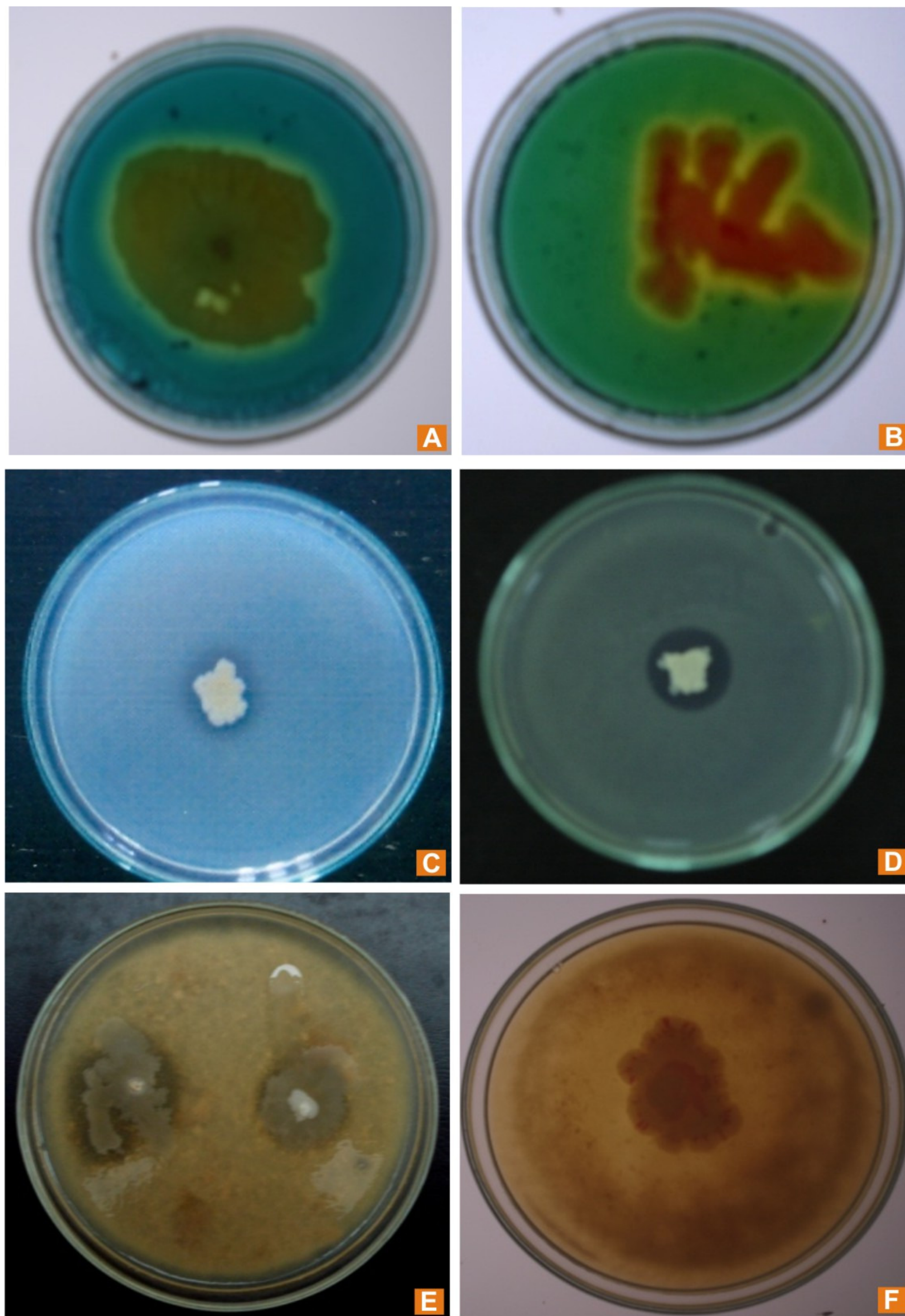


Figure 16: *In vitro* PGPR activities of *B. megaterium* (A,C&E) and *S. marcescens* (B,D&F). A&B-siderophore formation in CAS medium; C&D-phosphate solubilisation in PKV medium; E&F- protease production in skim milk agar.

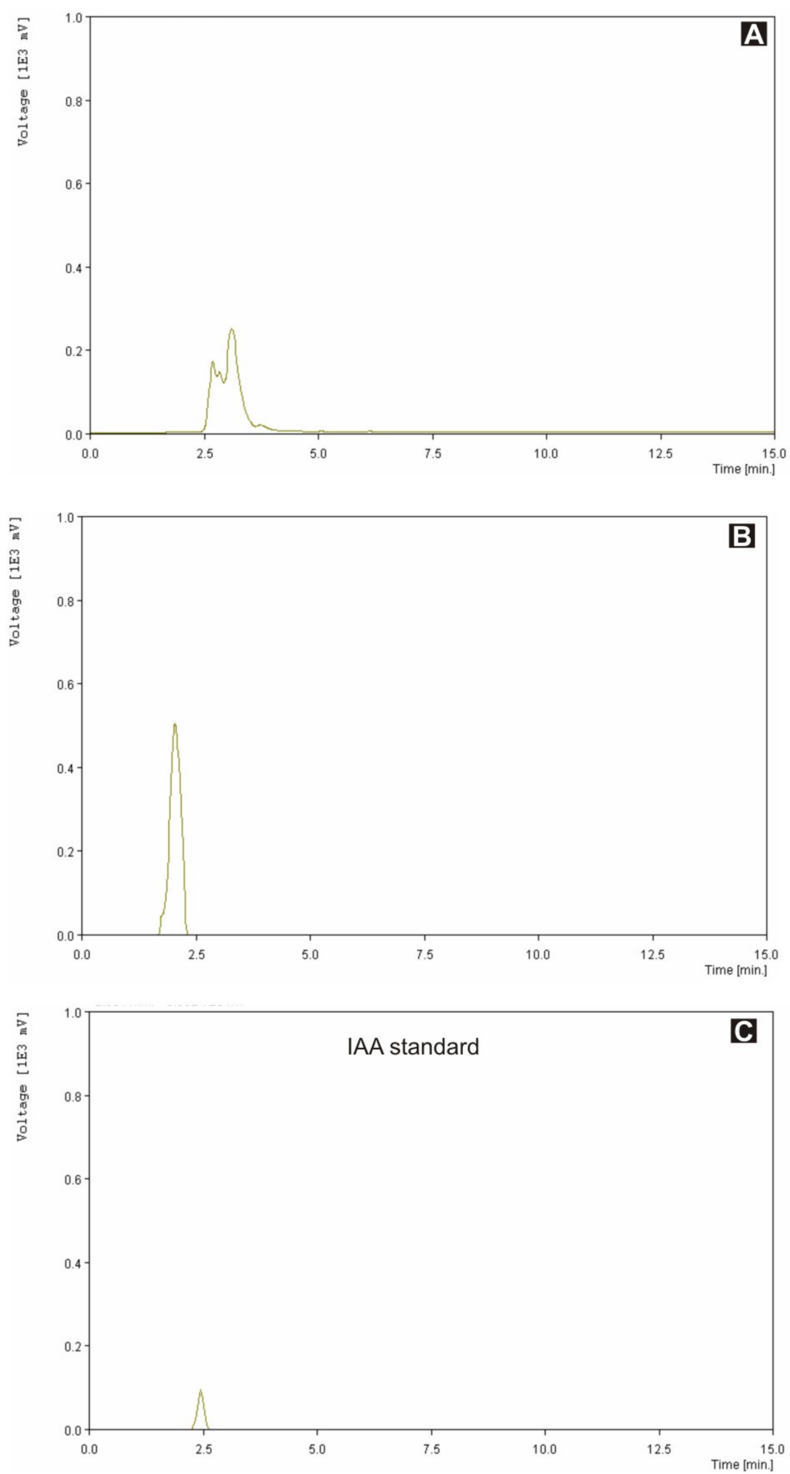


Figure 17: HPLC profile of IAA from *B. megaterium* (A) and *S. marcescens* (B) and standard IAA (C).

4.2.8. Antagonistic activity

The antagonistic effect of the two bacteria against several fungi were tested by dual culture methods in solid medium. In solid medium, inhibition of the growth of different fungal pathogens by the antagonistic bacteria and the zone of inhibition were recorded. The results revealed that *B. megaterium* inhibited the growth of all test pathogens *viz. Sclerotium rolfsii, Rhizoctonia solani, Fusarium solani* and *Fusarium graminearum. S.marcescens* also checked the growth of the tested pathogens significantly. All the tested fungi were inhibited to some degree, the percentage inhibition varying between 57-84% by *B. megaterium* and 27-75% by *S. marcescens* (Table 4; Figs. 18 & 19, A-I). Effect of culture filtrate of *B. megaterium* and *S. marcescens* in inhibiting one of the notorious root pathogen- *S. rolfsii* in terms of inhibition of germination of sclerotia was noted. Sclerotial germination of *S. rolfsii* with cell free culture filtrates of *B. megaterium* and *S. marcescens* showed about 90-95% inhibition in comparison to control (Fig. 20).

Table 4. *In vitro* antagonistic tests of *S. marcescens* and *B. megaterium* against test pathogens**.**

Test fungi	Paired with	Dia. of fungal growth (cm)*	Zone of inhibition (cm)	% of inhibition	Mycelial dry wt.(mg)
R. solani	-	9.2±0.21	-	-	251±3.02
	S. marcescens	2.1±0.22	1.7±0.09	75.0±2.91	97±2.65
	B. megaterium	1.5±0.09	2.0±0.25	83.5±2.66	102±2.23
F. oxysporum	-	8.6±0.20	-	-	281±3.23
	S. marcescens	3.8±0.09	1.6±0.09	58.9±2.27	170±2.89
	B. megaterium	2.9±0.20	1.7±0.05	71.6±2.80	103±2.56
F. graminearum	-	8.5±0.34	-	-	278±3.24
	S. marcescens	5.6±0.55	1.1±0.09	38.0±2.34	151±2.67
	B. megaterium	3.0±0.32	2.0±0.24	57.3±1.94	110±2.21
S. rolfsii	-	8.8±1.3	-	-	465±3.35
	S. marcescens	6.2±0.16	0.2±0.02	27.0±1.00	254±2.43
	B. megaterium	1.4±0.29	2.1±0.22	84.09±2.66	191±2.96

*After 7 days

4.3. Effect of application of bacteria on growth of tea plants

4.3.1. Saplings

The growth promotion of different varieties of tea sapling was observed in terms of increase in height of saplings, 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 months of application of bacteria to the soil were calculated. The results showed that both *B. megaterium* and *S. marcescens* efficiently promoted growth in tea plants irrespective of their variety. Since both the selected bacterial strains showed plant growth promoting activity, it was decided to co-inoculate the bacteria to determine if they show any synergistic growth promoting activity. Accordingly, bacteria grown in broth were applied individually as well as in different combinations to the young saplings of five varieties of tea (TV-18, TV-23, TV-25, TV-26 and T-17). The growth of the saplings was noted in each case and increase in growth rate was computed. It was observed that in relation to control, tea plants subjected to all treatments showed increased growth rate. It was further observed that percentage increase in height and number of leaves was greater when two bacteria were applied together in comparison to individual application. The growth was two folds higher than in control plants (Fig.21, A-H).

4.3.2. Potted plants

Bacterial suspensions of *B. megaterium* and *S. marcescens* were also applied to the rhizosphere of two year old potted plants at a regular interval of 7-10 days under same environment and physical conditions. Growth promotion of different varieties by individual bacterium was noted as compared to untreated control in terms of % increase in height, % increase in no of leaves and leaf dry mass. Observations were recorded after 2 months of 1st application. Results revealed an increase in all the parameters by single as well as dual application of bacteria. Statistical analysis (ANOVA) revealed that increase in all three parameters were significantly enhanced by the treatments. Initial differences among the plants selected for the study were not significant (Fig. 22, A-I; Fig. 23; Tables 5&6).

4.3.3. Field grown plants

Growth promotion was studied in terms of % increase in no of branches, height after 2 months of application and increase in leaf growth after 6 and 12 months of application in comparison to control plot in the experimental field. Each treatment was carried out under same physical and environmental condition. Results revealed that all 3 treatments increased no of branches, leaf growth as well as height of the plants. Analysis of variance showed significant differences between control and treatments, but there were no significant differences among the treatments (Fig. 24, A-H; Figs. 25&26; Table 7).

Figure 18: A-I: *In vitro* antagonistic tests of *S. marcescens* against test pathogens. *S. marcescens* paired with *Sclerotium rolfsii* (B), *Rhizoctonia solani* (D), *Fusarium graminearum* (F) and *F. oxysporum* (H) with A,C,E and G being respective controls.

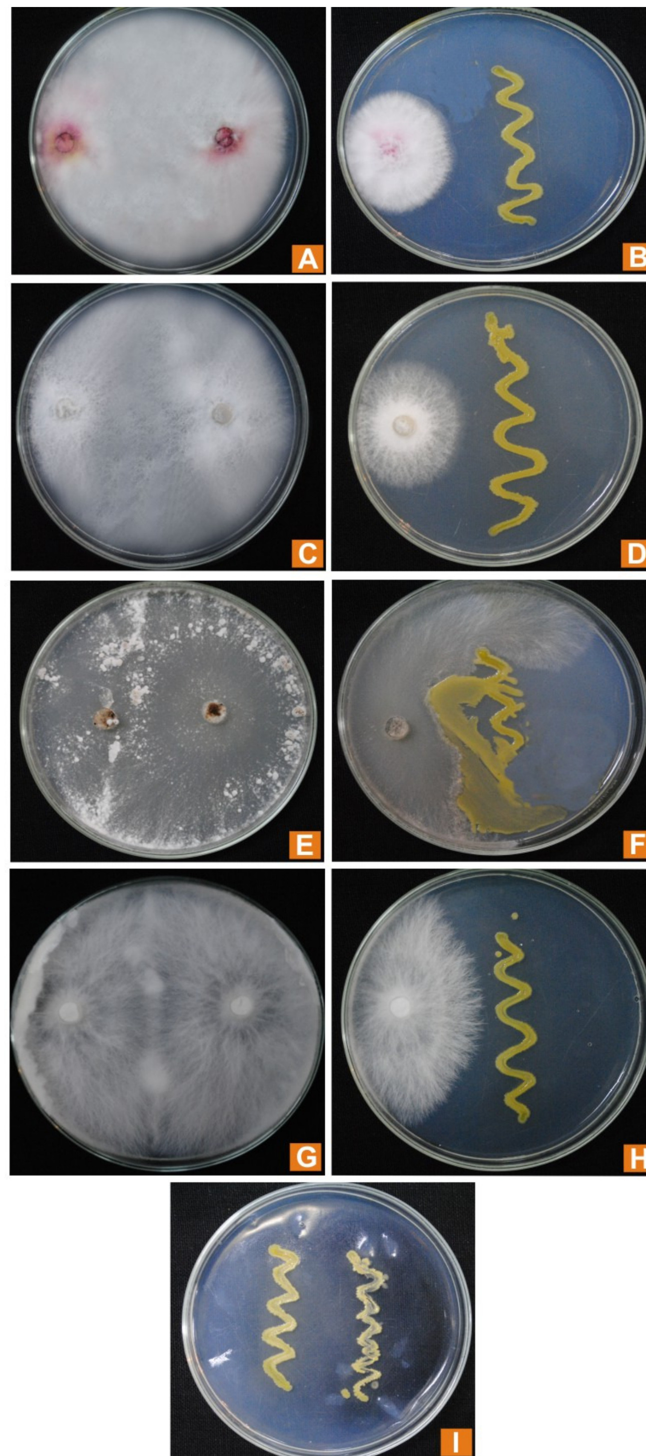


Figure 19: A-I: *In vitro* antagonistic tests of *B. megaterium* against test pathogens. *B. megaterium* paired with *Sclerotium rolfsii* (H), *Rhizoctonia solani* (F), *Fusarium graminearum* (B) and *F. oxysporum* (D) with G,E,A and C being respective controls.

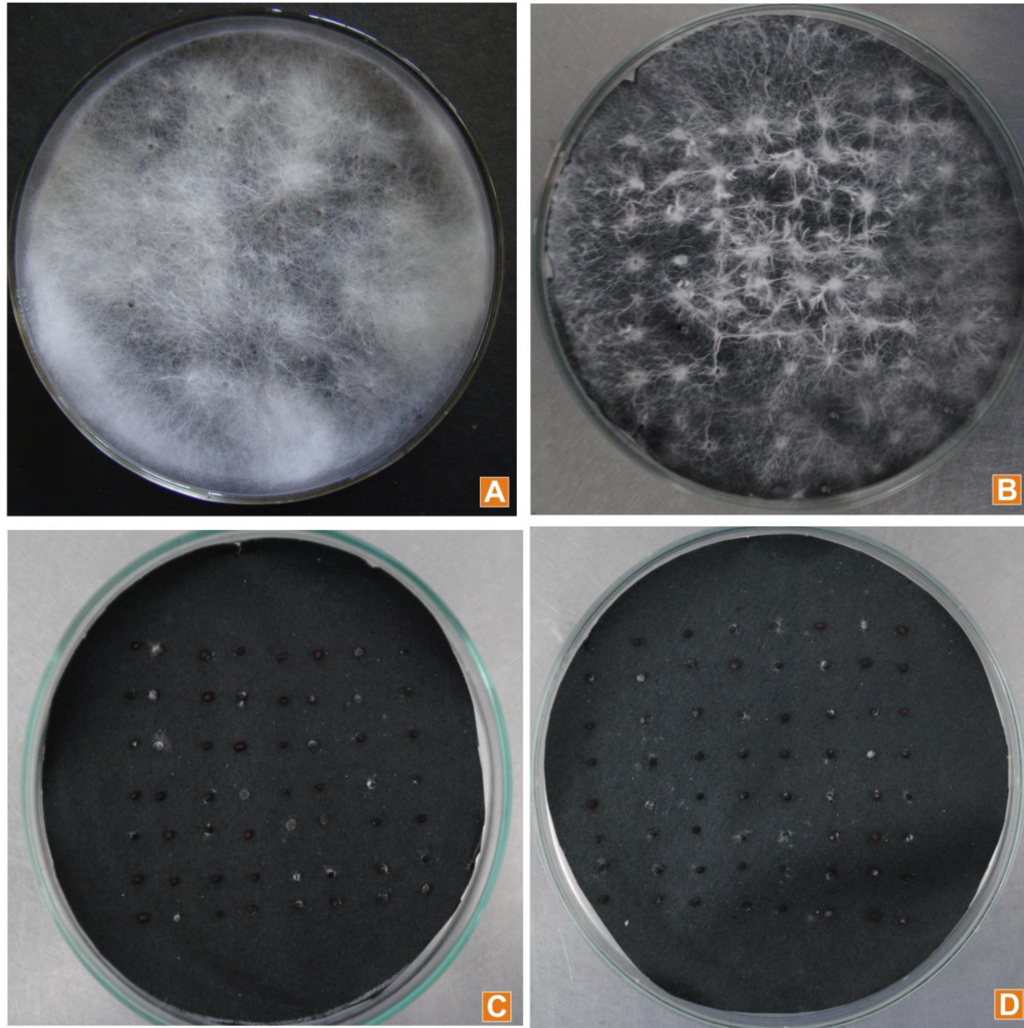


Figure 20: A-D: Effect of culture filtrates of *B. megaterium* and *S. marcescens* on sclerotial germination and mycelial growth of root pathogen- *Sclerotium rolfsii* . Water control (A); Media control(B); sclerotia treated with culture filtrate of *B. megaterium* (C) and sclerotia treated with culture filtrate of *S. marcescens* (D).

Figure 21: A-H: Effect of *B. megaterium* and *S. marcescens* on growth of tea saplings grown in sleeves. A&B:general view; C-E: Effect of bacteria on growth of TV-18 variety;F-H: Effect of *B. megaterium* and *S. marcescens* on growth of TV-23 variety.

Figure 22: A-I: Growth of tea plants in potted conditions following application of PGPR. TV-18(A-C), TV-23 (D-F) and T-17(G-I) varieties.

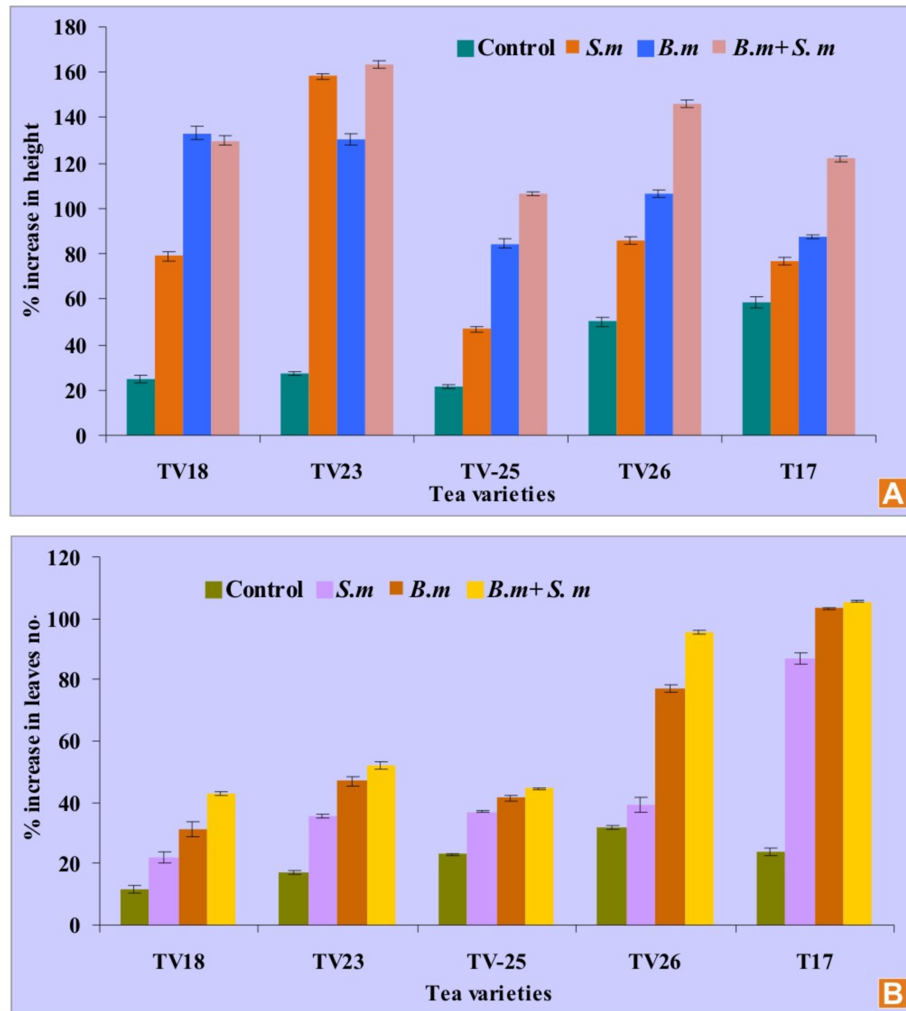


Figure 23: Effect of application of *B. megaterium* and *S. marcescens* on growth of potted tea plants after 2 months (% increase in height-A,% increase in leaf number-B).

Table 5: Effect of application of *Serratia marcescens* and *Bacillus megaterium*, singly or jointly on growth in potted tea plants

Tea varieties	Treatment	2 months after treatment		2 months after treatment	
		Initial height (cm)	**Final height(cm)**	**Initial leaf numbers**	**Final leaf numbers**
TV-18	Control	8.0±1.1	10.0±1.2 (2.0)	26.0±1.9	29.0± 1.6(3.0)
	S.marcescens	9.5±2.3	17.0± 1.4(7.5)	27.0±2.0	33.0± 1.2(6.0)
	B.megaterium	9.0±1.5	21.0± 1.5(12.0)	29.0±1.7	38.0±1.4(9.0)
	B.megaterium +S.marcescens	10.0±1.8	23.0± 1.5(13.0)	28.0±1.8	40.0± 2.8(12.0)
TV-23	Control	11.0±1.0	14.0± 1.3(3.0)	29.0±1.9	34.0± 1.8(5.0)
	S.marcescens	12.0±1.3	31.0± 2.1(19.0)	31.0±2.3	42.0± 2.6(11.0)
	B.megaterium	13.0±1.4	30.0± 1.8(17.0)	32.0±2.4	47.0± 2.3(15.0)
	B.megaterium +S.marcescens	11.0±1.2	29.0±2.2 (18.0)	33.0±1.8	49.5± 3.5(17.0)
TV-25	Control	14.0±1.5	17.0±1.1 (3.0)	26.0±1.8	32.0± 2.7(6.0)
	S.marcescens	15.0±1.8	22.0± 1.9(7.0)	27.0±1.9	37.0± 3.1(10.0)
	B.megaterium	13.0±1.5	24.0± 1.2(11.0)	29.0±2.7	41.0±2.9 (12.0)
	B.megaterium +S.marcescens	15.0±1.4	31.0± 2.3(16.0)	28.0±2.3	40.5± 3.0(12.5)
TV-26	Control	12.0±1.7	18.0± 1.5(6.0)	22.0±1.5	29.0± 2.4(7.0)
	S.marcescens	14.0±1.3	26.0±1.7(12.0)	23.0±1.2	32.0± 2.5(9.0)
	B.megaterium	15.0±1.1	31.0± 1.4(16.0)	22.0±1.1	39.0± 3.0(17.0)
	B.megaterium +S.marcescens	13.0±1.3	32.0± 2.1(19.0)	23.0±2.1	45.0± 3.1(22.0)
T-17	Control	10.2±1.1	16.2± 1.5(6.0)	17.1±1.4	21.2± 1.8(4.1)
	S.marcescens	11.5±1.3	20.3± 1.6(8.8)	16.1±1.3	30.1±1.5 (14.0)
	B.megaterium	12.0±1.4	22.5± 1.7(10.5)	17.2±1.5	35.0± 2.6(17.8)
	B.megaterium +S.marcescens	11.0±1.0	24.4±2.0(13.4)	18.0±1.1	37.0± 3.2(19.0)
CD (P=0.05) (Treatments) (Varieties)		1.144 1.280	3.582 4.005	1.117 1.248	2.570 2.874

Mean of 10 replicate plants / treatment; Figures in parenthesis indicate the increment over a period of 2 months

Table 5a: ANOVA of data presented in Table 5 (initial height)

Source of Variation	SS	df	MS	F
Rows	472.65	3	157.55	23.31196
Columns	221.972	4	55.493	8.211048
Error	81.1	12	6.758333	
Total	775.722	19		

Table 5b: ANOVA of data presented in Table 5 (final height)

Source of Variation	SS	Df	MS	F
Rows	6.176	3	2.058667	2.983935
Columns	65.253	4	16.31325	23.64525
Error	8.279	12	0.689917	
Total	79.708	19		

Table 5c: ANOVA of data presented in Table 5 (initial leaf no.)

Source of Variation	SS	Df	MS	F
Rows	12.1215	3	4.0405	6.148364
Columns	474.282	4	118.5705	180.4268
Error	7.886	12	0.657167	
Total	494.2895	19		

Table 5d: ANOVA of data presented in Table 5 (final leaf no.)

Source of Variation	SS	Df	MS	F
Rows	527.5855	3	175.8618	50.52654
Columns	318.613	4	79.65325	22.88503
Error	41.767	12	3.480583	
Total	887.9655	19		

Table 6 : Changes in biomass of tea leaves of potted tea plants (2 months after application following pruning)

Tea varieties	Treatment	Fresh weight (g)	Dry weight taken after 7 days (g)
TV-18	Control	04.5±1.5	2.4
	B.megaterium	15.0±1.4	7.4
	S.marcescens	17.0±2.1	8.0
	B.megaterium +S.marcescens	14.0±2.2	6.5
TV-23	Control	09.0±1.0	4.0
	B.megaterium	25.0±1.4	10.5
	S.marcescens	19.0±1.1	5.0
	B.megaterium +S.marcescens	21.0±1.3	8.5
TV-25	Control	10.0±1.0	6.5
	B.megaterium	18.0±1.2	8.8
	S.marcescens	19.0±1.1	8.0
	B.megaterium +S.marcescens	22.0±2.0	13.0
TV-26	Control	02.0±0.5	1.3
	B.megaterium	15.0±1.0	4.3
	S.marcescens	15.0±1.4	9.0
	B.megaterium +S.marcescens	13.0±1.1	5.2
T-17	Control	06.0±1.0	4.3
	B.megaterium	20.0±1.7	8.5
	S.marcescens	22.0±1.2	10.1
	B.megaterium +S.marcescens	23.0±1.6	11.5
CD (P=0.05) (Treatments)		2.929	2.731
(Varieties)		3.275	3.054

Mean of leaves from 10 plants

Table 6a : ANOVA of data presented in Table 6 (fresh wt. of leaves)

Source of Variation	SS	Df	MS	F
Rows	553.626	3	184.542	40.83616
Columns	179.423	4	44.85575	9.925851
Error	54.229	12	4.519083	
Total	787.278	19		

Table 6b : ANOVA of data presented in Table 6 (dry wt. of leaves)

Source of Variation	SS	Df	MS	F
Rows	82.128	3	27.376	6.966346
Columns	47.303	4	11.82575	3.009288
Error	47.157	12	3.92975	
Total	176.588	19		

Table 7: Effect of application of rhizobacteria on leaf number in tea plants

Tea varieties	Treatment	No. of leaves		
		Initial	Final after 6 months	Final after 12 months
TV-18	*Control*	11±0.8	47±1.4	51±1.6
	B.megaterium	12±0.9	51±1.5	61±1.5
	S.marcescens	12±0.7	49±2.3	55±1.4
	B.megaterium + S.marcescens	9±0.6	49±1.8	52±1.8
TV-23	*Control*	13±1.0	21±1.4	30±1.0
	B.megaterium	12±1.2	33±2.2	35±0.9
	S.marcescens	12±1.4	25±1.5	28±1.1
	B.megaterium + S.marcescens	13±0.8	31±2.6	35±1.4
TV-25	*Control*	12±0.7	29±1.7	31±0.8
	B.megaterium	9±0.5	79±1.7	80±0.7
	S.marcescens	12±1.0	67±1.1	69±1.3
	B.megaterium + S.marcescens	9±0.3	67±2.1	70±1.5
TV-26	*1.Control*	14±1.0	68±2.3	69±2.9
	2. B.megaterium	17±0.4	90±2.2	92±2.8
	3.S.marcescens	15±0.7	89±1.5	92±3.0
	4.B.megaterium + S.marcescens	16±0.8	90±2.7	92±2.2
T-17	*Control*	13±1.0	67±2.2	71±1.9
	B.megaterium	15±0.5	76±2.8	81±1.6
	S.marcescens	17±0.7	74±2.7	82±2.3
	B.megaterium + S.marcescens	16±0.9	76±1.9	79±2.5
CD (P=0.05) (Treatments)		2.058	11.302	11.836
(Varieties)		2.301	12.636	13.234

Table 7a: ANOVA of data presented in Table 7 (initial no. of leaves)

Source of Variation	SS	Df	MS	F
Rows	4.1335	3	1.377833	0.617194
Columns	80.583	4	20.14575	9.024189
Error	26.789	12	2.232417	
Total	111.5055	19		

Table 7b: ANOVA of data presented in Table 7 (no. of leaves after 6 months)

Source of Variation	SS	Df	MS	F
Rows	1071.058	3	357.0193	5.307148
Columns	7805.635	4	1951.409	29.00799
Error	807.257	12	67.27142	
Total	9683.95	19		

Table 7c : ANOVA of data presented in Table 7 (no. of leaves after 12 months)

Source of Variation	SS	Df	MS	F
Rows	1094.836	3	364.9453	4.946014
Columns	7298.003	4	1824.501	24.72701
Error	885.429	12	73.78575	
Total	9278.268	19		

4.3.4. Bushes

Bacterial suspensions of *B. megaterium* and *S. marcescens* were applied to the rhizosphere of 8-yrs old pruned plants of T-17/154 at a regular interval of 15 days in the experimental field of commercial tea garden (Hansqua Tea Garden). Aqueous suspensions of these bacteria were also sprayed on tea bushes after pruning under same physical and environmental conditions, under experimental design as mentioned in material and methods. Observations were recorded after 2 and 4 months of 1st application. Results showed a significant increase in no of leaves by application of the bacteria (Figs. 27, A-F & 28, A-J; Table 8).

4.4. Comparison of efficacy of isolated PGPR with other known strains

The growth promoting efficacy of *S. marcescens* (TRS 1) with other known MTCC strains of *S. marcescens* (MTCC 86, MTCC 97, MTCC 3124, MTCC 4301, MTCC 7298, MTCC 2645, MTCC 4822 and MTCC 7103) were compared in terms of % increase in height and no of leaves after 4 and 8 months of application in potted plants of five varieties of (TV-18, TV-23, TV-25, TV-26 and T-17) tea in comparison to untreated control plant. All the eight isolates along with *S. marcescens* (TRS 1)

Figure 24: Growth of tea plants following application of *B. megaterium* and *S. marcescens* after one year (A-D) and three years (E-H); control plot (A-E); *B. megaterium* treated plot(B); *S. marcescens* treated (C); Joint application of *B. megaterium* and *S. marcescens*(D); treated plants with *B. megaterium* (F); *S. marcescens* treated(G); *B. megaterium* and *S. marcescens* treated plot (H).

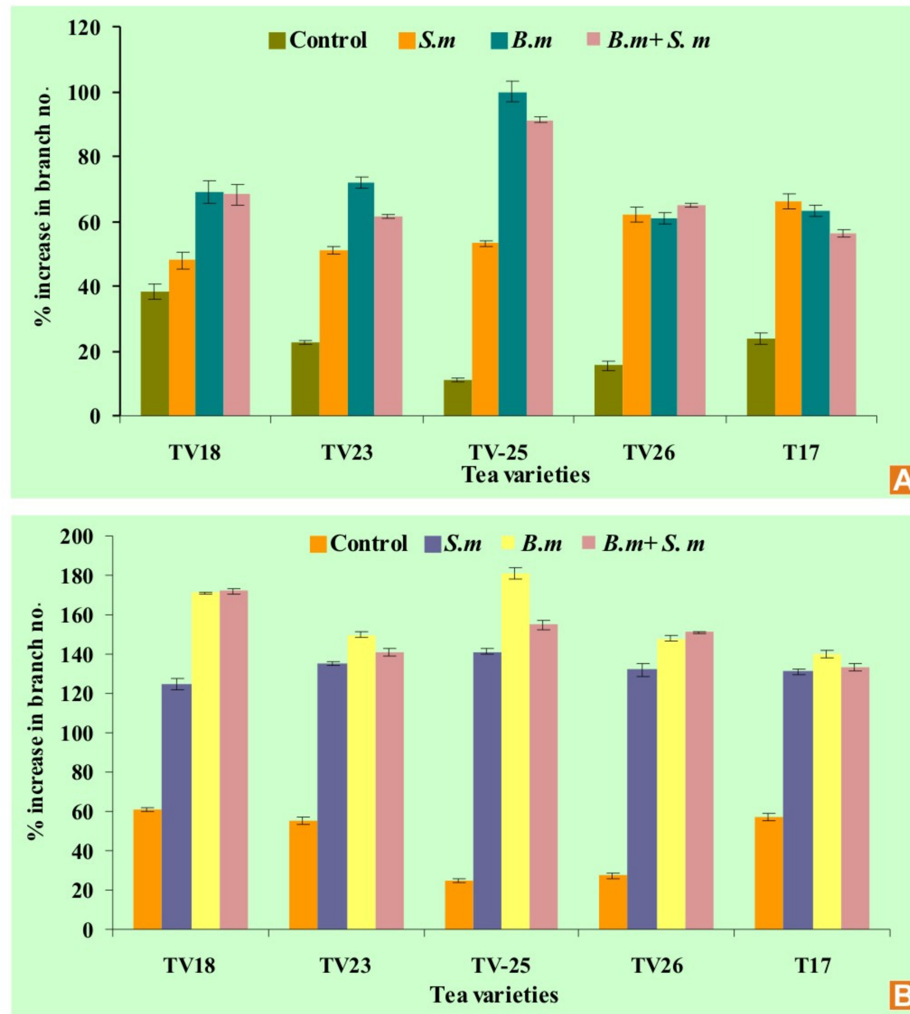


Figure 25: Effect of application of *B. megaterium* and *S. marcescens* on increase in number of branches of five different tea varieties after 6 (A) and 12 months (B).

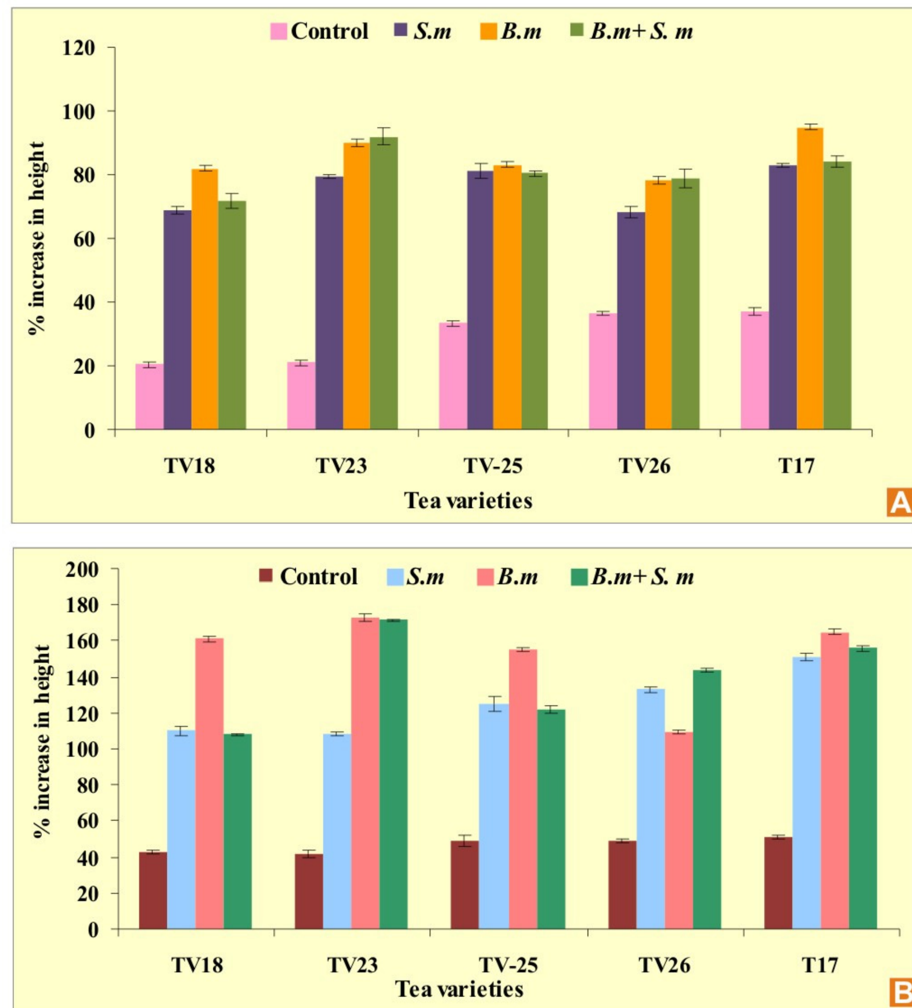


Figure 26: Effect of application of *B. megaterium* and *S. marcescens* on increase in height of five different tea varieties after 6 (A) and 12 months (B).

Figure 27: A-F: Pruned experimental field of Hansqua Tea Estate before treatments. General pruned field of T-17/154(A&B), pruned field of T-17/154 selected for experiments (C&D).

Figure 28: A-J: Pruned experimental field of Hansqua Tea Garden after treatments of T-17/154 with *B. megaterium* and *S. marcescens.* A&B-general view of treated plot, C&D-general (C) and single (D) view of untreated plant; E&F-general (E) and single (F) view of plant treated with *B. megaterium;* G&H- Plants treated with *S. marcescens;* I&J- Joint application of *B. megaterium* and *S. marcescens*.

showed significant increase in height and leaf numbers in all five varieties of tea but *S. marcescens* (TRS 1) comparatively promoted better plant growth in comparison to other isolates. Statistical analysis (ANOVA) showed significant differences between control and treatments (Table 9).

Table 8: Effect of rhizobacteria on leaf numbers of 8 yr old tea plants (T-17/1054) in Hansqua Tea Estate

Tea varieties	**Treatments**	**Mean leaf number after 2 months**	**Mean leaf number after 4 months**	**Increment in leaf number**
T-17/1054	Control	159±12.5	238±10.4	79
	T1 (*B.megaterium* treated)	184±13.2	314±12.9	130
	T2 (*S.marcescens* treated)	209±10.8	309±18.5	100
	T3 (*B.megaterium* +*S.marcescens* treated)	164±11.6	349±15.3	185

Mean leaf no. of 40 replicates; Treatments were applied to 8 yr. old bushes soon after pruning; Differences between control and treatments significant at P=0.01 4 months after 1st treatment

4.5. Phosphate solubilization *in vivo* by application of bacteria

Both *B. megaterium* and *S. marcescens* could solubilize phosphate *in vitro* as evidenced by the appearance of halo zone around the inocula in PVK medium. After *in vitro* confirmation, bacteria were applied to the rhizospheric soil in order to determine their *in vivo* potentiality as phosphate solubilizers.

4.5.1. Soil phosphate content

Soil P content decreased following application of *S. marcescens* and *B. megaterium* singly or jointly both in treated potted and field grown tea plants in comparison to control. Changes in phosphate contents following the treatments were statistically significant (Table 10).

4.5.2. Leaf and root phosphate content

Increase in root and leaf phosphate contents was observed following the application of bacteria in five varieties of tea both in potted as well as field conditions. Maximum phosphate content was obtained in the leaf tissues. Statistical analysis (ANOVA) revealed the significant increase in phosphate contents (Tables 11&12).

Table 9: Comparison of growth promotion efficacy of isolated *S. marcescens* (TRS1) with other known strains in tea varieties, grown in potted conditions.

Tea varieties	Treatment	% increase in height after 04 months	% increase in height after 08 months	% increase in no of leaves after 04 months	% increase in no of leaves after 08 months
TV-18	Control	20.0±1.20	43.0±2.88	11.0±1.15	25.0±1.154
	S.m(TRS1)	55.0±1.732	76.0±0.115	33.3±1.64	41.5±0.692
	MTCC 86	51.0±0.75	73.0±0.461	27.0±0.58	35.5±1.212
	MTCC 97	44.0±0.58	66.0±0.51	21.9±1.73	31.0±1.732
	MTCC 3124	37.0±1.15	56.0±1.27	16.0±0.57	26.5±0.750
	MTCC 4301	36.0±0.69	57.5±1.15	19.2±0.173	29.0±0.577
	MTCC 7298	33.0±0.230	51.0±0.692	15.0±0.288	23.3±0.750
	MTCC 2645	34.0±0.635	55.0±1.154	23.0±1.732	33.0±0.577
	MTCC 4822	45.0±0.577	66.0±1.64	20.8±0.577	38.0±1.154
	MTCC 7103	31.0±0.288	51.2±0.461	15.0±0.461	35.0±0.577
TV-23	Control	22.0±0.75	42.5±0.92	9.0±0.58	15.0±2.30
	S.m(TRS1)	41.0±1.154	62.0±0.346	12.6±0.230	31.2±0.173
	MTCC 86	34.5±2.30	55.0±0.115	25.8±0.404	32.2±0.577
	MTCC 97	34.0±1.732	54.5±0.69	16.5±0.1732	25.5±0.230
	MTCC 3124	28.0±0.92	49.0±0.635	13.0±1.154	22.0±0.58
	MTCC 4301	37.0±2.88	58.0±2.30	17.7±0.404	27.0±1.73
	MTCC 7298	32.5±0.86	53.0±0.173	13.3±0.519	24.5±0.288
	MTCC 2645	35.0±0.63	53.0±0.404	17.0±2.30	29.0±1.154
	MTCC 4822	31.0±0.80	49.0±1.732	10.3±0.115	24.5±0.230
	MTCC 7103	33.3±0.92	52.7±2.30	11.0±0.288	31.5±0.173
TV-25	Control	27.0±0.8	61.0±0.75	9.0±1.73	17.0±0.577
	S.m(TRS1)	56.0±0.7	85.0±0.288	31.2±0.58	46.9±0.635
	MTCC 86	49.0±0.6	79.0±1.50	29.0±1.154	40.0±1.732
	MTCC 97	48.0±1.4	77.0±1.32	28.0±0.923	31.0±0.577
	MTCC 3124	41.0±0.8	72.0±0.230	15.0±0.173	26.5±0.692
	MTCC 4301	46.0±0.3	75.5±1.21	21.4±0.346	32.5±1.27
	MTCC 7298	37.0±0.7	66.6±0.173	22.5±0.692	35.0±1.154
	MTCC 2645	35.0±0.9	64.0±0.64	22.0±0.866	37.8±0.750
	MTCC 4822	32.0±1.2	60.5±2.80	17.6±1.154	31.0±1.154
	MTCC 7103	37.5±0.5	59.0±1.15	14.5±0.577	34.0±0.577
TV-26	Control	32.5±2.7	53.0±1.4	15.0±2.30	32.0±1.732
	S.m(TRS1)	49.0±3.0	71.0±1.5	48.0±1.27	61.2±0.230
	MTCC 86	48.0±2.2	70.0±2.2	43.3±1.32	59.9±0.461
	MTCC 97	45.0±1.5	68.0±1.1	28.5±0.346	49.0±0.519
	MTCC 3124	43.0±1.3	66.5±1.3	27.0±0.461	41.0±0.692
	MTCC 4301	47.0±0.7	67.7±1.1	36.6±1.732	46.0±1.154
	MTCC 7298	48.5±0.8	71.5±1.2	39.0±0.635	45.5±2.30
	MTCC 2645	39.0±1.1	63.3±1.7	29.9±0.750	49.8±0.577
	MTCC 4822	36.5±1.4	59.9±0.5	22.5±0.577	48.0±0.288
	MTCC 7103	38.8±2.3	67.0±0.6	25.0±0.115	43.0±0.404
T-17	Control	31.0±3.46	51.5±2.1	7.0±1.154	14.0±1.15
	S.m(TRS1)	63.0±4.04	83.0±2.2	47.5±0.58	59.0±2.88
	MTCC 86	56.0±1.15	78.0±1.2	49.0±0.57	59.3±0.57
	MTCC 97	45.0±2.30	75.5±1.6	26.0±1.154	35.5±0.115
	MTCC 3124	43.0±2.02	66.6±1.73	38.0±2.30	44.5±1.32
	MTCC 4301	39.0±0.75	61.2±2.02	21.6±0.17	35.5±1.50
	MTCC 7298	37.0±1.73	59.0±1.15	22.5±0.115	33.0±0.57
	MTCC 2645	41.5±0.58	62.4±1.4	32.0±0.577	42.0±1.73
	MTCC 4822	38.0±0.577	59.8±0.5	25.0±0.692	37.0±0.58
	MTCC 7103	40.5±0.98	61.0±0.9	21.0±0.58	33.5±0.404
CD (P=0.05)	(Treatments)	5.85	6.21	6.88	6.15
	(Varieties)	4.14	4.39	4.86	4.35

Table 9a : ANOVA of data presented in Table 9 (% increase in height after 4 mo)

Source of Variation	SS	Df	MS	F
Rows	2246.3858	9	249.59842	11.9663455
Columns	725.9868	4	181.4967	8.70138601
Error	750.9012	36	20.858367	
Total	3723.2738	49		

Table 9b: ANOVA of data presented in Table 9 (% increase in height after 8 mo)

Source of Variation	SS	Df	MS	F
Rows	2301.4098	9	255.7122	10.8905718
Columns	1788.9708	4	447.2427	19.047698
Error	845.2852	36	23.480144	
Total	4935.6658	49		

Table 9c: ANOVA of data presented in Table 9 (% increase in no of leaves after 4 mo)

Source of Variation	SS	df	MS	F
Rows	2442.984	9	271.44267	9.42923971
Columns	1889.672	4	472.418	16.4106204
Error	1036.344	36	28.787333	
Total	5369	49		

Table 9d: ANOVA of data presented in Table 9 (% increase in no of leaves after 8 mo)

Source of Variation	SS	Df	MS	F
Rows	2524.7928	9	280.53253	12.1830415
Columns	2645.8268	4	661.4567	28.7259175
Error	828.9532	36	23.026478	
Total	5999.5728	49		

4.5.3. Phosphatase activities

Changes in acid and alkaline soil phosphatase activities were determined in rhizosphere soil of five varieties of potted and field grown tea plants after application of *B. megaterium* and *S. marcescens.*

4.5.3.1. Acid phosphatase

Acid phosphatase activities in rhizosphere soil of all five varieties were enhanced following application of bacteria. Analysis of variance of results showed that differences of control were significantly different from all 3 treatments, but no significant differences were evident among the treatments (Fig. 29).

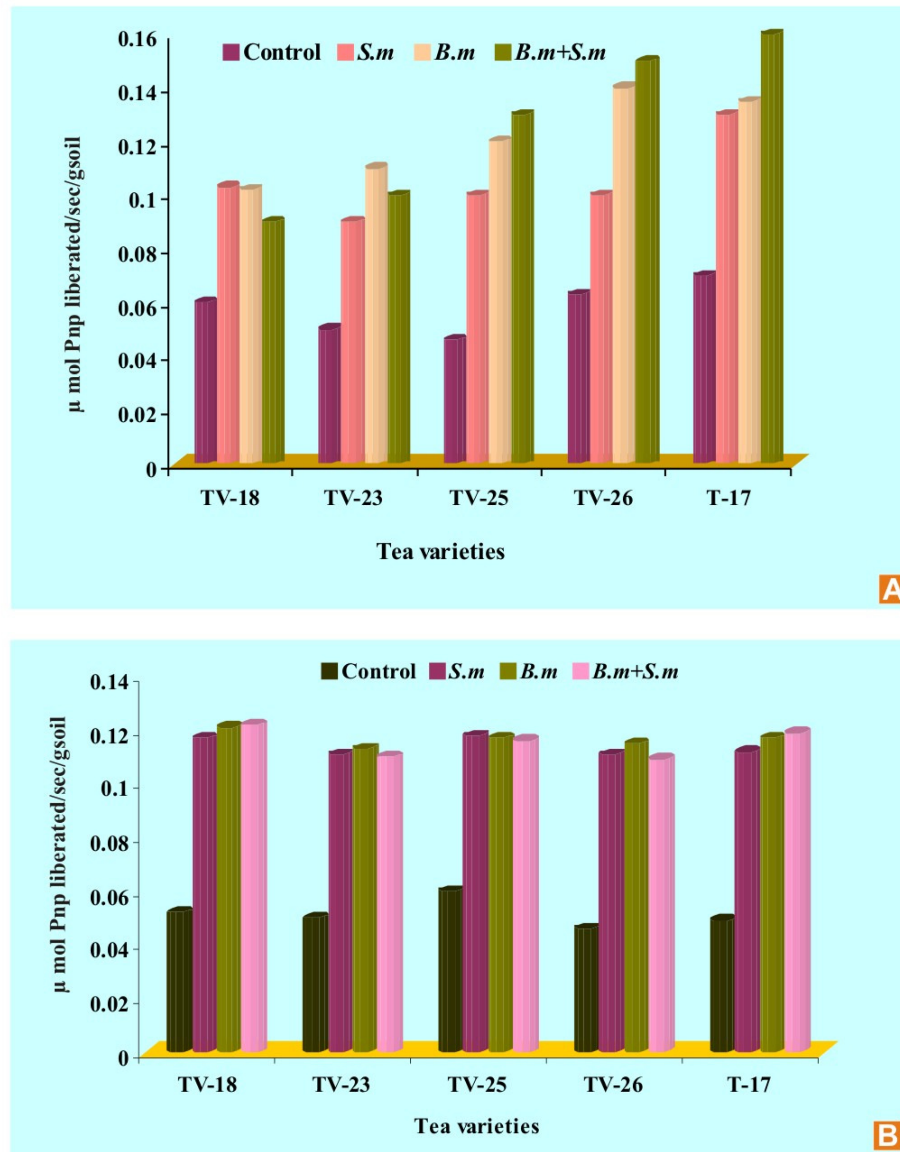


Figure 29: Acid phosphatase activities in rhizosphere soil of tea plants subjected to treatments in field (A) and pots (B).

Table 10: Phosphate content from soil of treated and control plants in field grown and potted plants

Tea varieties	Treatment	Soil phosphate (µg/g)	
		Field	Pot
TV-18	Control	121.2±2.4	156.3±1.8
	S.marcescens	76.3±1.8	92.7±2.3
	B.megaterium	77.2±1.7	101.7±1.9
	B.megaterium +S.marcescens	79.0±1.6	105.6±2.4
TV-23	Control	76.4±1.8	92.7±2.7
	S.marcescens	26.9±1.0	37.4±1.4
	B.megaterium	27.5±1.4	37.1±1.3
	B.megaterium +S.marcescens	32.0±1.7	36.2±1.8
TV-25	Control	92.6±2.4	116.6±1.4
	S.marcescens	62.8±2.7	60.2±2.0
	B.megaterium	70.0±2.8	62.2±1.9
	B.megaterium +S.marcescens	56.0±2.9	68.0±1.7
TV-26	Control	72.0±1.8	81.0±1.1
	S.marcescens	49.2±1.1	75.4±1.7
	B.megaterium	34.5±1.3	78.1±1.9
	B.megaterium +S.marcescens	38.4±1.2	78.0±1.8
T-17	Control	134.6±1.4	135.8±2.4
	S.marcescens	116.7±1.8	106.2±2.8
	B.megaterium	110.2±1.4	107.5±2.9
	B.megaterium +S.marcescens	97.5±1.9	105.0±2.7
CD (P=0.05) (Treatments)		12.377	16.243
(Varieties)		14.397	18.160

Average of 3 replicate sets

Table 10a: ANOVA of data presented in Table 10 (soil phosphate in field)

Source of Variation	SS	Df	MS	F
Rows	4876.474	3	1625.491	32.5434
Columns	14517.28	4	3629.319	72.66134
Error	599.381	12	49.94842	
Total	19993.13	19		

Table 10b: ANOVA of data presented in Table 10 (soil phosphate in pot)

Source of Variation	SS	Df	MS	F
Rows	5964.37	3	1988.123	14.36235
Columns	11728.4	4	2932.101	21.18171
Error	1661.113	12	138.4261	
Total	19353.89	19		

Table 11: Phosphate content from root of treated and control plants in field grown and potted plants

Tea varieties	Treatment	Root phosphate (µg/g)	
		Field	**Pot**
TV-18	Control	103.2±1.3	115.8±1.9
	S.marcescens	194.5±.2.5	216.9±1.5
	B.megaterium	192.0±2.7	215.4±1.4
	B.megaterium +S.marcescens	182.0±2.1	214.3±1.9
TV-23	Control	41.8±1.2	74.4±1.8
	S.marcescens	76.3±1.6	142.8±2.0
	B.megaterium	66.5±1.3	146.7±2.7
	B.megaterium +S.marcescens	71.2±1.8	158.0±2.5
TV-25	Control	35.9±1.3	74.1±1.1
	S.marcescens	65.8±1.1	146.3±1.9
	B.megaterium	72.5±1.8	149.5±1.5
	B.megaterium +S.marcescens	67.5±1.9	156.2±2.1
TV-26	Control	62.8±2.0	73.3±1.8
	S.marcescens	79.2±2.7	76.3±1.7
	B.megaterium	81.0±2.8	75.2±1.9
	B.megaterium +S.marcescens	75.0±2.8	78.0±1.3
T-17	Control	149.6±2.9	151.8±2.4
	S.marcescens	246.8±3.8	255.8±2.9
	B.megaterium	235.0±4.2	258.0±2.7
	B.megaterium +S.marcescens	242.0±2.8	256.0±2.9
CD (P=0.05)	(Treatments)	24.838	30.399
	(Varieties)	27.770	33.987

Average of 3 replicate sets

Table 11a : ANOVA of data presented in Table 11 (root phosphate in field)

Source of Variation	SS	Df	MS	F
Rows	9878.37	3	3292.79	10.13862
Columns	82745.83	4	20686.46	63.69432
Error	3897.325	12	324.7771	
Total	96521.52	19		

Table 11b : ANOVA of data presented in Table 11 (root phosphate in pot)

Source of Variation	SS	Df	MS	F
Rows	19402.9	3	6467.633	15.28529
Columns	57361.69	4	14340.42	33.89146
Error	5077.535	12	423.1279	
Total	81842.13	19		

Table 12: Phosphate content of leaves of treated and control plants in field grown and potted plants

Tea varieties	Treatment	Leaf phosphate (µg/g)	
		Field	Pot
TV-18	Control	200.4±3.6	203.4±2.4
	S.marcescens	276.7±2.9	272.3±3.2
	B.megaterium	265.0±2.8	270.0±3.5
	B.megaterium +S.marcescens	266.0±3.1	265.0±3.9
TV-23	Control	191.5±1.7	194.5±1.9
	S.marcescens	291.7±2.5	285.7±1.8
	B.megaterium	285.0±2.9	272.2±2.8
	B.megaterium +S.marcescens	288.1±2.8	271.1±2.9
TV-25	Control	181.0±2.3	190.0±3.0
	S.marcescens	297.7±2.9	293.2±2.9
	B.megaterium	279.0±2.5	290.0±2.5
	B.megaterium +S.marcescens	277.0±3.2	293.2±2.6
TV-26	Control	190.0±2.8	196.0±1.8
	S.marcescens	293.2±2.9	287.2±1.5
	B.megaterium	284.0±3.0	278.8±1.9
	B.megaterium +S.marcescens	290.1±3.1	270.0±2.8
T-17	Control	193.0±1.9	191.5±1.8
	S.marcescens	285.7±2.3	270.8±2.2
	B.megaterium	284.5±2.7	268.2±2.9
	B.megaterium +S.marcescens	278.0±2.9	266.5±3.5
CD (P=0.05) (Treatments)		10.656	10.158
(Varieties)		11.913	11.357

Average of 3 replicate sets

Table 12a: ANOVA of data presented in Table 12 (leaf phosphate in field)

Source of Variation	SS	df	MS	F
Rows	31755.05	3	10585.02	177.403
Columns	402.742	4	100.6855	1.687471
Error	715.998	12	59.6665	
Total	32873.79	19		

Table 12b: ANOVA of data presented in Table 12 (leaf phosphate in pot)

Source of Variation	SS	df	MS	F
Rows	25330.61	3	8443.536	156.1216
Columns	687.367	4	171.8417	3.177366
Error	648.997	12	54.08308	
Total	26666.97	19		

4.5.3.2. Alkaline phosphatase

A significant increase in alkaline phosphatase activities in soil of potted as well as field grown tea plants of five varieties was also observed (Fig. 30).

4.6. Biochemical changes in tea leaves induced by bacteria

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

4.6.1. Enzymes

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

4.6.1.1. Peroxidase

Peroxidase activities were assayed and isozyme analysis was done from control and bacteria inoculated tea leaves, as described in Materials amd Methods.

4.6.1.1.1. Assay of activity

Application of bacterial suspension in the rhizosphere of potted and field grown tea plants resulted in a significant increase in peroxidase activity in treated plants compared to untreated control plants (Fig. 31).

4.6.1.1.2. Isozyme profile

Native PAGE analysis of peroxidase showed the existence of three isoforms in all the varieties of tea. All the isoforms which were present in untreated control plants were also present in treated ones. However, there was clear distinction between the treated and untreated control plants as far as intensities were considered. Maximum intensity of bands was noticed in the treated plants with Rm (Relative mobility) values of 0.883, 0.58, 0.348 in T-17 and 0.85, 0.574, 0.404, 0.255 in TV-25 varieties (Fig. 32, A&B).

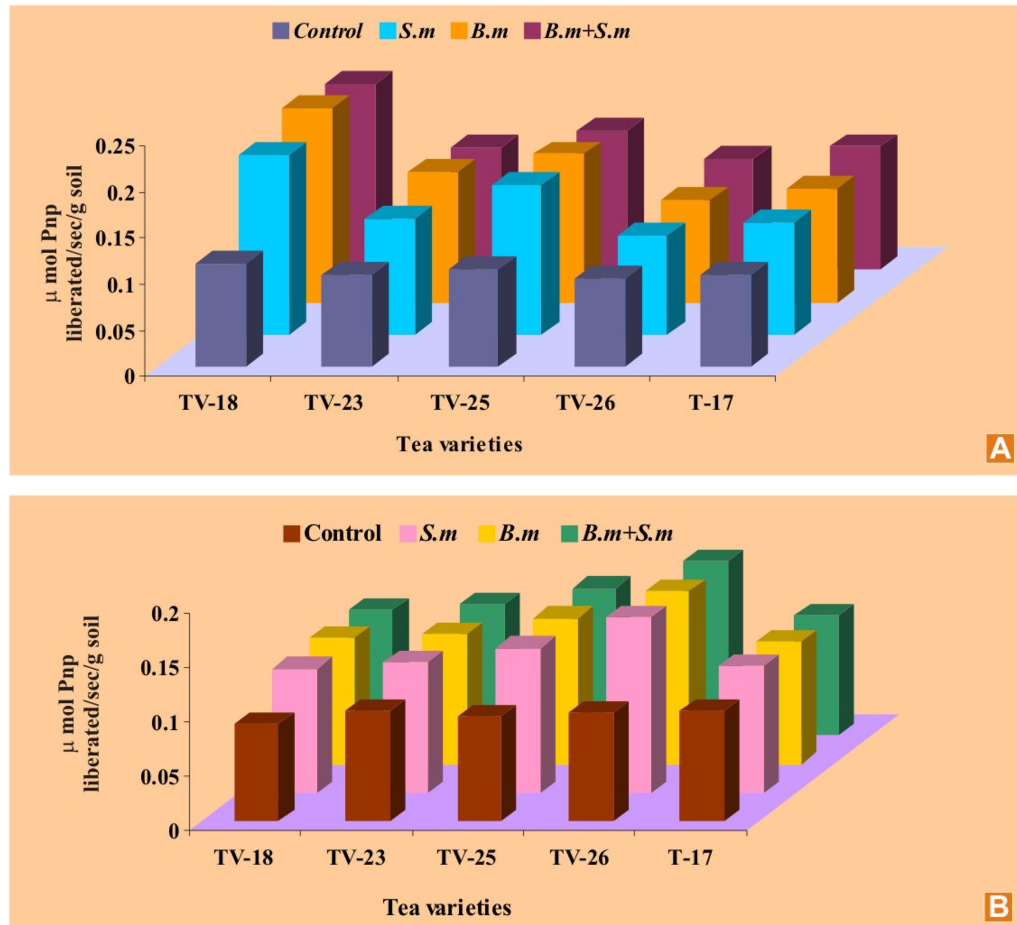
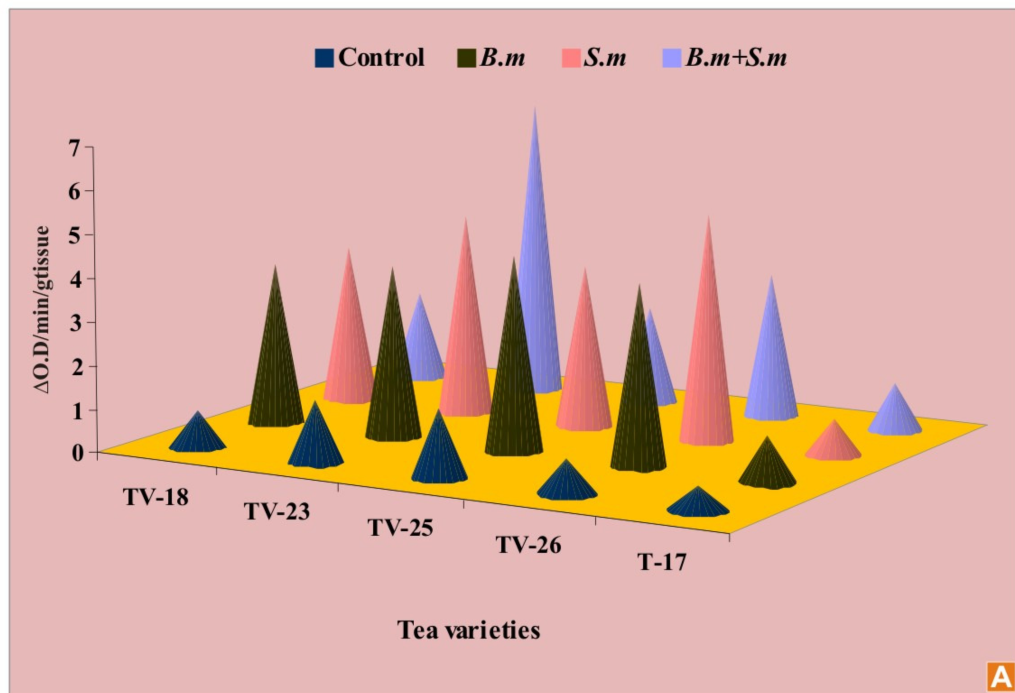


Figure 30: Alkaline phosphatase activities in rhizosphere soil of tea plants subjected to treatments in field (A) and pots (B).



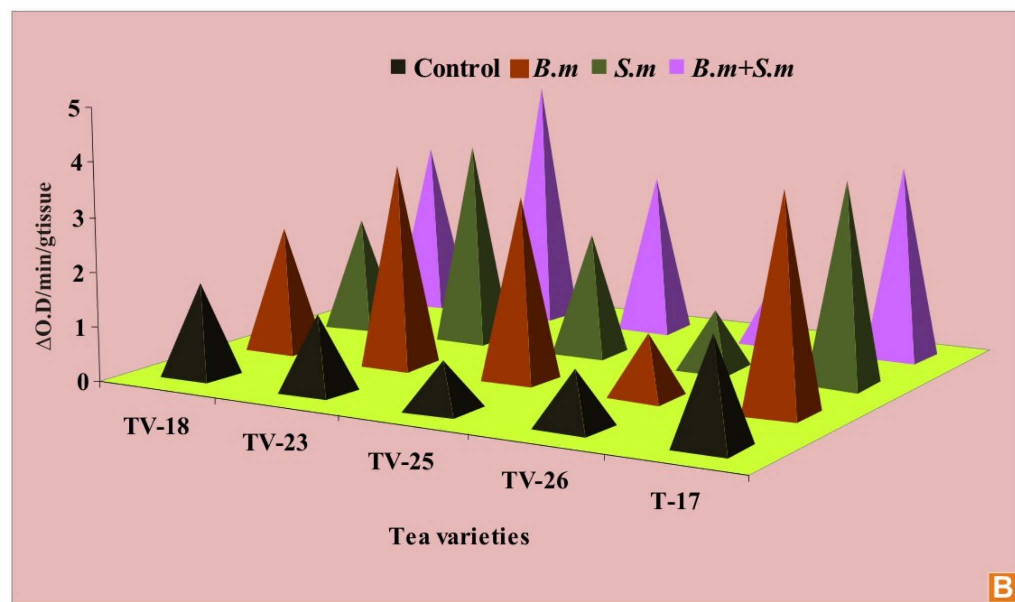


Figure 31: Peroxidase activities in leaves of field grown (A) and potted (B) tea varieties treated with *B. megaterium* and *S. marcescens*.

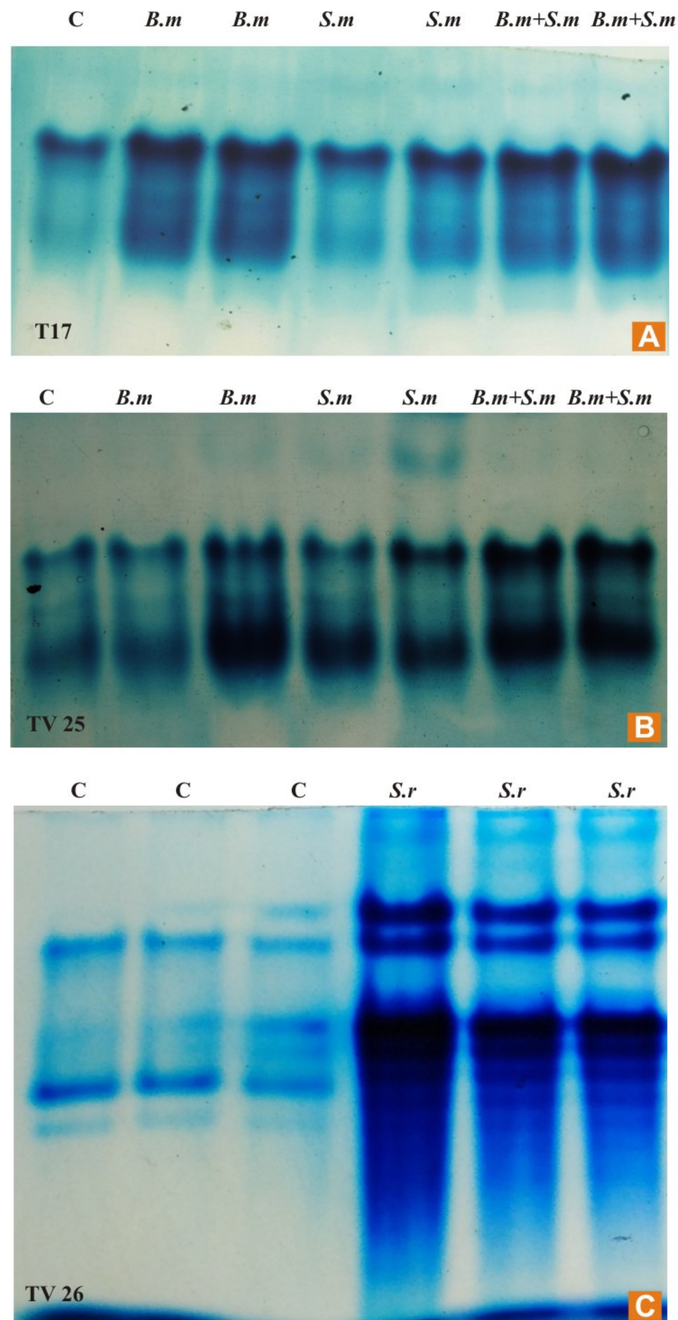


Figure 32: PAGE analysis of peroxidase isozyme in tea variety; A:T17 and B:TV25 subjected to the treatment by *B.megaterium* and *S.marcescens*; C: TV26 subjected to the treatment by *Sclerotium rolfsii*.

4.6.1.2. Chitinase

When the chitinase activity was assayed after the application of PGPR in the rhizosphere of tea plants, significant increase in activity of chitinase enzyme was observed in all the five varieties. *B. megaterium* elicited highest production of chitinase in T-17 both in potted and field grown plants whereas *S. marcescens* in T-17 and TV-18 in case of potted and field grown tea plants. In TV-25, TV-26, T-17 induction of chitinase was higher by *B. megaterium* whereas in TV-18 and TV-23 by *S. marcescens* in tea plants grown in field conditions. Similarly, single application of *B. megaterium* in rhizosphere soil of potted tea plants showed higher induction of chitinase in TV-23, TV-26 and T-17 than *S. marcescens* except TV-18 and TV-25. Joint application of bacteria also increased chitinase activities significantly in comparison to control in all selected five varieties of tea (Fig. 33).

4.6.1.3. β-1,3 glucanase

β-1,3-glucanase activity was observed in all the five varieties of plants. However, there was significant increase in the activity of β-1,3-glucanase in bacteria treated plants and activity was lower in untreated control plants. In all varieties of potted plants, *B. megaterium* induced enzyme activity was more than in *S. marcescens* induced except TV-18. Similar trends were also observed in field grown plants except TV-25 and T-17. Joint application also showed significant increase in enzyme activities but single application of bacteria gave better responses (Fig. 34).

4.6.1.4. Phenyl alanine ammonia lyase

Application of *B. megaterium* and *S. marcescens* significantly increased the PAL enzyme activity in five varieties of tea in comparison to control plants. Dual application of bacteria showed more increase in PAL activities in TV-18, T-17 in case of field grown plants whereas in TV-23 in potted tea plants than single application of *B. megaterium* and *S. marcescens* (Fig. 35).

4.6.2. Phenols

4.6.2.1. Total and O-phenol contents

Both the total and O- dihydroxy phenol contents of the tea leaves were increased significantly after single as well as combined application of *B. megaterium* and *S. marcescens* as compared to untreated control in different varieties of tea. Results revealed that maximum amount of total phenol accumulated in TV-26 and TV-23 where as O-phenol maximum in TV-23 and TV-26 in case of potted and field grown plants (Figs. 36 & 37).

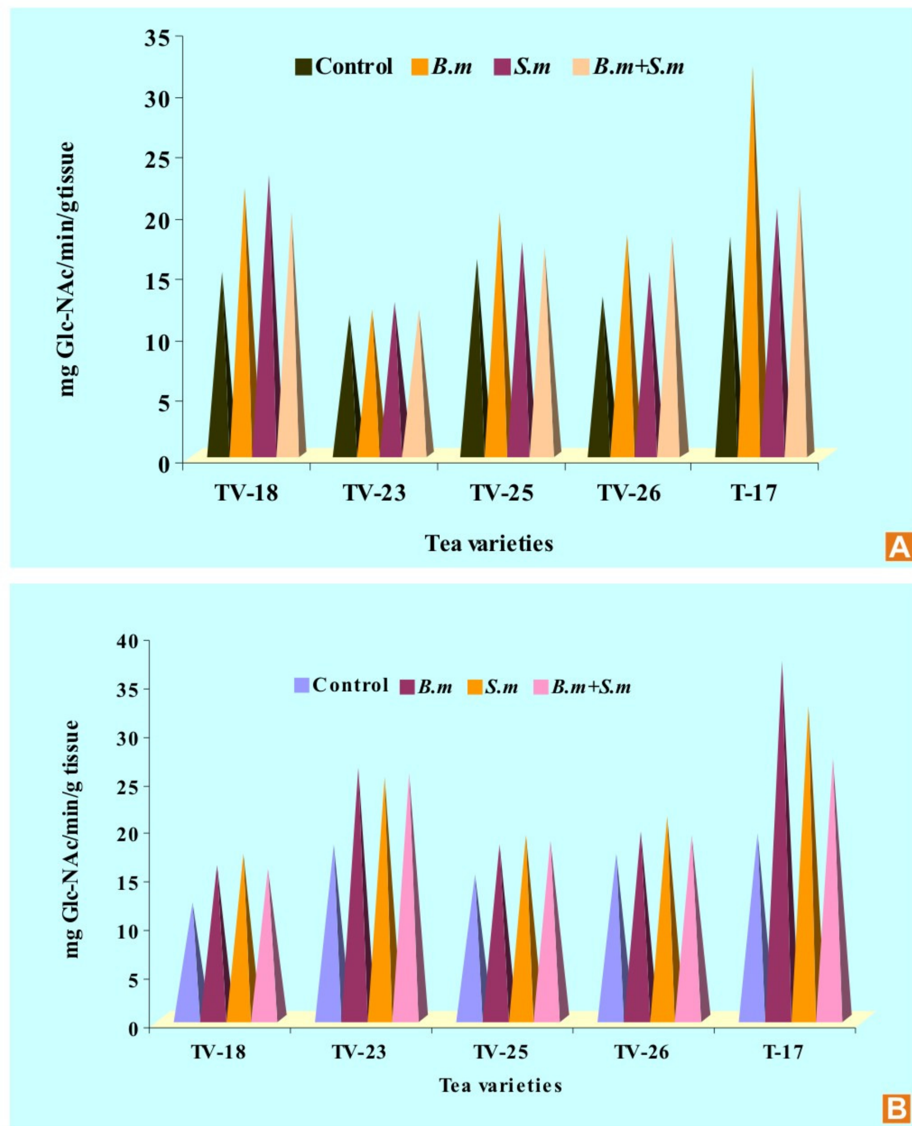


Figure 33: Chitinase activities in leaves of field grown (A) and potted (B) tea varieties treated with *B. megaterium* and *S. marcescens*.

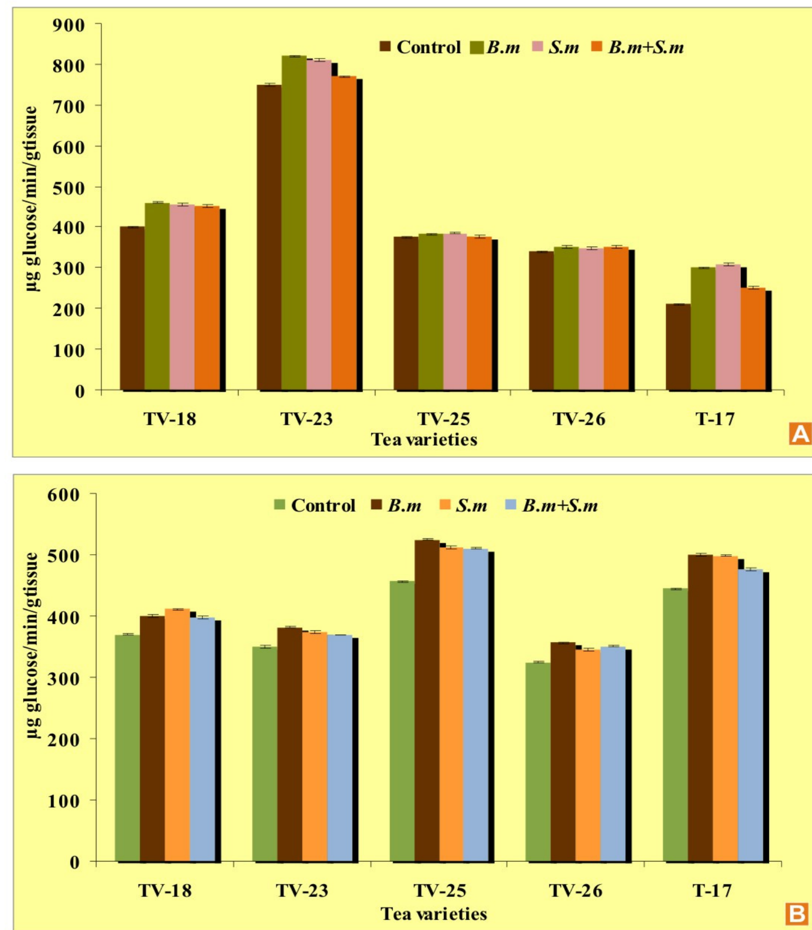


Figure 34: β-1,3 glucanase activities in leaves of field grown (A) and potted (B) tea varieties treated with *B. megaterium* and *S. marcescens*.

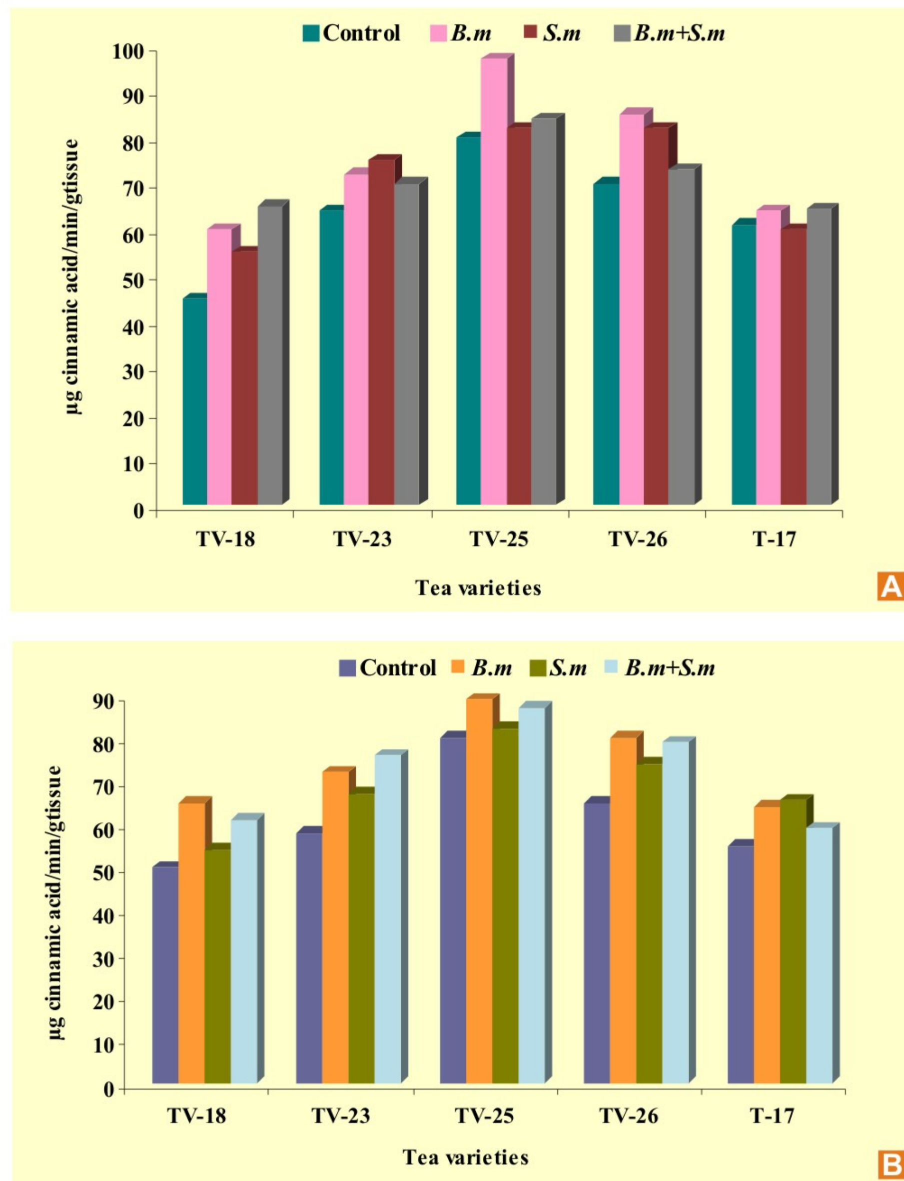


Figure 35: Phenyl alanine ammonia lyase activities in leaves of field grown (A) and potted (B) tea varieties treated with *B. megaterium* and *S. marcescens*.

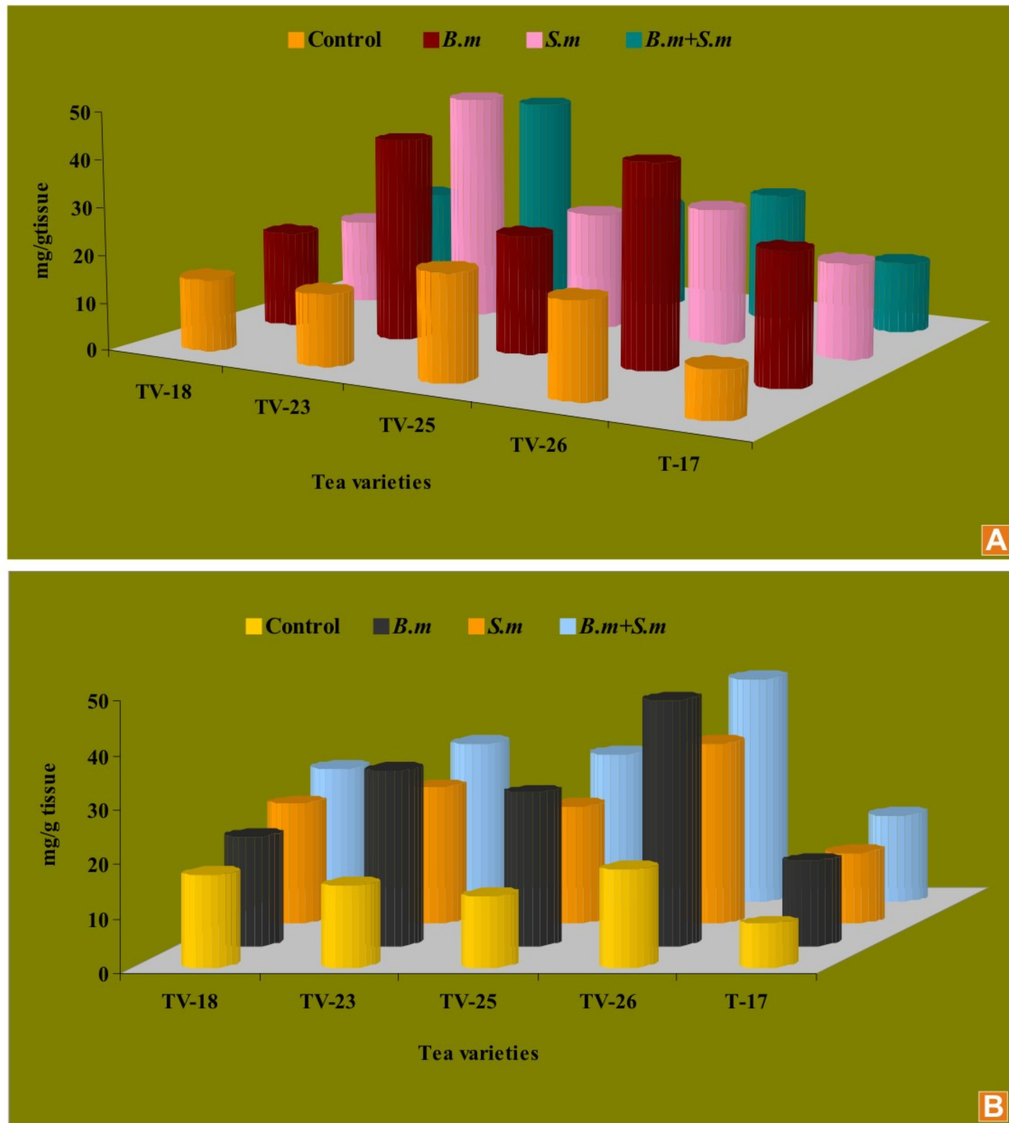


Figure 36: Changes in total phenol contents in leaves of field grown (A) and potted (B) tea varieties treated with *B. megaterium* and *S. marcescens*.

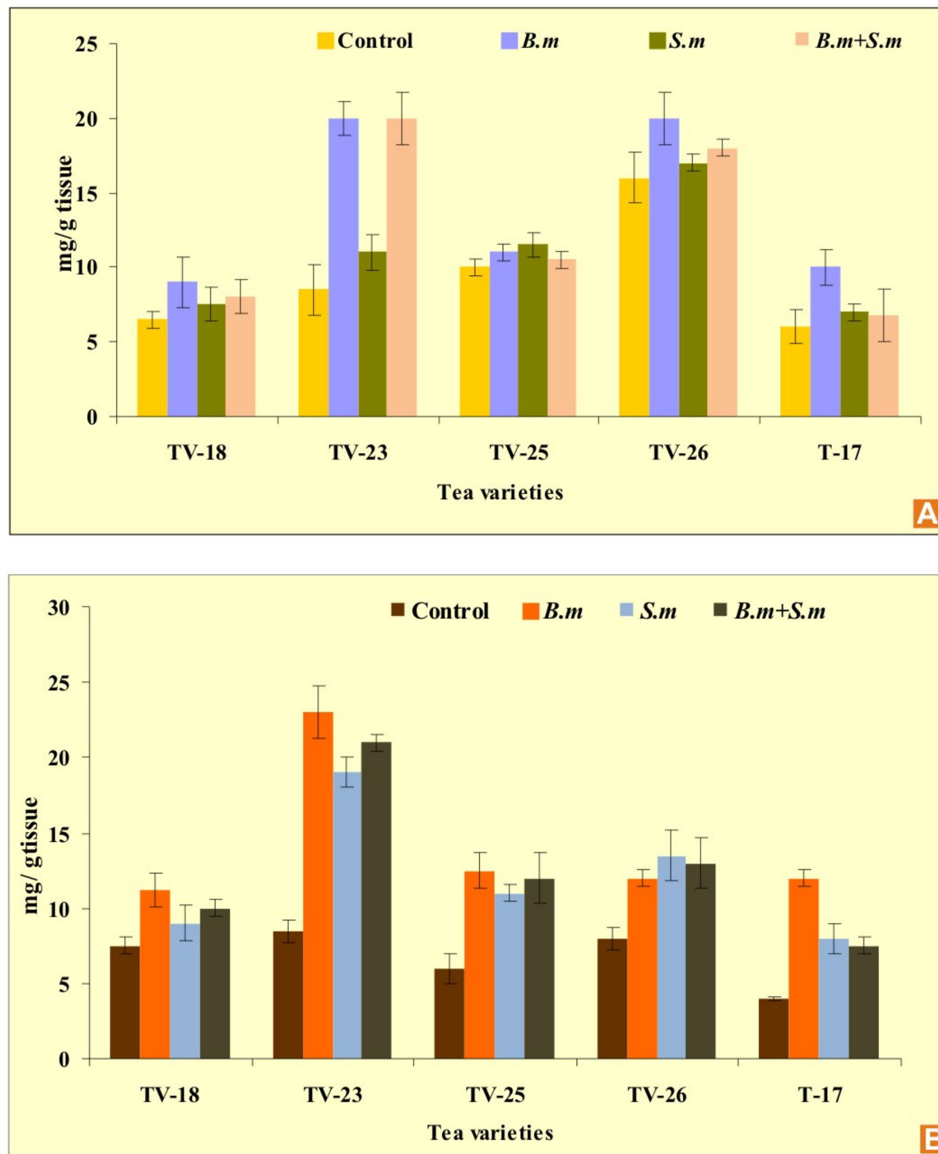


Figure 37: Changes in ortho-dihydroxy phenol contents in leaves of field grown (A) and potted (B) tea varieties treated with *B. megaterium* and *S. marcescens.*

4.6.3. Chlorophylls

Chlorophyll content of leaves from plants grown in control and treated plots and pots were estimated following standard procedure. Results revealed significant increase in total chlorophyll in all treatments (Table 13).

Table 13: Chlorophyll content of leaves of tea varieties following treatment with *B.megaterium* and *S.marcescens*

Tea varieties	Treatment	Total chlorophyll (mg/gm tissue)	
		Field grown plants	Potted plants
TV-18	Control	0.601±0.09	2.348±0.06
	S.marcescens	0.689±0.02	2.685±0.01
	B.megaterium	0.979±0.11	2.495±0.03
	B.megaterium +S.marcescens	0.998±0.12	2.470±0.02
TV-23	Control	1.050±0.10	0.539±0.05
	S.marcescens	1.240±0.11	2.485±0.07
	B.megaterium	1.320±0.09	2.540±0.09
	B.megaterium +S.marcescens	0.981±0.07	2.280±0.08
TV-25	Control	1.040±0.04	1.330±0.12
	S.marcescens	1.100±0.08	2.120±0.23
	B.megaterium	1.320±0.05	2.360±0.45
	B.megaterium +S.marcescens	1.680±0.06	2.260±0.50
TV-26	Control	1.340±0.10	1.090±0.13
	S.marcescens	1.370±0.13	2.220±0.12
	B.megaterium	1.970±0.12	2.520±0.15
	B.megaterium +S.marcescens	1.780±0.15	2.450±0.20
T-17	Control	1.000±0.08	2.157±0.23
	S.marcescens	0.838±0.09	2.260±0.21
	B.megaterium	1.940±0.05	2.437±0.15
	B.megaterium +S.marcescens	1.300±0.02	2.405±0.13
CD (P=0.05)	(Treatments)	0.303	0.520
	(Varieties)	0.338	0.582

Mean of 3 replicates

Table 13a: ANOVA of data presented in Table 13 (chlorophyll content of field grown plants)

Source of Variation	SS	df	MS	F
Rows	3.119395	3	1.039798	7.28589
Columns	0.82571	4	0.206428	1.446442
Error	1.712568	12	0.142714	
Total	5.657673	19		

Table13b: ANOVA of data presented in Table 13 (chlorophyll content of potted plants)

Source of Variation	SS	df	MS	F
Rows	0.866654	3	0.288885	5.97018
Columns	1.321199	4	0.3303	6.826079
Error	0.580655	12	0.048388	
Total	2.768507	19		

4.6.4. Proteins

4.6.4.1. Protein content

Soluble protein extracted from control and bacteria treated plants from five different tea varieties were assayed. Results indicated that soil application of *B. megaterium* and *S. marcescens* resulted in significant increase in the protein content of the plant (Table 14).

Table 14: Changes in protein contents of leaves of tea varieties following treatment with aqueous suspensions of *B. megaterium* and *S. marcescens*

Tea varieties	Treatments	Protein content(mg/gm tissue)
TV 18	Control	095±1.15
	B.megaterium	190±0.58
	S.marcescens	185±3.46
	B.megaterium+S. marcescens	210±1.73
TV 23	Control	070±0.57
	B.megaterium	175±0.33
	S.marcescens	171±2.88
	B.megaterium+S. marcescens	195±2.30
TV 25	Control	060±1.73
	B.megaterium	140±4.04
	S.marcescens	139±1.15
	B.megaterium+S. marcescens	189±2.30
TV 26	Control	075±0.58
	B.megaterium	120±0.60
	S.marcescens	119±0.80
	B.megaterium+S. marcescens	180±0.86
T17	Control	095±0.57
	B.megaterium	145±1.15
	S.marcescens	151±4.04
	B.megaterium+S. marcescens	197±1.03
CD (P=0.05)	(Treatments)	18.00
	(Varieties)	20.13

Mean of 3 replicates

Table 14 a : ANOVA of data presented in Table 14 (Changes in protein contents in tea leaves)

Source of Variation	SS	df	MS	F
Rows	34608.15	3	11536.05	67.54128
Columns	5281.2	4	1320.3	7.730094
Error	2049.6	12	170.8	
Total	41938.95	19		

4.6.4.2. Protein pattern

The soluble proteins extracted from control and bacteria treated tea plants were analysed by SDS-PAGE. Upon electrophoretic comparison, proteins from control and treated plants revealed a number of protein bands on staining with coomassie brilliant blue. Almost all the proteins detected as bands were present with molecular weights of (15, 57, 68, 96, 97) KDa in TV-18, (15, 60, 70, 97, 99) KDa in TV-23, (14.3, 43, 67, 68, 70, 94, 95) KDa in T-17 and (13, 38, 58, 70, 95, 96, 97) KDa in TV-26 varieties treated with *B. megaterium* and *S. marcescens* with respective to control, however, the level of expressions of some proteins were higher in bacteria treated plants (Fig. 38, A-D).

4.7. Effect of PGPR on tea catechins

Catechins derived from leaves of plants whose rhizosphere was soil drenched with bacteria were analysed in HPLC. Analysis revealed that a few isomers were enhanced by the treatments, a few new ones developed and few were lost. New isoforms and increase of isomers were observed in *B. megaterium* treated plants where as few were lost or there was suppression of few isomers by the treatment of *S. marcescens*. Similar trends were observed in TV26, TV18, TV25 and T17. But in T-17/154 some new isomers were enhanced by the treatment of *S. marcescens*. In TV-25 variety, in control one isomer- gallo catechin gallate (GCG) with retention time of 13.13 min, whereas, in *B.megaterium* treatment, two isomers- gallocatechin (GC) and gallo catechin gallate (GCG) with retention times of 4.59 and 13.36 min were detected. Similarly, in *S. marcescens* and *B.megaterium+S.marcescens* treated TV-25 variety, gallo catechin gallate (GCG) and epigallo catechin gallate (EPC) with

retention times of 13.36 and 10.95 min were detected. In TV-18 variety, in addition to gallo catechin gallate (GCG) with retention time of 13.36 min, another isomer-epigallo catachin (EGC)- 5.922 was detected in control, *B. megaterium* and *B.megaterium+S.marcescens* treated plants. Similarly, in T-17 and TV-26 varieties, gallo catechin (GC) - (4.59-control; 4.59- *B. megaterium* treated), gallo catechin gallate(GCG)-(13.36- in control; *B. megaterium* and *B.megaterium+S.marcescens* treated) and epigallo catechin (EGC) with retn. time- 5.92 (in *B.megaterium+S.marcescens* treated plants) were predicted as isomers of catechin. However, no major loss of isomers were noted due to treatments indicating that flavor components were not lost (Figs. 39-43 A-D; Tables 15-19 a-d).

4.8. Studies on survivability of bacteria in bioformulations

4.8.1. *In vitro* survivability

The viability of *B.megaterium* and *S. marcescens* in formulations was tested during the storage period of 9 months at 1 month interval. Results revealed that *B. megaterium* could survive in the range of $6.1\times\log_{10}$ cfu/ml in bioformulations of saw dust, rice husk and $6.98\times\log_{10}$ cfu/ml in tea waste respectively where as *S. marcescens* could survive in the range of 7.12, 7.11 and $7.2\times\log_{10}$ cfu/ml in saw dust, rice husk and tea waste formulations (Fig. 44; Fig. 45, A-G).

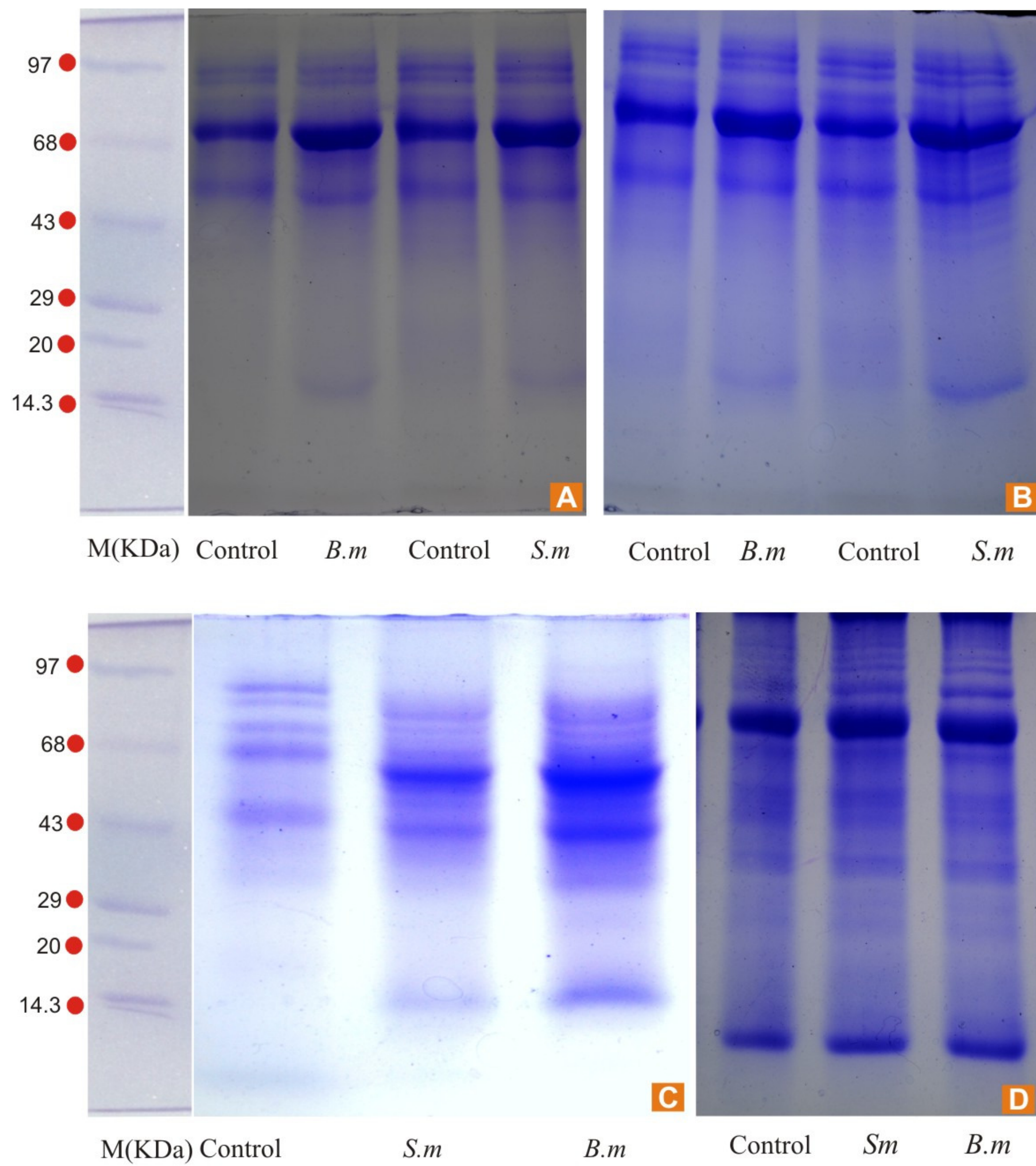


Figure 38: A-D: SDS-PAGE analysis of proteins from leaves of tea plants of different varieties treated with *B. megaterium* and *S. marcescens.* A-TV-18; B-TV-23; C- T-17 and D- TV-26.

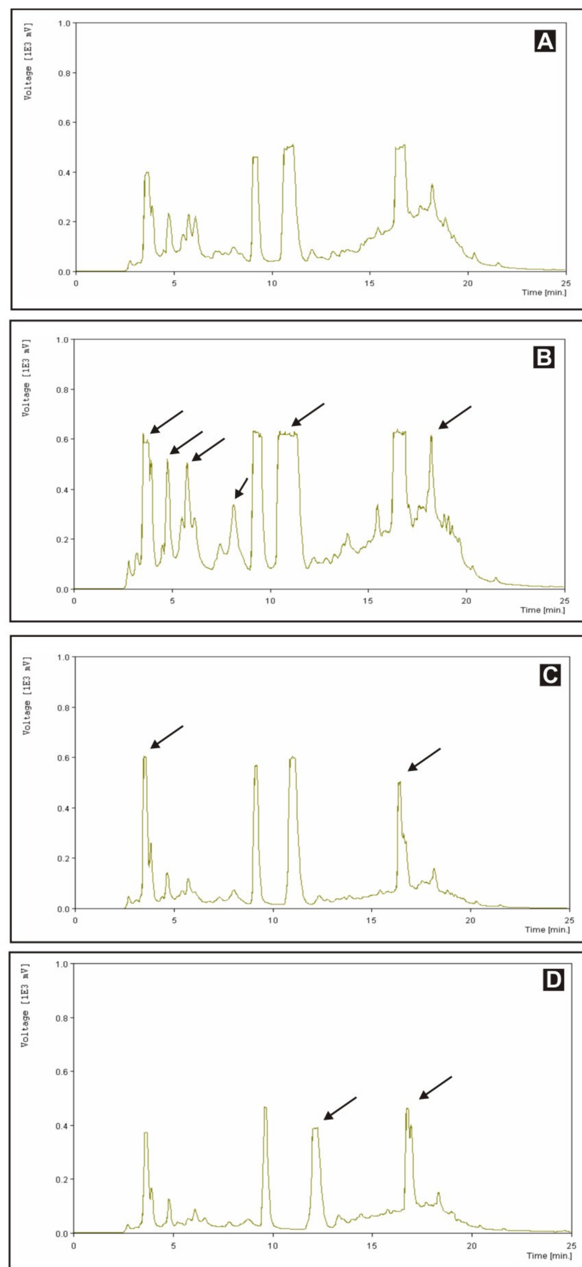


Figure 39: HPLC profiles of catechins of leaves of tea plants treated with *B. megaterium* (B), *S. marcescens* (C) and *B. megaterium*+ *S. marcescens* (D) in comparison to untreated leaves (A) of TV 26, (Arrows indicate new isoforms and increase of isomers in treated plants).

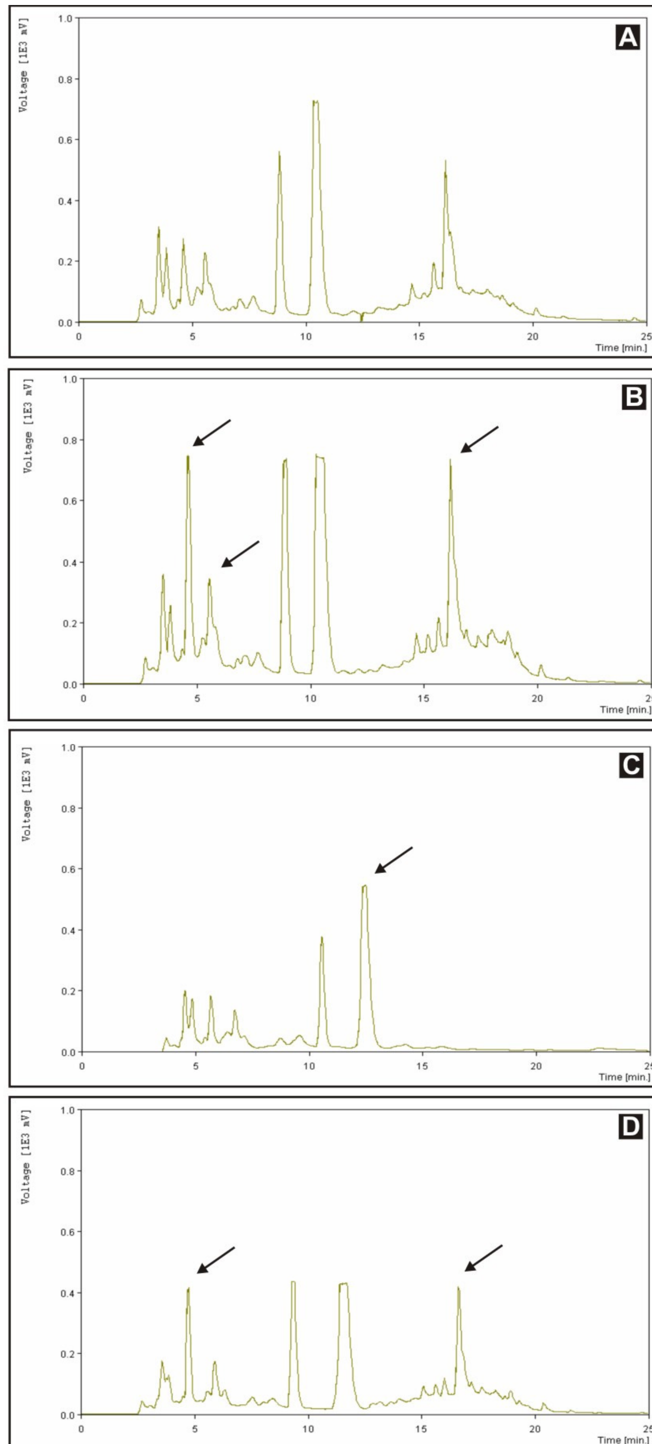


Figure 40: HPLC profiles of catechins of leaves of tea plants treated with *B. megaterium* (B), *S. marcescens* (C) and *B. megaterium*+ *S. marcescens* (D) in comparison to untreated leaves (A) of T 17, (Arrows indicate new isoforms and increase of isomers in treated plants).

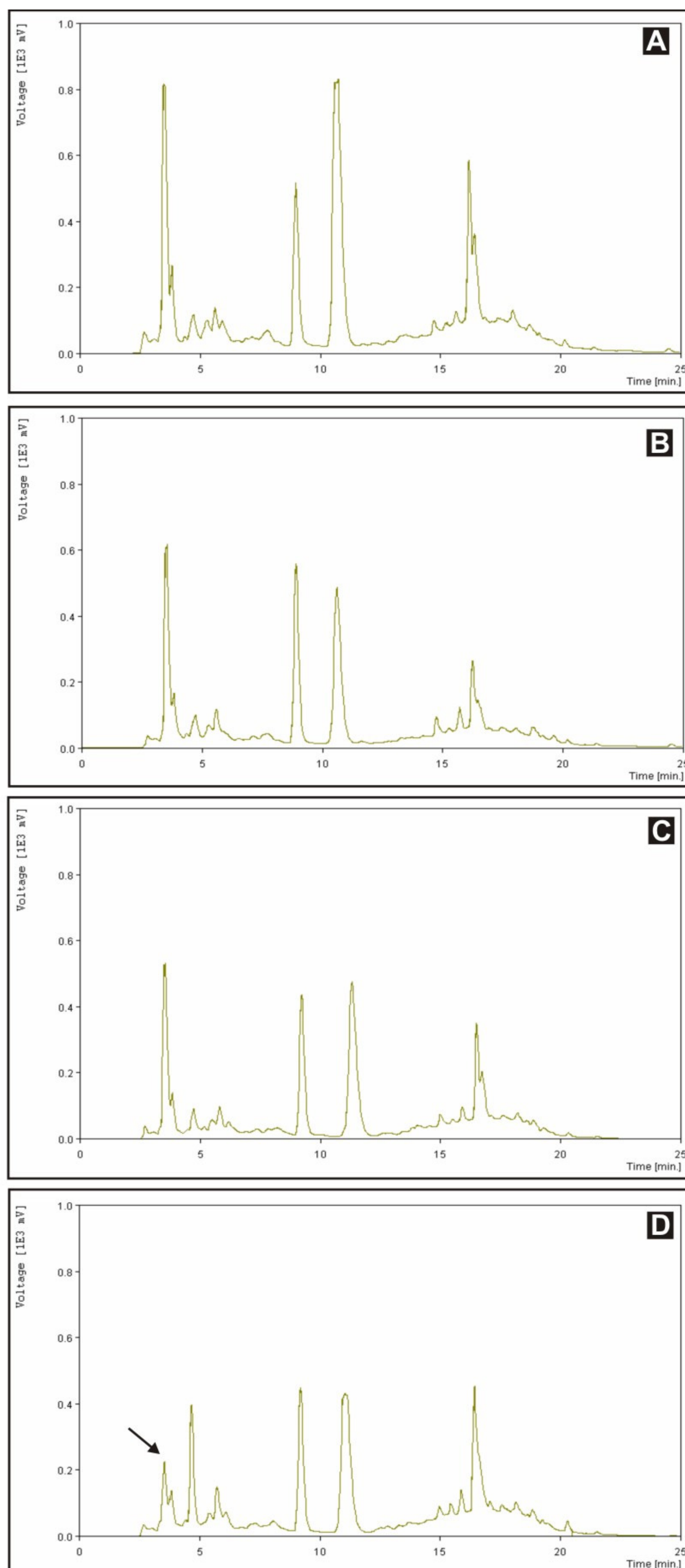


Figure 41: HPLC profiles of catechins of leaves of tea plants treated with *B. megaterium* (B), *S. marcescens* (C) and *B. megaterium+S. marcescens* (D) in comparison to untreated leaves (A) of TV 18, (Arrows indicate new isoforms and increase of isomers in treated plants).

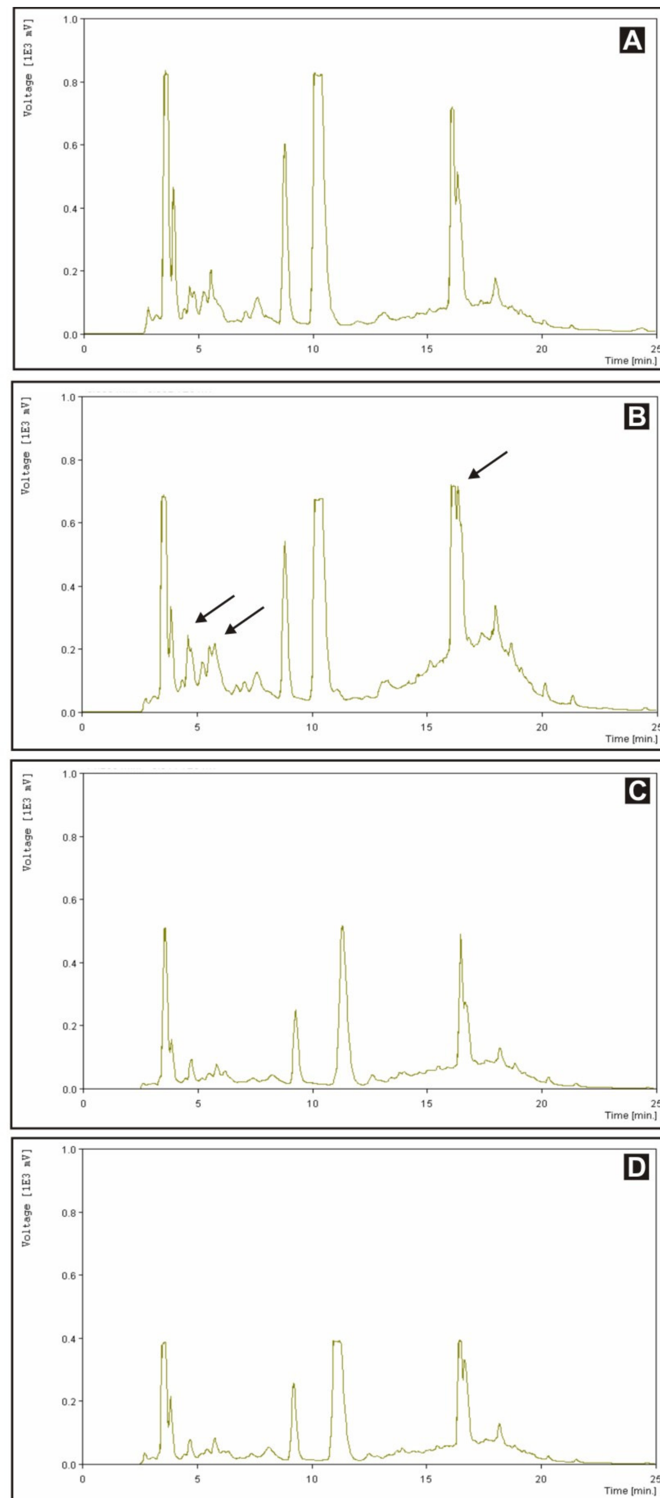


Figure 42: HPLC profiles of catechins of leaves of tea plants treated with *B. megaterium* (B), *S. marcescens* (C) and *B. megaterium*+*S. marcescens* (D) in comparison to untreated leaves (A) of TV 25, (Arrows indicate new isoforms and increase of isomers in treated plants).

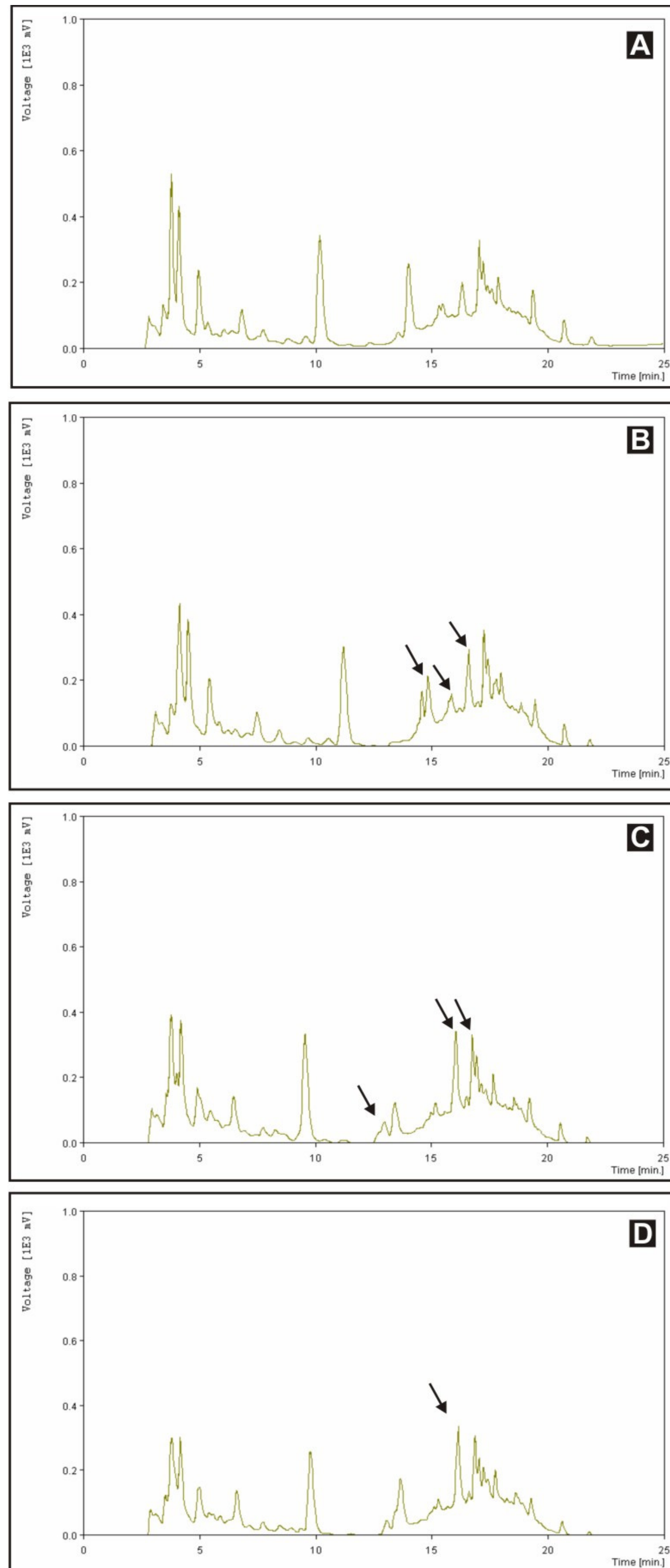


Figure 43: HPLC profiles of catechins from 8 yrs old tea plants of T17/154 treated with foliar spray of *B. megaterium* (B), *S. marcescens* (C) and *B. megaterium*+ *S. marcescens* (D) in comparison to untreated (A) leaves, (Arrows indicate new isoforms and increase of isomers in treated plants).

Table 15 a: Peak result of HPLC analysis of catechin extracts from leaves of untreated control. (cv. TV-26)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	2.740	590.1520	40.825	0.365	1.106
2	3.670	12722.5907	398.654	0.500	7.874
3	4.700	5811.0867	233.824	0.300	3.597
4	5.740	12776.1564	228.421	0.920	7.907
5	7.120	3561.0949	80.059	2.204	2.169
6	8.040	4391.8700	95.650	2.718	2.591
7	9.040	12202.0500	457.589	0.380	7.552
8	11.050	24748.7733	510.511	0.720	15.317
9	12.040	2267.0321	85.013	1.403	2.303
10	12.530	1489.7794	53.348	0.922	1.445
11	13.860	6078.2293	84.226	3.762	2.282
12	14.950	4708.7662	131.623	0.760	2.914
13	15.420	5171.0675	174.406	0.590	3.200
14	16.760	29749.8524	507.733	0.700	18.413
15	17.570	9479.3642	261.195	0.660	5.867
16	18.190	25825.8939	347.920	1.050	15.985

Table 15 b: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Bacillus megaterium*. (cv. TV-26)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	2.770	1390.6953	112.542	0.506	1.853
2	3.180	2298.4376	143.713	0.837	2.366
3	3.500	20019.4371	617.316	7.287	10.163
4	4.740	12517.9887	515.993	4.556	8.495
5	5.750	22863.1957	503.242	8.322	8.285
6	7.430	6008.6941	178.511	2.187	2.939
7	8.120	12534.9687	336.524	4.562	5.541
8	9.110	23621.9931	630.417	8.598	10.379
9	10.490	47348.2430	628.679	17.233	10.351
10	12.240	3360.0023	122.606	1.223	2.019
11	12.850	4147.6470	118.849	1.510	1.957
12	13.260	2690.7192	135.447	0.979	2.230
13	13.950	10087.0800	218.041	3.671	3.590
14	15.480	14497.6329	335.020	5.277	5.516
15	15.890	5094.8732	230.599	1.854	3.797
16	16.510	34974.7055	635.842	12.730	10.468
17	18.220	51290.5618	610.536	18.668	10.051

Table 15 c: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Serratia marcescens*. (cv. TV-26)

Peak no	Retn time (min)	Area (mVs)	Height (mV)	Area(%)	Height (%)
1	2.680	646.6168	50.319	0.648	1.311
2	3.090	607.9323	36.357	0.609	0.947
3	3.470	12878.1841	610.034	0.250	12.907
4	4.390	718.7049	49.757	0.720	1.297
5	4.640	2571.7516	145.085	0.250	2.577
6	5.390	1850.6808	73.181	1.855	1.907
7	5.710	2008.9874	120.983	0.360	2.013
8	6.040	1845.5117	68.265	1.850	1.779
9	6.780	544.2133	29.867	0.545	0.778
10	7.280	1354.6690	47.139	1.358	1.228
11	8.010	3228.0214	75.032	3.235	1.955
12	9.130	11232.3518	570.778	0.280	11.257
13	10.990	20274.2066	608.175	0.500	20.319
14	12.350	1448.8349	51.470	1.452	1.341
15	12.760	739.9397	36.422	0.742	0.949
16	13.240	768.4746	39.050	0.770	1.018
17	13.630	848.7430	46.541	0.851	1.213
18	13.880	1223.3927	52.106	1.226	1.358
19	14.290	709.8130	41.604	0.711	1.084
20	14.740	953.4495	47.807	0.956	1.246
21	15.420	2779.1116	72.762	2.785	1.896
22	15.820	1367.5925	68.041	1.371	1.773
23	16.460	14843.1584	506.239	0.460	14.876
24	17.570	4165.5867	111.523	0.700	4.175
25	18.170	4707.2220	158.713	0.450	4.718
26	18.840	3821.2119	73.939	3.830	1.927
27	20.310	891.7997	28.200	0.894	0.735
28	21.530	702.5757	14.837	0.704	0.387
29	24.330	15.3148	0.947	0.015	0.025
30	24.700	32.4674	2.607	0.031	0.066

Table 15 d: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with combined application of *B. megaterium* and *S. marcescens*. (cv. TV-26)

Peak no	Retn time (min)	Area(mVs)	Height (mV)	Area(%)	Height(%)
1	2.700	417.5193	28.562	0.508	1.078
2	3.350	593.0862	34.997	0.721	1.320
3	3.610	8441.8778	373.113	0.280	10.266
4	4.780	2142.1280	125.737	0.220	2.605
5	5.230	977.0494	37.955	1.188	1.432
6	6.080	2619.4997	86.591	3.186	3.267
7	6.570	1740.8535	53.279	2.117	2.010
8	7.440	377.4445	22.820	0.459	0.861
9	7.790	1130.3766	39.000	1.375	1.471
10	8.770	2272.4421	48.318	2.764	1.823
11	9.590	9479.5613	467.601	0.270	11.528
12	12.230	13153.3113	387.896	0.500	15.996
13	13.310	2063.4406	65.731	2.509	2.480
14	14.010	909.0662	48.378	1.106	1.825
15	14.440	2195.8963	68.394	2.670	2.580
16	14.970	970.3846	63.254	1.180	2.387
17	15.780	3569.8493	84.016	4.341	3.170
18	16.770	15916.6358	462.227	0.420	19.356
19	18.340	13167.2840	148.834	1.290	16.013
20	24.710	92.7161	3.752	0.112	0.143

Table 16 a: Peak result of HPLC analysis of catechin extracts from leaves of untreated control. (cv. T-17)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	2.730	923.8596	73.150	0.895	1.556
2	3.050	555.2647	32.947	0.538	0.701
3	3.490	3825.9065	312.279	3.707	6.643
4	3.840	3473.6993	245.323	0.200	3.365
5	4.350	916.2130	73.319	0.888	1.560
6	4.590	4279.0588	273.969	0.240	4.146
7	5.200	2434.4760	114.271	0.410	2.359
8	5.550	5681.1259	227.982	0.390	5.504
9	6.480	799.3863	43.253	0.774	0.920
10	6.780	772.0844	48.934	0.748	1.041
11	7.080	1646.8483	73.765	1.595	1.569
12	7.670	3294.4882	83.474	3.192	1.776
13	8.830	9583.4546	560.258	0.240	9.285
14	10.350	20538.8525	728.574	0.420	19.898

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
15	11.270	667.7300	27.929	0.647	0.594
16	12.100	1298.8390	34.259	1.258	0.729
17	12.580	595.1502	28.113	0.577	0.598
18	13.200	1788.3005	45.829	1.733	0.975
19	13.710	501.0161	40.179	0.485	0.855
20	14.130	1408.1426	54.924	1.364	1.168
21	14.680	3425.3424	123.577	0.490	3.319
22	15.220	1637.8450	93.554	1.587	1.990
23	15.640	3672.2302	193.776	0.340	3.558
24	15.940	910.7418	109.266	0.140	0.882
25	16.160	14671.9112	531.194	0.360	14.214
26	17.360	3308.1391	104.687	0.580	3.205
27	18.010	3070.4064	105.222	0.550	2.975
28	18.450	1046.6204	83.125	1.014	1.768
29	18.660	1800.5683	84.123	1.744	1.789
30	19.130	2250.7584	59.974	2.181	1.276
31	20.160	1054.8918	42.759	1.022	0.910
32	20.910	296.0453	12.932	0.287	0.275
33	21.360	616.9233	16.289	0.598	0.346
34	22.790	299.2851	6.123	0.290	0.130
35	24.030	43.5617	1.827	0.042	0.039
36	24.500	129.5209	9.782	0.124	0.208

Table 16 b: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Bacillus megaterium.* (cv. T-17)

Peak no	Retn time (min)	Area(mVs)	Height (mV)	Area(%)	Height(%)
1	2.710	1138.6316	85.844	0.767	1.340
2	3.030	907.8586	52.192	0.612	0.815
3	3.480	4739.4171	360.210	0.200	3.194
4	3.800	4057.6172	257.431	0.230	2.734
5	4.340	1661.8404	113.188	0.300	1.120
6	4.570	11821.6223	748.179	0.230	7.966
7	5.230	2902.8866	149.618	0.390	1.956
8	5.540	8785.3124	344.754	0.430	5.920
9	6.410	1075.8097	59.820	0.725	0.934
10	6.780	1232.7278	80.906	0.831	1.263
11	7.090	2310.7570	92.230	1.557	1.440
12	7.670	4178.6942	101.947	0.610	2.816
13	8.910	15306.9925	735.823	0.300	10.315
14	10.250	26696.0087	751.815	0.550	17.990
15	11.440	1251.8605	42.751	0.844	0.667
16	12.110	1471.8943	46.906	0.992	0.732
17	12.620	1131.4545	43.931	0.762	0.686
18	13.190	2120.9157	60.214	1.429	0.940

Peak no	Retn time (min)	Area(mVs)	Height (mV)	Area(%)	Height(%)
19	13.740	781.8650	53.424	0.527	0.834
20	14.130	2024.1207	73.392	1.364	1.146
21	14.670	3540.8945	164.625	0.330	2.386
22	14.970	984.4875	104.768	0.160	0.663
23	15.180	2695.0434	160.452	0.350	1.816
24	15.650	4087.7892	214.796	0.370	2.755
25	16.180	17012.5935	736.152	0.330	11.464
26	16.870	4112.5540	175.386	0.490	2.771
27	17.400	4002.4467	155.719	0.500	2.697
28	18.010	7198.5724	174.821	0.820	4.851
29	18.710	6664.3467	171.466	0.420	4.491
30	20.170	1506.4383	60.073	1.015	0.938
31	21.380	612.6690	18.719	0.413	0.292
32	22.860	232.4250	5.258	0.157	0.082
33	24.070	37.3578	1.552	0.025	0.024
34	24.540	108.4814	8.571	0.075	0.133

Table 16 c: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Serratia marcescens*. (cv. T-17)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	3.700	658.2046	47.614	1.442	2.286
2	4.030	387.9846	22.828	0.850	1.096
3	4.520	2678.2978	202.315	0.210	5.866
4	4.830	2740.2886	176.704	0.230	6.001
5	5.400	596.3365	48.530	1.306	2.330
6	5.660	2768.5244	185.246	0.220	6.063
7	6.390	1708.4239	66.726	3.742	3.204
8	6.720	2263.0383	137.578	0.280	4.956
9	7.130	1290.6704	53.139	2.827	2.552
10	8.280	745.6566	21.027	1.633	1.010
11	8.710	1322.3674	45.312	2.896	2.176
12	9.560	2355.9378	53.723	5.160	2.580
13	10.550	6460.6885	377.769	0.230	14.149
14	12.460	15093.1706	548.485	0.410	33.055
15	14.250	1322.7162	23.275	2.897	1.118
16	15.230	459.0735	13.160	1.005	0.632
17	15.840	784.3271	16.496	1.718	0.792
18	17.220	495.3626	7.229	1.085	0.347
19	18.470	271.2241	5.957	0.594	0.286
20	19.730	266.1827	4.680	0.583	0.225
21	20.630	215.8298	3.669	0.473	0.176
22	22.830	412.6512	9.824	0.904	0.472
23	23.370	200.7257	6.698	0.440	0.322
24	24.130	163.2018	4.434	0.355	0.212

Table 16 d: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with dual application of *B. megaterium* and *S. marcescens* . (cv. T-17)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	2.670	611.9642	41.567	0.752	1.140
2	3.020	462.8038	27.251	0.569	0.747
3	3.550	4761.5013	174.024	5.849	4.772
4	4.180	492.1371	40.596	0.605	1.113
5	4.470	655.3570	57.515	0.805	1.577
6	4.730	6045.2400	413.254	7.427	11.332
7	5.250	635.0962	44.612	0.780	1.223
8	5.550	1322.1169	73.307	1.624	2.010
9	5.870	2955.4777	172.419	0.280	3.631
10	6.310	1980.1466	78.325	2.433	2.148
11	7.010	482.5280	28.278	0.593	0.775
12	7.510	1564.3415	53.212	1.922	1.459
13	8.020	663.8236	38.144	0.816	1.046
14	8.420	1923.0318	50.071	2.362	1.373
15	9.290	9086.3800	435.765	0.290	11.163
16	11.660	16495.1204	431.004	0.560	20.264
17	12.860	714.8459	33.825	0.878	0.928
18	13.160	881.6440	39.051	1.083	1.071
19	13.640	796.4171	38.918	0.978	1.067
20	14.020	1343.2634	48.660	1.650	1.334
21	14.720	1395.7748	50.600	1.715	1.388
22	15.080	2055.5491	90.227	2.525	2.474
23	15.630	2008.3836	93.908	2.467	2.575
24	16.010	2020.8516	116.338	0.410	2.483
25	16.630	8972.4737	416.573	0.260	11.023
26	17.210	1925.9989	104.178	0.380	2.366
27	17.660	2246.3222	87.891	2.760	2.410
28	18.110	635.3257	67.984	0.780	1.864
29	18.270	1613.4939	76.816	1.982	2.106
30	18.690	634.9311	57.562	0.780	1.578
31	18.940	1372.8409	73.961	1.687	2.028
32	19.310	1430.0013	42.060	1.757	1.153
33	20.390	803.0056	34.203	0.986	0.938

Table 17 a: Peak result of HPLC analysis of catechin extracts from leaves of untreated control. (cv. TV-18)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	2.640	1058.5157	64.966	0.969	1.433
2	3.070	758.1258	43.975	0.694	0.970
3	3.470	15313.8499	819.853	0.230	14.018
4	4.340	682.8369	49.535	0.625	1.093
5	4.700	2584.6250	117.747	0.380	2.366
6	5.270	2119.1078	99.097	1.940	2.186
7	5.600	1979.3081	139.076	0.300	1.812
8	5.910	2429.1389	98.098	2.224	2.164
9	6.540	683.1326	38.493	0.625	0.849
10	6.910	717.3183	44.817	0.657	0.989
11	7.150	1169.5335	49.747	1.071	1.098
12	7.780	3016.7294	68.522	2.762	1.512
13	8.970	9481.9812	517.646	0.240	8.680
14	10.730	22773.3968	832.887	0.420	20.847
15	14.740	2342.7852	98.479	2.145	2.173
16	15.230	1709.2886	89.997	1.565	1.986
17	15.660	2919.5299	125.657	0.490	2.673
18	16.200	15376.6721	585.366	0.390	14.076
19	17.410	4175.7869	104.643	0.710	3.823
20	18.030	4501.2949	129.707	0.800	4.121
21	18.710	4331.0385	85.287	3.965	1.882

Table 17 b: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Bacillus megaterium.* (cv. TV-18)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	3.520	10844.4371	618.435	0.210	15.874
2	4.700	2041.8730	98.327	2.989	3.049
3	5.260	1366.1923	68.431	2.000	2.122
4	5.580	3038.0797	117.364	0.280	4.447
5	8.890	9096.5958	558.570	0.240	13.316
6	10.600	11078.1562	484.786	0.330	16.216
7	14.750	2256.5553	92.106	3.303	2.856
8	15.300	977.1638	58.489	1.430	1.814

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
9	15.720	2328.7234	123.263	0.290	3.409
10	16.250	6487.2648	262.104	0.370	9.496
11	16.940	1428.5522	59.651	2.091	1.850
12	17.460	1820.3471	61.152	2.665	1.896
13	18.060	2113.9462	59.485	3.094	1.845
14	18.800	2108.9704	60.895	3.087	1.888

Table 17 c: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Serratia marcescens*. (cv. TV-18)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	3.540	9406.6806	533.144	0.210	14.103
2	4.710	2010.0994	92.416	3.014	2.990
3	5.800	1546.5727	100.826	0.270	2.319
4	6.180	1482.5404	53.170	2.223	1.720
5	9.210	7591.3571	441.171	0.240	11.381
6	11.310	11653.4481	476.128	0.360	17.472
7	15.000	2039.0612	75.283	3.057	2.435
8	15.530	1185.5679	61.689	1.777	1.996
9	15.930	1927.9808	98.347	2.891	3.181
10	16.520	9103.1640	353.298	0.210	13.648
11	17.600	2575.6766	72.742	3.862	2.353
12	18.220	2059.1230	80.880	3.087	2.616
13	18.610	861.7767	57.287	1.292	1.853
14	18.870	1147.9306	59.329	1.721	1.919

Table 17 d: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Bacillus megaterium* and *Serratia marcescens*. (cv. TV-18)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	3.510	3024.3928	226.453	0.220	3.843
2	3.810	2429.1077	138.699	0.260	3.087
3	4.650	5196.3155	398.848	0.190	6.603
4	5.390	1624.5667	70.453	2.064	1.952
5	5.710	2365.2179	150.305	0.270	3.006
6	9.190	7656.8056	449.990	0.240	9.730
7	11.030	13131.8767	433.201	0.440	16.688

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
8	14.980	2333.4112	90.903	2.965	2.519
9	15.450	2274.2960	98.060	2.890	2.717
10	15.890	2525.8460	139.816	0.380	3.210
11	16.440	12587.4405	454.480	0.320	15.996
12	17.590	2410.4633	95.417	3.063	2.644
13	18.170	3018.0151	103.316	0.620	3.835
14	18.660	680.0561	66.501	0.864	1.842
15	18.850	1629.1170	81.068	2.070	2.246

Table 18 a: Peak result of HPLC analysis of catechin extracts from leaves of untreated control. (cv. TV-25)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	2.780	1048.4266	83.990	0.789	2.064
2	3.140	1065.4175	59.455	0.801	1.461
3	3.560	19206.9925	836.852	0.260	14.449
4	5.540	12201.7505	201.791	0.390	9.179
5	7.060	2606.2911	70.482	1.961	1.732
6	7.560	4774.7326	113.769	0.530	3.592
7	8.760	10906.6113	602.561	0.250	8.205
8	10.080	30207.5050	827.462	0.550	22.724
9	11.950	1818.4621	36.126	1.368	0.888
10	13.130	3409.1141	64.275	2.565	1.580
11	14.150	1978.7946	51.636	1.489	1.269
12	14.580	1954.3046	61.903	1.470	1.521
13	15.090	1448.4897	74.576	1.090	1.833
14	15.710	2316.5957	76.867	1.743	1.889
15	16.090	21447.7069	716.813	0.510	16.134
16	17.980	15782.4345	173.083	1.040	11.872
17	21.980	460.5916	7.023	0.346	0.173
18	24.420	298.6112	10.108	0.223	0.250

Table 18 b: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Bacillus megaterium*. (cv. TV-25)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	2.740	502.5549	42.927	0.294	0.699
2	3.110	965.0245	50.116	0.565	0.816
3	3.520	11939.7275	689.376	0.280	6.990
4	3.850	4169.9682	334.648	0.200	2.441
5	4.350	1553.2268	101.186	0.310	0.909
6	4.590	5191.5414	243.526	0.370	3.039

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
7	5.220	2875.4318	157.154	0.380	1.683
8	5.530	2579.3947	209.184	0.250	1.510
9	5.770	6234.7684	217.155	0.430	3.650
10	6.690	1613.8096	84.594	0.945	1.377
11	7.050	1846.1717	94.193	1.081	1.533
12	7.590	5733.1556	124.448	0.890	3.357
13	8.800	10561.7127	540.048	0.240	6.183
14	10.350	24266.5949	676.025	0.550	14.207
15	11.060	1867.3542	70.074	1.093	1.140
16	11.900	1565.6545	43.892	0.917	0.714
17	12.380	1236.4239	47.486	0.724	0.773
18	13.050	1938.5276	93.531	1.135	1.522
19	13.290	3134.4566	98.124	1.835	1.597
20	14.210	2816.9280	96.544	1.649	1.571
21	14.600	1808.0795	116.700	0.310	1.059
22	15.140	5524.3421	161.645	0.700	3.234
23	15.660	3384.3584	160.948	0.370	1.981
24	16.080	31706.1031	715.623	0.600	18.563
25	17.380	9695.3419	249.366	0.710	5.676
26	18.000	11755.2106	334.511	0.820	6.882
27	18.670	9958.7926	215.872	0.710	5.830
28	20.150	2369.2374	87.683	1.387	1.427

Table 18 c: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Serratia marcescens*. (cv. TV-25)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	2.590	203.6235	17.751	0.272	0.562
2	3.040	458.4001	17.911	0.612	0.567
3	3.530	9342.7823	513.677	0.230	12.474
4	4.450	458.2654	34.719	0.612	1.099
5	4.690	1584.3929	94.744	2.115	3.000
6	5.160	520.9088	34.945	0.695	1.107
7	5.470	894.5168	50.167	1.194	1.589
8	5.800	1326.3412	78.603	1.771	2.489
9	6.160	1567.2746	56.951	2.093	1.803
10	6.900	505.0251	23.650	0.674	0.749
11	7.410	1023.0048	34.893	1.366	1.105

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
12	8.220	2273.2847	44.701	3.035	1.415
13	9.250	5311.2389	251.515	0.250	7.091
14	11.290	13006.3682	520.790	0.360	17.365
15	12.610	1144.0835	44.145	1.528	1.398
16	12.970	479.9595	26.647	0.641	0.844
17	13.440	675.5071	35.802	0.902	1.134
18	13.810	813.6923	50.862	1.086	1.611
19	14.010	1235.5853	53.717	1.650	1.701
20	14.680	1578.4988	52.795	2.108	1.672
21	15.030	753.8359	58.824	1.006	1.863
22	15.200	557.7064	58.439	0.745	1.850
23	15.500	1534.5991	72.263	2.049	2.288
24	15.930	1447.5672	68.792	1.933	2.178
25	16.480	12983.1398	491.789	0.410	17.334
26	17.580	3250.4568	91.141	4.340	2.886
27	18.190	4266.6008	129.197	0.780	5.697
28	18.850	3798.2125	81.687	5.071	2.587
29	20.310	1032.6641	36.533	1.379	1.157

Table 18 d: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Bacillus megaterium* and *Serratia marcescens*. (cv. TV-25)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	2.690	430.4852	35.918	0.599	1.586
2	3.070	400.8424	22.389	0.557	0.989
3	3.570	9280.9945	389.386	0.280	12.904
4	4.680	1711.7022	78.215	2.380	3.453
5	5.750	4154.4133	83.398	5.776	3.682
6	6.880	366.1677	20.857	0.509	0.921
7	7.370	968.0418	33.623	1.346	1.485
8	8.100	2373.0443	54.892	3.299	2.424
9	9.200	4802.5770	257.904	0.250	6.677
10	10.950	14564.3647	394.621	0.550	20.249
11	12.500	1351.2924	33.042	1.879	1.459
12	13.950	2425.6830	51.626	3.372	2.279
13	14.450	1441.5963	41.671	2.004	1.840
14	15.120	1129.8041	45.503	1.571	2.009
15	15.490	1078.1832	57.456	1.499	2.537
16	15.860	1133.6470	57.457	1.576	2.537
17	16.460	13165.9622	395.750	0.490	18.305
18	17.600	2910.5835	79.506	4.047	3.510
19	18.200	8180.8587	129.145	0.510	11.374
20	24.680	55.6738	2.542	0.077	0.112

Table 19 a: Peak result of HPLC analysis of catechin extracts from leaves of untreated control. (cv. Pruned T-17/154- Hansqua Tea Estate field)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	2.790	2550.3325	106.216	0.500	2.896
2	3.410	1976.0318	139.313	0.260	2.244
3	3.760	6285.8247	537.951	0.170	7.137
4	4.090	6911.1867	439.800	0.190	7.848
5	4.940	3753.7289	244.792	0.230	4.262
6	5.340	1433.4135	87.287	1.628	2.155
7	5.700	754.6841	49.224	0.857	1.216
8	6.040	1119.2727	63.134	1.271	1.559
9	6.380	1264.2516	58.889	1.436	1.454
10	6.810	2444.6859	122.559	0.300	2.776
11	7.510	774.7684	40.228	0.880	0.993
12	7.720	1199.4439	60.260	1.362	1.488
13	8.160	740.2660	26.136	0.841	0.645
14	8.790	1045.6932	33.030	1.187	0.816
15	9.560	952.5099	39.733	1.082	0.981
16	10.170	6702.1247	346.067	0.280	7.610
17	11.410	467.5759	13.699	0.531	0.338
18	12.340	462.5123	17.669	0.525	0.436
19	13.550	1495.4603	48.690	1.698	1.202
20	14.000	5425.7790	256.362	0.290	6.161
21	14.820	1585.7327	68.515	1.801	1.692
22	15.480	4527.4297	133.136	0.700	5.141
23	15.880	1621.3021	101.274	0.280	1.841
24	16.320	5220.2966	198.533	0.440	5.928
25	17.050	11495.2400	326.418	0.540	13.053
26	17.880	10086.0949	211.344	0.820	11.453
27	19.380	4069.2167	171.770	0.230	4.621
28	20.720	1240.4428	79.897	1.409	1.973
29	21.910	457.7670	27.186	0.520	0.671

Table 19 b: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Bacillus megaterium*. (cv. Pruned T-17/154- Hansqua Tea Estate field)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	3.080	1431.5233	112.496	0.240	3.782
2	3.350	1346.6680	75.953	3.558	3.023
3	3.750	1652.8127	128.208	0.230	4.367
4	4.110	6582.0028	431.344	0.220	17.391
5	4.490	5931.5726	378.826	0.200	15.672
6	5.410	3104.6515	191.591	0.240	8.203
7	5.830	724.4694	55.570	1.914	2.212
8	6.210	427.9222	26.234	1.131	1.044
9	6.530	391.9569	26.846	1.036	1.069
10	7.070	282.5418	16.437	0.747	0.654
11	7.460	1319.7396	84.974	3.487	3.382
12	8.410	613.6818	38.793	1.621	1.544
13	9.090	101.6364	6.401	0.269	0.255
14	9.660	343.8009	19.301	0.908	0.768
15	10.530	343.0705	18.897	0.906	0.752
16	11.190	5465.3507	299.491	0.290	14.441
17	12.500	44.5354	3.475	0.118	0.138
18	13.320	60.8659	5.200	0.161	0.207
19	13.830	30.7434	2.614	0.081	0.104
20	14.580	1514.6885	128.213	0.160	4.002
21	14.840	2352.2463	166.689	0.210	6.215
22	15.360	113.0078	13.342	0.299	0.531
23	15.860	1408.9289	76.168	3.723	3.032
24	16.220	222.9383	20.018	0.589	0.797
25	16.600	2035.7533	185.121	0.170	5.379

Table 19 c: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Serratia marcescens*. (cv. Pruned T-17/154- Hansqua Tea Estate field)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	2.920	1206.7108	113.086	0.190	1.318
2	3.150	1829.6633	92.891	1.998	3.196
3	3.760	8605.1507	400.691	0.260	9.398
4	4.180	6297.1337	383.467	0.220	6.877
5	4.900	4136.2803	178.177	0.390	4.517
6	5.460	3257.0031	107.220	0.650	3.557
7	6.130	735.5392	61.138	0.803	2.103
8	6.470	3185.0991	150.179	0.300	3.479
9	6.930	1008.0948	45.140	1.101	1.553
10	7.440	565.2819	33.038	0.617	1.137
11	7.730	1232.3787	54.355	1.346	1.870
12	8.260	1829.0858	47.479	1.998	1.633
13	9.540	6648.0358	342.772	0.270	7.261
14	10.380	503.4254	17.025	0.550	0.586
15	11.190	526.7582	15.723	0.575	0.541

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
16	11.970	278.0371	8.618	0.304	0.296
17	12.980	1824.2652	71.594	1.992	2.463
18	13.430	3133.0311	130.504	0.320	3.422
19	15.190	6009.1639	130.633	0.700	6.563
20	15.610	1629.0145	103.112	0.280	1.779
21	16.070	35712.0173	351.053	0.260	39.003
22	20.590	1406.5983	68.146	1.536	2.344
23	23.470	5.6112	0.701	0.150	0.025

Table 19 d: Peak result of HPLC analysis of catechin extracts from leaves of tea plants treated with *Bacillus megaterium* and *Serratia marcescens*. (cv. Pruned T-17/154- Hansqua Tea Estate field)

Peak no	Retn time (min)	Area(mVs)	Height(mV)	Area(%)	Height(%)
1	2.860	885.0965	86.919	1.071	2.305
2	3.100	1443.4552	73.333	1.747	1.944
3	3.500	1349.1120	131.857	0.180	1.632
4	3.770	5781.2247	308.306	0.380	6.995
5	4.150	5309.3047	311.592	0.230	6.424
6	4.970	3331.6736	155.365	0.330	4.031
7	5.400	1790.9842	76.242	2.167	2.021
8	5.870	1166.5521	65.804	1.412	1.745
9	6.590	3769.4049	144.223	0.280	4.561
10	7.130	859.9041	37.520	1.040	0.995
11	7.710	1356.3631	47.943	1.641	1.271
12	8.440	916.1236	36.683	1.108	0.973
13	8.940	627.5156	29.238	0.759	0.775
14	9.370	440.4616	27.850	0.533	0.738
15	9.760	4886.8132	267.297	0.270	5.913
16	10.490	427.5532	12.664	0.517	0.336
17	11.530	370.4569	11.229	0.448	0.298
18	12.300	104.8946	5.177	0.127	0.137
19	13.060	1036.5787	52.037	1.254	1.380
20	13.650	4370.4264	182.037	0.310	5.288
21	14.560	1724.1926	55.177	2.086	1.463
22	15.110	2266.4540	95.021	2.742	2.519
23	15.290	1967.2604	119.498	0.330	2.380
24	15.690	1528.4274	96.279	1.849	2.553
25	16.150	7043.6194	344.019	0.260	8.523
26	16.620	1743.8455	142.924	0.230	2.110
27	16.860	10551.4131	314.308	0.720	12.767
28	17.750	6883.1532	209.637	0.880	8.328
29	18.640	4087.8737	139.057	0.640	4.946
30	19.290	3467.5663	120.393	0.300	4.196
31	20.630	871.4697	50.222	1.054	1.332
32	21.600	41.0104	4.468	0.050	0.118
33	21.810	240.0382	16.658	0.290	0.442
34	23.500	5.7991	0.665	0.160	0.020

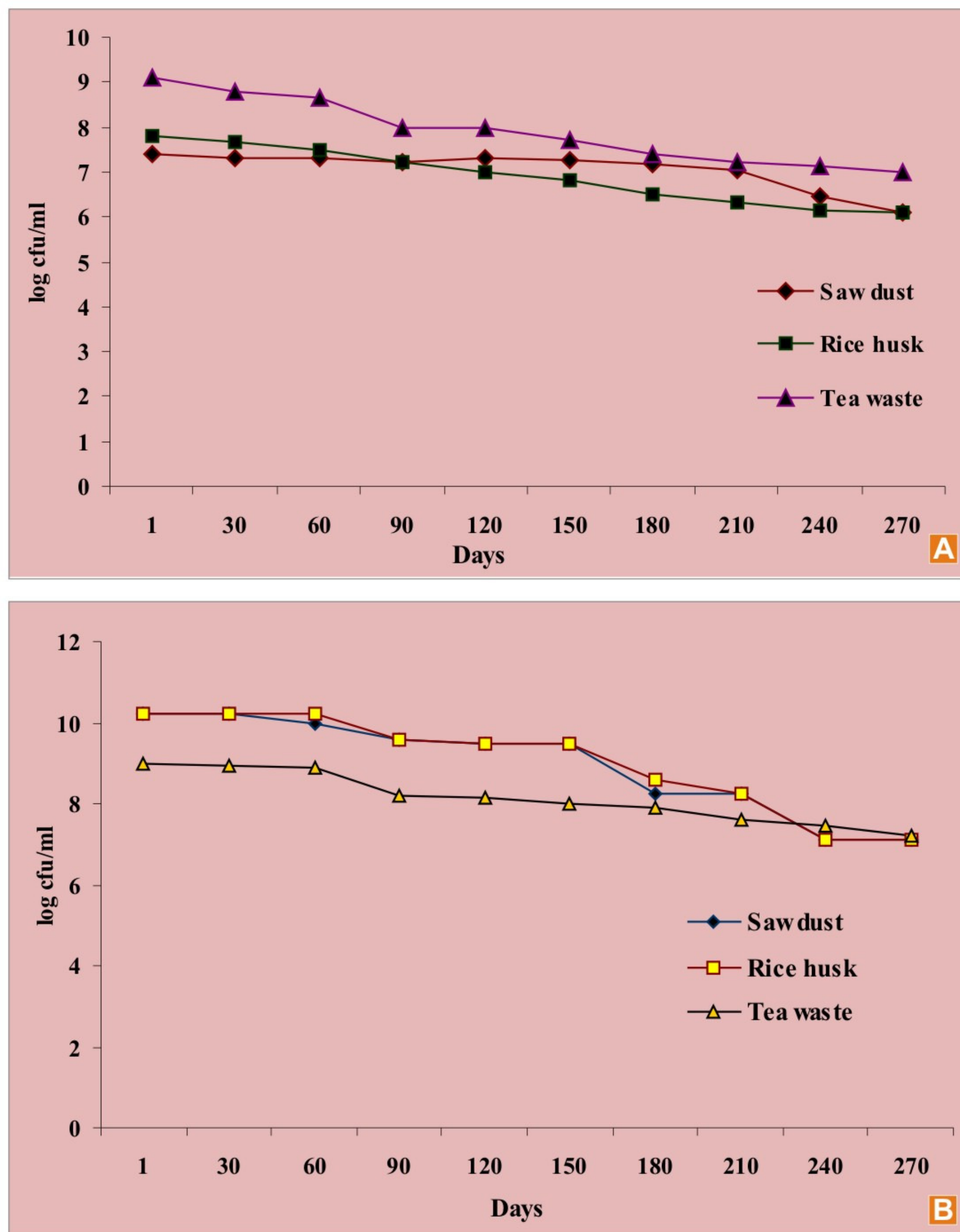


Figure 44: Growth of bacteria in bioformulations at different periods of incubation A- *B.megaterium* and B-*S.marcescens*.

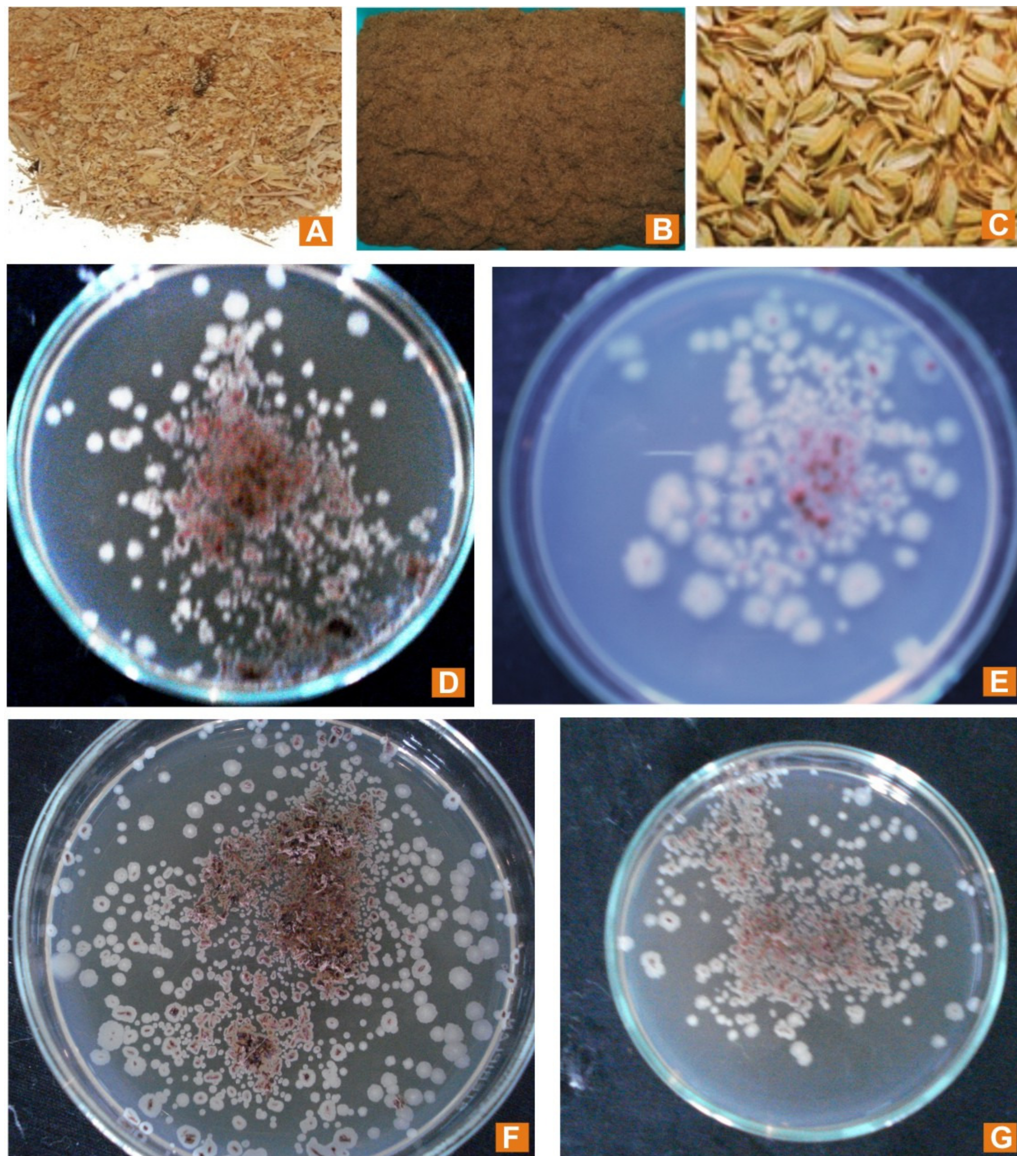


Figure 45: Bioformulation materials- A- saw dust; B- tea waste; C- rice husk. *In vitro* growth of *B. megaterium* (D&E) and *S. marcescens* (F&G) in saw dust (D&E) and tea waste (F&G) formulations after 8 months.

4.9. Effect of application of *B. megaterium* and *S. marcescens* as bioformulations on growth of tea plants

Besides aqueous suspensions, the single and joint effects of *S. marcescens* and *B. megaterium* in various bioformulations were also tested on growth. Application of *B. megaterium* and *S. marcescens* singly or jointly, in formulations of saw dust, rice husk and tea waste led to significant increase in growth of tea plants. The growth was measured in terms of increase in height and the number of leaves of plants one and two months after application. The observed increase was similar to that observed by application of aqueous suspension of the bacteria. Statistical analysis (ANOVA) revealed that there was no significant difference among the aqueous suspension or different bioformulations although all of them were significantly higher than control (Tables 20-22; Figs. 46 & 47, A-L).

4.10. Biochemical changes induced in tea leaves by application of bioformulations

Biochemical changes in terms of activities of defense enzymes and phenolics in tea leaves of five different varieties were noticed following the application of aqueous suspensions as well as different bioformulations of *S. marcescens* and *B. megaterium.*

4.10.1. Enzymes

Young leaves were selected from potted tea plants of all five varieties. Analyses were performed immediately from leaves after 72 h of bacterial inoculation as aqueous suspensions or bioformulations of bacteria to the rhizosphere.

4.10.1.1. Peroxidase and Chitinase

Peroxidase and Chitinase activities of leaves of tea varieties following treatment with aqueous suspensions as well as bioformulations of *B.megaterium* and *S.marcescens* in potted plants were assayed. Significant increase in activities of PO and CHT was observed in treated tea plants in comparison to untreated control plant (Tables 23 & 24).

Table 20: *In vivo* application of bioformulation of *S. marcescens* in potted plants

Tea varieties	Treatment	2 months after treatment			
		Initial height	**Final Height**	**Initial leaf numbers**	**Final leaf numbers**
TV-18	Control	19.0±1.3	26.0± 1.5(7.0)	19.3±1.8	24.5± 1.2(5.2)
	S. marcescens	22.0±2.1	30.2± 1.8(8.2)	19.1±1.6	25.5± 1.4(6.4)
	S.m+saw dust	21.2±2.0	28.3± 1.7(7.1)	18.5±0.9	26.0± 1.4(7.5)
	S.m+rice husk	20.0±1.5	31.0± 2.1(11.0)	20.4±1.1	26.1±0.9 (5.7)
	S.m+tea waste	19.0±1.0	27.2± 1.9(8.2)	21.3±0.8	27.5± 1.1(6.2)
TV-23	Control	22.4±0.9	25.0± 1.7(2.6)	17.1±1.2	24.0± 1.0(6.9)
	S. marcescens	24.5±1.6	32.2± 2.1(7.7)	18.4±1.3	25.2±1.7 (6.8)
	S.m+saw dust	24.2±1.4	37.3± 2.4(13.1)	18.5±1.4	25.0± 1.3(6.5)
	S.m+rice husk	23.1±1.6	35.0± 1.8(11.9)	19.6±0.8	26.2± 2.0(6.6)
	S.m+tea waste	22.0±1.9	35.5±2.0 (13.5)	18.3±1.6	26.0±1.5 (7.7)
TV-25	Control	21.4±2.0	24.5±1.9 (3.1)	19.5±1.0	25.2±1.8 (5.7)
	S.marcescens	22.5±2.3	27.5± 2.0(5.0)	20.0±1.8	28.0± 1.1(8.0)
	S.m+saw dust	23.3±1.9	33.0± 1.8(9.7)	20.2±1.9	29.1± 2.0(8.9)
	S.m+rice husk	22.2±1.7	35.3± 1.7(13.1)	21.4±1.2	27.2± 1.6(5.8)
	S.m+tea waste	21.0±0.9	29.0± 1.4(8.0)	22.3±2.0	28.5± 1.1(6.2)
TV-26	Control	22.4±1.8	25.5±2.3 (3.1)	17.5±2.0	21.0± 1.5(3.5)
	S. marcescens	23.2±1.4	28.5± 2.1(5.3)	18.2±1.9	30.3± 1.3(12.1)
	S.m+saw dust	24.5±1.5	28.2± 2.4(3.7)	19.4±1.5	31.1± 1.0(11.7)
	S.m+rice husk	23.0±1.2	27.2± 2.7(4.2)	18.1±1.7	29.0± 1.9(10.9)
	S.m+tea waste	23.2±1.8	27.5± 1.8(4.3)	19.2±1.5	31.0± 1.7(11.8)
T-17	Control	24.0±2.4	27.2± 1.2(3.2)	18.5±1.5	20.4±1.8 (1.9)
	S. marcescens	24.2±2.2	33.1± 1.7(8.9)	18.2±1.9	25.5± 1.0(7.3)
	S.m+saw dust	23.5±2.1	33.2± 2.0(9.7)	19.0±1.2	26.0± 1.1(7.0)
	S.m+rice husk	25.2±2.0	34.2± 1.4(9.0)	19.2±1.7	27.2± 1.4(8.0)
	S.m+tea waste	26.1±2.3	32.5± 1.1(6.4)	19.5±1.8	25.3± 1.1(5.8)
CD (P=0.05)	(Treatments)	1.233	2.875	0.892	2.186
	(Varieties)	1.233	2.875	0.892	2.186

Average of 10 replicate pots/treatment; Figures in parenthesis indicate increment in final over initial values

Table 20a : ANOVA of data presented in Table 20 (Initial height of tea plants)

Source of Variation	SS	Df	MS	F
Rows	8.3896	4	2.0974	2.479489301
Columns	53.2496	4	13.3124	15.73755763
Error	13.5344	16	0.8459	
Total	75.1736	24		

Table 20b : ANOVA of data presented in Table 20 (Final height of tea plants)

Source of Variation	SS	Df	MS	F
Rows	147.6616	4	36.9154	8.024825277
Columns	110.2136	4	27.5534	5.98967425
Error	73.6024	16	4.60015	
Total	331.4776	24		

Table 20c: ANOVA of data presented in Table 20 (Initial leaf no. of tea plants)

Source of Variation	SS	Df	MS	F
Rows	9.9464	4	2.4866	5.608661329
Columns	18.7504	4	4.6876	10.57313635
Error	7.0936	16	0.44335	
Total	35.7904	24		

Table 20d : ANOVA of data presented in Table 20 (Final leaf no. of tea plants)

Source of Variation	SS	Df	MS	F
Rows	74.4304	4	18.6076	6.998363954
Columns	47.7824	4	11.9456	4.49276943
Error	42.5416	16	2.65885	
Total	164.7544	24		

4.10.1.2. β-1,3 glucanase and Phenyl alanine ammonia lyase

β-1,3-glucanase and Phenyl alanine ammonia lyase activities increased due to single and joint application of bacteria as aqueous suspensions or formulations of saw dust, rice husk and tea waste. ANOVA also revealed that although the enzyme activity in all treatments were significantly higher than control but there was no significant difference among the varieties (Tables 25 & 26).

4.10.2. Phenolics

Total and O-phenol contents in leaves of different varieties of potted tea plants were increased significantly after treatment of the rhizosphere with aqueous suspensions and bioformulation of *B.megaterium* and *S.marcescens* (Tables 27&28).

Table 21: *In vivo* application of bioformulation of *B. megaterium* in potted plants

Tea varieties	Treatment	2 months after treatment			
		Initial height	**Final Height**	**Initial leaf numbers**	**Final leaf numbers**
TV-18	Control	19.0±1.3	26.0± 1.5(7.0)	19.3±1.8	24.5± 1.2(5.2)
	B.megaterium	25.0±1.5	30.0±2.1 (5.0)	19.0±1.3	26.0±2.0(7.0)
	B.m+saw dust	20.0±1.9	27.0± 1.2(7.0)	17.0±0.9	24.0± 1.8(7.0)
	B.m+rice husk	19.5±1.5	32.0± 1.1(12.5)	18.1±1.0	28.0± 2.1(9.9)
	B.m+tea waste	21.0±1.8	29.0±1.8 (8.0)	22.0±1.2	27.0±2.4(5.0)
TV-23	Control	22.4±0.9	25.0± 1.7(2.6)	17.1±1.2	24.0± 1.0(6.9)
	B. megaterium	24.5±1.5	33.0±2.1(8.5)	19.0±1.1	28.0±1.9 (9.0)
	B.m+saw dust	24.0±1.4	38.0± 2.5(14.0)	21.0±1.7	32.0± 2.3(11.0)
	B.m+rice husk	23.0±2.1	39.0.± 2.7(16.0)	18.0±1.0	31.0± 2.4(13.0)
	B.m+tea waste	22.0±2.2	36.0± 1.9(14.0)	19.5±2.0	30.2±1.4(10.7)
TV-25	Control	21.4±2.0	24.5±1.9 (3.1)	19.5±1.0	25.2±1.8 (5.7)
	B. megaterium	29.0±1.9	32.0±2.1 (3.0)	34.2±2.0	45.0±1.1(10.8)
	B.m+saw dust	27.0±2.1	29.0± 1.3(2.0)	34.0±2.1	45.2± 2.1(11.2)
	B.m+rice husk	26.1±2.0	28.1± 1.1(2.0)	31.5±1.9	42.5±1.8 (11.0)
	B.m+tea waste	28.0±2.2	32.0± 2.1(4.0)	30.2±1.3	41.0±11.9(10.8)
TV-26	Control	22.4±1.8	25.5±2.3 (3.1)	17.5±2.0	21.0± 1.5(3.5)
	B. megaterium	25.0±1.7	33.0± 1.7(8.0)	19.0±1.5	32.0±2.2 (13.0)
	B.m+saw dust	22.0±1.1	29.2± 1.1(7.2)	17.0±1.0	30.0±2.4(13.0)
	B.m+rice husk	21.0±1.7	31.0± 1.8(10)	16.0±1.2	27.0± 1.9(11.0)
	B.m+tea waste	21.2± 1.9	30.2± 1.2(9.0)	18.0±1.1	26.2± 1.7(8.2)
T-17	Control	24.0±2.4	27.2± 1.2(3.2)	18.5±1.5	20.4±1.8 (1.9)
	B. megaterium	25.0±1.5	34.0± 2.1(9.0)	18.0±1.6	26.0±1.5(8.0)
	B.m+saw dust	22.0±1.4	33.0± 2.3(11)	21.0±2.0	26.5±1.7(5.5)
	B.m+rice husk	25.2±1.3	32.5±2.2 (7.3)	19.0±1.6	27.0±1.8(8.0)
	B.m+tea waste	27.0±2.0	31.0±1.9 (4.0)	20.0±1.5	28.3±1.1 (8.3)
CD (P=0.05)	(Treatments)	2.58	2.98	3.84	2.85
	(Varieties)	2.58	2.98	3.84	2.85

Average of 10 replicate pots/treatment; Figures in parenthesis indicate increment in final over initial values

Table 21a: ANOVA of data presented in Table 21 (Initial height of tea plants)

Source of Variation	SS	Df	MS	F
Rows	52.1664	4	13.0416	3.519904996
Columns	134.4344	4	33.6086	9.07090227
Error	59.2816	16	3.7051	
Total	245.8824	24		

Table 21b: ANOVA of data presented in Table 21 (Final height of tea plants)

Source of Variation	SS	Df	MS	F
Rows	140.7616	4	35.1904	7.103933463
Columns	150.7576	4	37.6894	7.60840996
Error	79.2584	16	4.95365	
Total	370.7776	24		

Table 21c: ANOVA of data presented in Table 21 (Initial leaf no. of tea plants)

Source of Variation	SS	Df	MS	F
Rows	48.3176	4	12.0794	1.472128111
Columns	509.9336	4	127.4834	15.53652473
Error	131.2864	16	8.2054	
Total	689.5376	24		

Table 21d: ANOVA of data presented in Table 21 (Final leaf no. of tea plants)

Source of Variation	SS	Df	MS	F
Rows	153.008	4	38.252	8.416744595
Columns	129.476	4	32.369	7.122283954
Error	72.716	16	4.54475	
Total	355.2	24		

Table 22: *In vivo* application of combined bioformulation of *B. megaterium* and *S. marcescens* in potted plants

Tea varieties	Treatment	2 months after treatment			
		Initial height	Final Height	Initial leaf numbers	Final leaf numbers
TV-18	Control	19.0±1.3	26.0± 1.5(7.0)	19.3±1.8	24.5± 1.2(5.2)
	B.m+S.m	28.0±1.4	33.0± 2.1(5.0)	21.0±1.6	27.2±1.1(6.2)
	B.m+ S.m+saw dust	23.0±1.4	26.2± 1.6(3.2)	20.0±1.1	23.5±1.4(3.5)
	B.m+S.m+rice husk	19.0±1.8	30.0±1.5(11.0)	18.0±1.7	26.6±1.9(8.6)
	B.m+S.m+tea waste	22.0±2.0	27.0±1.3(5.0)	25.0±2.1	28±1.8(3.0)
TV-23	Control	22.4±0.9	25.0± 1.7(2.6)	17.1±1.2	24.0± 1.0(6.9)
	B.m+S.m	25.0±1.9	31.0±2.1(6.0)	21.0±1.7	29.9±1.1(8.9)
	B.m+ S.m+saw dust	22.0±1.8	36.7±2.0(14.7)	20.0±1.6	31.0±1.2(11.0)
	B.m+S.m+rice husk	23.0±2.0	37.0±1.9(14.0)	18.5±2.1	27.7±2.0(9.2)
	B.m+S.m+tea waste	22.1±1.5	35.5±2.3(13.4)	19.0±1.9	30.0±1.5(11.0)
TV-25	Control	21.4±2.0	24.5±1.9 (3.1)	19.5±1.0	25.2±1.8 (5.7)
	B.m +S.m	22.5±2.0	29.8±2.1(7.3)	21.0±2.0	35.5± 2.1(14.5)
	B.m+ S.m+saw dust	21.2±1.7	28.3±1.9(7.1)	23.2±1.8	35.2±1.8(12.01)
	B.m+S.m+rice husk	22.0±1.0	31.0±1.7(9.0)	21.0±1.7	33.4±2.2(12.4)
	B.m+S.m+tea waste	25.2±1.8	32.2±1.4(7.0)	23.8±2.0	31.3±1.9(7.5)
TV-26	Control	22.4±1.8	25.5±2.3 (3.1)	17.5±2.0	21.0± 1.5(3.5)
	B.m +S.m	25.9±1.6	35.0±2.1(9.1)	19.0±1.5	37.0±2.1(18.0)
	B.m+ S.m+saw dust	22.3±2.0	29.0± 1.8(6.7)	17.7±1.0	34.0±2.4(16.3)
	B.m+S.m+rice husk	18.8±1.9	34.0±2.1(15.2)	16.3±1.3	26.3±2.5(10.0)
	B.m+S.m+tea waste	21.0±1.1	32.2±1.7(11.2)	15.0±1.1	26.0±1.8(11.0)
T-17	Control	24.0±2.4	27.2± 1.2(3.2)	18.5±1.5	20.4±1.8 (1.9)
	B.m +S.m	24.0±1.7	36.0±2.0(12.0)	18.54±1.4	26.5±2.2(8.0)
	B.m+ S.m+saw dust	21.0±2.0	32.2±1.9(11.2)	21.5±1.0	26.0± 1.5(5.5)
	B.m+S.m+rice husk	25.0±1.9	32.5± 1.1(7.5)	18.0±1.4	28.6±2.1 (10.6)
	B.m+S.m+tea waste	27.2±1.8	34.1± 1.8(6.9)	21.0±1.8	28.5±1.8(7.5)
CD (P=0.05)	(Treatments)	3.28	3.47	2.17	3.87
	(Varieties)	3.28	3.47	2.17	3.87

Average of 10 replicate pots/treatment; Figures in parenthesis indicate increment in final over initial values

Table 22a: ANOVA of data presented in Table 22 (Initial height of tea plants)

Source of Variation	SS	Df	MS	F
Rows	33.822464	4	8.455616	1.404887022
Columns	24.481264	4	6.120316	1.016880677
Error	96.299456	16	6.018716	
Total	154.603184	24		

Table 22b: ANOVA of data presented in Table 22 (Final height of tea plants)

Source of Variation	SS	Df	MS	F
Rows	160.8496	4	40.2124	6.000820755
Columns	182.9536	4	45.7384	6.825455332
Error	107.2184	16	6.70115	
Total	451.0216	24		

Table 22c: ANOVA of data presented in Table 22 (Initial leaf no. of tea plants)

Source of Variation	SS	Df	MS	F
Rows	27.012576	4	6.753144	2.562334
Columns	60.104576	4	15.026144	5.70134439
Error	42.168704	16	2.635544	
Total	129.285856	24		

Table 22d: ANOVA of data presented in Table 22 (Final leaf no. of tea plants)

Source of Variation	SS	Df	MS	F
Rows	196.757016	4	49.189254	5.890193936
Columns	128.904616	4	32.226154	3.858938313
Error	133.616664	16	8.3510415	
Total	459.278296	24		

4.11. Effect of insecticides on growth of *B.megaterium* and *S. marcescens*

Since insecticides/fungicides are applied in tea gardens, the ability of the bacteria to grow in their presence was also investigated. Common insecticides viz. Acephate (organophosphate insecticide), Confidor (systemic neuroactive insecticide, class- Imidacloprid), Ethion 50EC (Organophosphorus insecticide and miticide) were selected. Besides, other two common fungicides Calixin (common name- Tridemorph, class- Morpholine) and Contaf 5E (class- Triazole) were also selected and solutions of the chemicals were made according to desired concentrations.

Figure 46: A-L: Effect of aqueous suspensions and bioformulations of *B. megaterium* and *S. marcescens* on growth of TV-23 variety. A-C: changes in growth pattern by application of aqueous suspension of *B. megaterium* (A); *S. marcescens* (B) and *B. megaterium* + *S. marcescens* (C); D-F: saw dust formulation; G-I: tea waste formulation; J-L: rice husk formulation.

Figure 47: A-L: Effect of single as well as joint application of *B. megaterium* and *S. marcescens* on growth of T-17 variety. A-C: aqueous suspensions of *B. megaterium* (A); *S. marcescens* (B) and *B. megaterium*+ *S. marcescens* (C); D-F: saw dust formulation; G-I: formulation of tea waste; J-L: rice husk formulation.

Table 23: Peroxidase activities of leaves of tea varieties following treatment with aqueous suspensions as well as bioformulation of *B.megaterium* and *S.marcescens* in potted plants

Treatment	POX activity (Δ A_{465}/min/gtissue)				
	TV-18	TV-23	TV-25	TV-26	T-17
Control	1.5±0.28	1.4±0.57	0.9±0.05	1.0±0.12	1.7±0.57
B.megaterium	2.5±0.57	3.4±0.80	3.6±0.69	1.3±0.06	4.2±0.60
S. marcescens	2.0±0.28	3.6±0.65	2.4±0.57	1.2±0.57	3.7±0.58
B.megaterium+S.marcescens	3.0±1.15	4.5±0.57	3.7±0.75	1.4±0.63	3.9±0.11
B.m+Saw dust	2.4±0.57	3.0±0.57	3.6±0.57	1.0±0.28	4.0±0.28
B.m +Rice husk	2.3±0.58	3.1±0.63	3.5±0.80	1.2±0.58	3.9±0.58
B.m+ Tea waste	2.6±0.83	3.3±0.58	3.3±1.15	1.5±0.69	4.1±1.15
S.m+Saw dust	2.0±0.80	3.2±0.69	2.5±0.69	1.1±0.57	3.3±0.69
S.m+ Rice husk	1.9±0.57	3.5±0.63	2.3±0.58	1.0±0.23	3.5±1.15
S.m+Tea waste	1.8±0.80	3.3±0.63	2.4±0.57	1.3±0.57	3.6±0.23
B.m+*S.m*+Saw dust	3.1±1.15	4.0±1.15	3.5±0.80	1.2±0.03	3.8±0.17
B.m+*S.m*+Rice husk	3.2±0.57	4.2±1.21	3.7±1.15	1.3±0.005	3.9±0.69
B.m+*S.m*+Tea waste	3.6±1.73	4.1±1.15	3.6±0.63	1.4±0.60	4.0±0.11
CD (P=0.05) (Treatments)	0.508				
(Varieties)	0.315				

Average of 10 replicate pots/treatment

Table 23a: ANOVA of data presented in Table 23 (Peroxidase activities in tea leaves)

Source of Variation	SS	df	MS	F
Rows	17.79354	12	1.482795	9.259479
Columns	49.21025	4	12.30256	76.82472
Error	7.686628	48	0.160138	
Total	74.69042	64		

4.11.1. *In vitro*

For *in vitro* tolerance, both the bacteria were allowed to grow at different concentrations of selected insecticides and fungicides by adding these to the liquid medium after autoclaving. Results revealed that the bacteria could tolerate much higher concentrations of insecticides. *B. megaterium* could tolerate acephate concentration of 1250mg/ml, confidor, ethion 50EC ,calixin concentrations of 1000 µl/ml and contaf 5E conc. of 500 µl/ml; whereas *S. marcescens* could tolerate acephate concentration of 625mg/ml, confidor, ethion 50EC ,calixin concentrations of 250, 1000 and 500 µl/ml and contaf 5E concentrations of 125 µl/ml (Figs. 48& 49).

Table 24: Chitinase assay from leaves of tea varieties following treatment with aqueous suspensions as well as bioformulation of *B.megaterium* and *S.marcescens* in potted plants

Treatment	CHT activity (mg Glc-NAc/min/gtissue)				
	TV-18	TV-23	TV-25	TV-26	T-17
Control	12±0.57	18±1.15	14±0.57	16±0.28	17±0.58
B.megaterium	16±2.30	26±1.73	19±1.73	19±0.32	36±3.5
S. marcescens	18±4.61	25±3.5	21±0.58	20±0.33	32±0.57
B.megaterium+S.marcescens	19±1.15	27±0.58	20±2.30	17±3.46	37±1.15
B.m+Saw dust	13±1.73	23±0.33	18±1.15	19±1.15	33±2.30
B.m +Rice husk	14±1.73	24±1.15	17±0.57	19±0.57	34±1.15
B.m+ Tea waste	15±0.57	25±4.04	20±2.30	19±2.88	36±0.44
S.m+Saw dust	18±4.04	21±0.57	19±0.58	21±1.15	31±1.15
S.m+ Rice husk	21±0.32	19±1.15	19±0.33	20±0.58	31±1.73
S.m+Tea waste	22±1.73	22±0.58	21±1.58	18±1.15	29±0.57
B.m+S.m+Saw dust	19±0.58	27±2.30	17±0.33	19±1.73	35±3.5
B.m+S.m+Rice husk	20±1.15	29±1.73	17±0.37	21±0.58	35±0.58
B.m+S.m+Tea waste	21±1.73	25±4.61	16±0.57	22±0.66	32±0.57
CD (P=0.05) (Treatments)		3.40			
(Varieties)		2.11			

Mean of 10 replicate pots/treatment

Table 24a: ANOVA of data presented in Table 24 (Chitinase activities in tea leaves)

Source of Variation	SS	Df	MS	F
Rows	301.5782	12	25.13151	3.505277
Columns	1909.982	4	477.4955	66.59982
Error	344.1418	48	7.169622	
Total	2555.702	64		

Table 25: β-1,3 glucanase assay from leaves of tea varieties following treatment with aqueous suspensions as well as bioformulation of *B.megaterium* and *S.marcescens* in potted plants

Treatment	GLU activity (µg glucose/min/gtissue)				
	TV-18	TV-23	TV-25	TV-26	T-17
Control	155±0.57	147±0.58	161±2.88	148±1.15	164±0.58
B.megaterium	159±1.15	156±1.73	164±0.57	153±0.58	169±1.73
S. marcescens	161±2.30	148±2.90	170±2.30	149±3.46	170±4.04
B.megaterium+S.marcescens	158±2.30	159±4.04	174±0.58	165±1.73	183±3.50
B.m+Saw dust	159±1.73	154±1.15	162±1.73	151±1.15	163±1.15
B.m +Rice husk	160±0.33	149±1.73	161±0.58	149±0.57	165±0.57
B.m+ Tea waste	162±1.15	148±2.30	163±1.73	152±0.33	166±1.73
S.m+Saw dust	157±0.33	149±0.32	170±0.33	155±2.30	171±0.33
S.m+ Rice husk	159±4.04	155±0.58	177±0.57	157±0.58	169±3.46
S.m+Tea waste	157±1.15	154±1.73	176±2.30	154±1.73	167±1.15
B.m+S.m+Saw dust	162±2.30	152±2.88	172±4.61	166±0.57	181±2.88
B.m+S.m+Rice husk	161±2.88	155±0.57	174±2.30	162±4.04	180±2.30
B.m+S.m+Tea waste	157±1.73	156±5.77	170±2.88	157±3.46	175±0.57
CD (P=0.05) (Treatments)		4.72			
(Varieties)		2.93			

Mean of 10 replicate pots/treatment

Table 25a: ANOVA of data presented in Table 25 (β-1,3 glucanase activities in tea leaves)

Source of Variation	SS	Df	MS	F
Rows	952.2462	12	79.35385	5.73988
Columns	3511.6	4	877.9	63.5009
Error	663.6	48	13.825	
Total	5127.446	64		

Table 26: Phenyl alanine ammonia lyase activities of leaves of tea varieties following treatment with aqueous suspensions as well as bioformulation of *B.megaterium* and *S.marcescens* in potted plants

Treatment	PAL activity (µg cinnamic acid/gtissue/min)				
	TV-18	TV-23	TV-25	TV-26	T-17
Control	49±1.15	55±0.58	77±1.15	63±1.15	56±2.30
B.megaterium	61±1.73	71±1.15	82±0.57	83±2.88	65±0.57
S. marcescens	54±3.46	69±0.57	92±2.30	77±0.57	66±1.15
B.megaterium+S.marcescens	59±1.15	73±1.73	88±1.15	79±1.73	67±1.15
B.m+Saw dust	52±0.33	69±1.15	79±1.73	72±0.58	66±2.90
B.m +Rice husk	53±1.15	62±0.57	84±0.57	70±1.15	64±0.58
B.m+ Tea waste	49±0.57	63±2.30	78±3.46	75±0.57	57±1.73
S.m+Saw dust	52±1.15	61±1.15	79±1.73	71±1.15	61±2.30
S.m+ Rice husk	58±1.73	59±0.57	77±0.57	69±2.88	62±0.58
S.m+Tea waste	52±0.58	57±1.15	83±1.73	73±1.73	68±2.30
B.m+*S.m*+Saw dust	53±1.15	72±0.57	87±1.15	71±1.73	61±3.46
B.m+*S.m*+Rice husk	57±1.73	70±2.30	85±0.58	65±0.58	63±0.57
B.m+*S.m*+Tea waste	58±0.57	68±0.58	82±2.30	73±2.02	64±0.57
CD (P=0.05) (Treatments)	4.46				
(Varieties)	2.76				

Average of 10 replicate pots/treatment.

Table 26a: ANOVA of data presented in Table 26 (PAL activities in tea leaves)

Source of Variation	SS	df	MS	F
Rows	792.5538	12	66.04615	5.368487
Columns	5803.077	4	1450.769	117.9241
Error	590.5231	48	12.30256	
Total	7186.154	64		

Table 27: Total Phenol contents in leaves of different varieties of potted tea plants after treatment of the rhizosphere with aqueous suspensions and bioformulation of *B.megaterium* and *S.marcescens*.

Treatment	**Total phenol content(mg/gtissue)**				
	TV-18	TV-23	TV-25	TV-26	T-17
Control	16±0.60	14±0.60	11±0.58	17±1.15	16±0.57
B.megaterium	21±0.57	31±1.15	27±1.73	45±1.73	26±1.15
S. marcescens	23±1.73	26±0.58	22±2.88	37±3.46	24±0.60
B.megaterium+S.marcescens	25±2.88	28±2.88	29±3.46	43±2.30	27±0.58
B.m+Saw dust	19±0.58	29±1.73	26±0.33	41±0.58	29±4.04
B.m +Rice husk	20±1.15	32±3.46	25±2.30	40±1.15	28±0.69
B.m+ Tea waste	18±1.73	30±0.58	22±0.57	39±4.61	29±0.80
S.m+Saw dust	22±3.46	26±0.33	19±0.58	33±0.57	23±0.86
S.m+ Rice husk	22±0.57	27±0.33	18±1.73	34±0.58	22±0.69
S.m+Tea waste	21±1.15	26±0.57	17±3.5	35±2.88	22±0.98
B.m+*S.m*+Saw dust	25±1.73	27±2.30	29±1.15	42±2.30	27±2.30
B.m+*S.m*+Rice husk	25±0.57	22±4.04	32±0.58	41±0.33	27±1.03
B.m+*S.m*+Tea waste	26±0.33	23±0.57	33±4.61	40±0.57	28±0.69
CD (P=0.05) (Treatments)	4.24				
(Varieties)	2.63				

Average of 10 replicate pots/treatment.

Table 27a: ANOVA of data presented in Table 27 (Total phenol contents in tea leaves)

Source of Variation	**SS**	**df**	**MS**	**F**
Rows	1114.51	12	92.87579	8.355805
Columns	1961.882	4	490.4705	44.12642
Error	533.5258	48	11.11512	
Total	3609.918	64		

Table 28: O-Phenol contents in leaves of different varieties of potted tea plants after treatment of the rhizosphere with aqueous suspensions and bioformulation of *B.megaterium* and *S.marcescens*.

Treatment	**O-dihydroxy phenol content(mg/gtissue)**				
	TV-18	TV-23	TV-25	TV-26	T-17
Control	06±0.75	08±1.73	09±0.57	10±0.57	07±2.88
B.megaterium	11±0.69	13±2.30	14±1.73	12±2.30	15±0.69
S. marcescens	09±1.15	12±2.88	10±3.46	11±1.15	11±1.96
B.megaterium+S.marcescens	13±0.98	16±1.44	18±1.15	14±1.73	19±2.30
B.m+Saw dust	09±1.15	12±2.30	12±1.73	13±1.15	13±1.44
B.m +Rice husk	10±0.63	12±1.27	11±3.50	12±0.58	14±1.38
B.m+ Tea waste	08±0.58	12±0.57	12±0.33	12±1.15	14±1.15
S.m+Saw dust	09±3.46	11±1.15	09±0.58	11±2.88	10±0.80
S.m+ Rice husk	08±0.33	11±1.44	10±1.78	12±1.09	09±2.88
S.m+Tea waste	08±1.09	12±1.32	10±1.84	14±1.21	08±1.73
B.m+*S.m*+Saw dust	11±3.46	16±2.30	17±1.90	14±0.33	15±0.57
B.m+*S.m*+Rice husk	12±0.75	17±1.44	18±2.07	13±2.02	16±2.30
B.m+*S.m*+Tea waste	13±1.21	17±1.50	18±1.73	12±2.30	18±1.44
CD (P=0.05) (Treatments)		1.84			
(Varieties)		1.14			

Table 28a: ANOVA of data presented in Table 28 (O-dihydroxy phenol contents in tea leaves)

Source of Variation	SS	df	MS	F
Rows	389.9462	12	32.49551	15.42861
Columns	94.63908	4	23.65977	11.23347
Error	101.0969	48	2.106186	
Total	585.6822	64		

4.11.2. PGPR and insecticide application on growth and insect attack

Application of *B. megaterium* to the rhizosphere of five varieties of tea plants in the experimental field resulted in an increase in growth in terms of increase in leaf numbers and number of branches. Increase in number of leaves was significant after 12 months of application, whereas initial leaf number and increase in leaf number after 6 months of 1st application were insignificant. Similar trend was also observed in case of increase in branch numbers. But insect (*Helopeltis theivora*) attack in newly emerged leaves after single application of *B. megaterium* was still observed (% of insect attack ranging from 44-58%) and was not reduced by the application of PGPR to any great extent. So, combined application of *B. megaterium* and low dose of acephate (1:2000-0.5mg/ml), commonly used in tea gardens was tried in order to reduce insect attack keeping the growth promotion achieved by *B. megaterium*. Results revealed that significant growth promotion was achieved in both cases, but in case of combined application, leaves were healthier with a significant reduction in insect attack (% of insect attack reduced to 16-22% after foliar spray of acephate). Results revealed that initial leaf and branch no were insignificant but increase in leaf and branch numbers were found to be significant after 6 and 12 months only in *B. megaterium* and *B. megaterium*+acephate treated tea leaves in comparison to control and control + acephate treatments (Table 29). Similarly, significant increase in leaf, branch numbers and reduction in % of insect attack (14-21% from 47-56% after foliar spray of acephate with aqueous suspensions of *S. marcescens*; 14-20% from 48-57% after foliar spray of acephate with aqueous suspensions of *S. marcescens* and *B. megaterium*) were also observed after combined application of *S. marcescens* alone or with *B. megaterium* and low dose of acephate (1:2000-0.5mg/ml) (Tables 30&31).

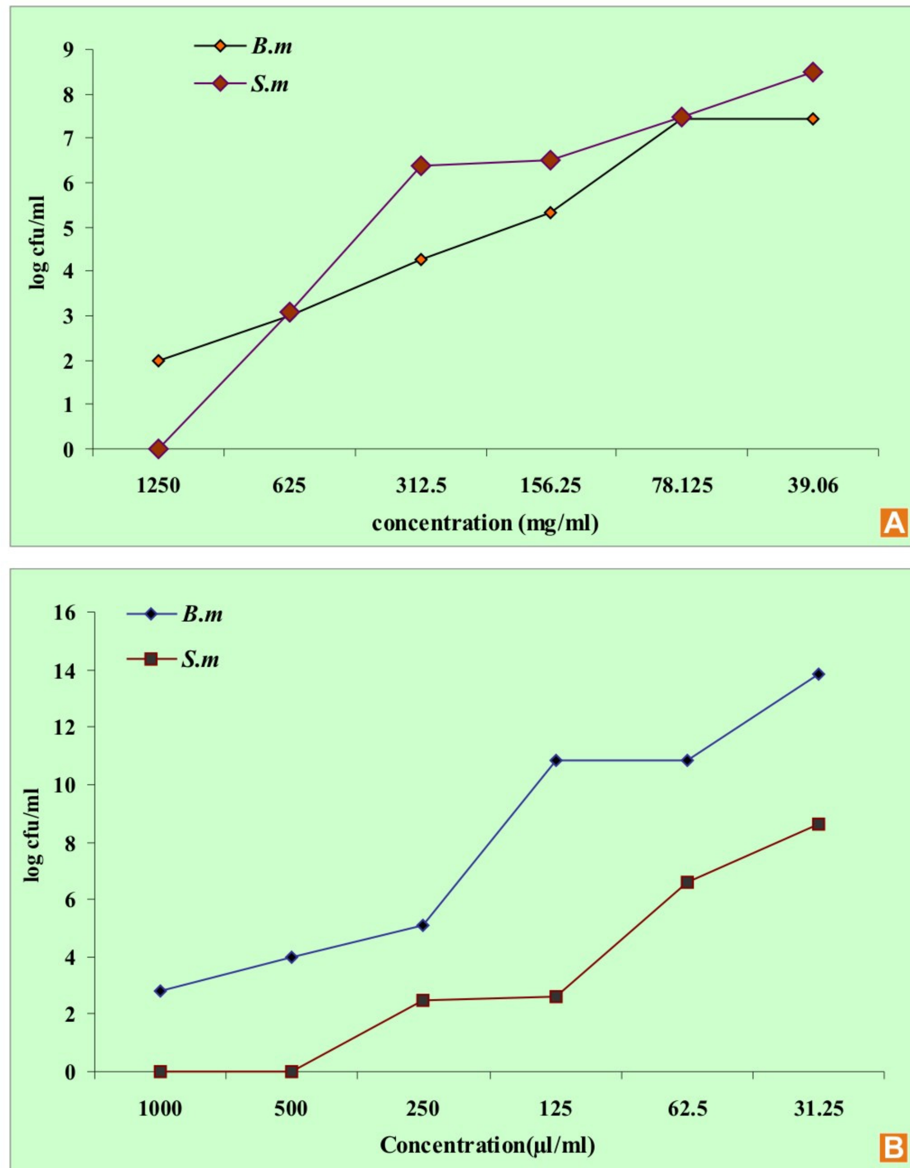


Figure 48: Growth of *B. megaterium* and *S.marcescens* in medium amended with different concentrations of Acephate (A) and Confidor (B) *in vitro.*

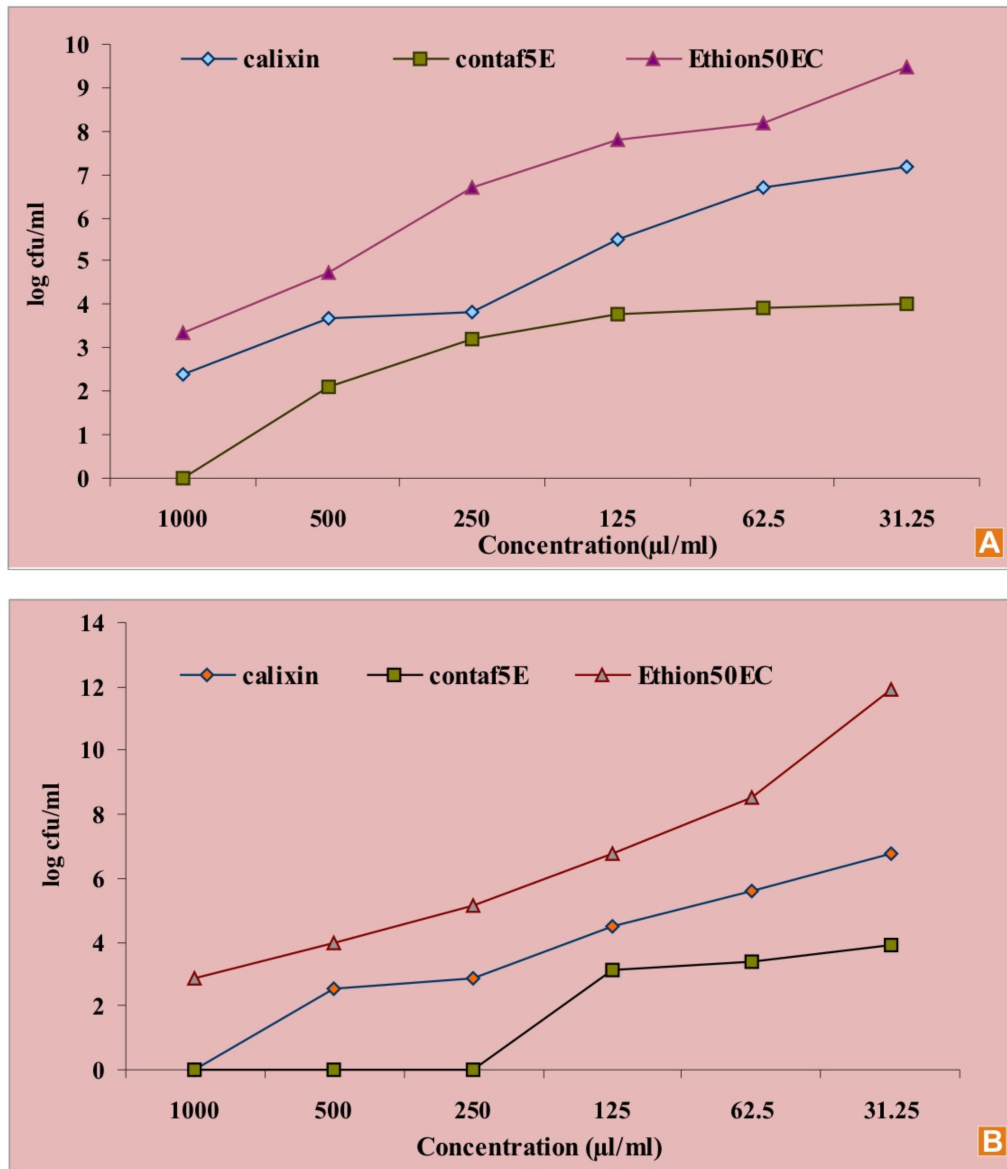


Figure 49: Growth of *B. megaterium* and *S.marcescens* in medium amended with different concentrations of Calixin,Contaf 5E and Ethion 50EC *in vitro.*

Table 29: Effect of combined application of *B. megaterium* and low dose of acephate (1:2000-0.5mg/ml) on number of leaves, branches and insect attack of tea plants

Tea varieties	Treatment	No. of leaves			No. of branches			Insect attack (%)
		Initial	(6mo)	(12 mo)	Initial	(6 mo)	(12 mo)	
TV-18	Control	11±0.8	47±1.4	51±1.6	3±0.5	04±0.5	08±1.2	57
	Control+acephate	10±0.7	48±1.4	52±1.4	4±0.9	06±1.5	10±1.5	21
	B.megaterium	12±0.9	51±1.5	61±1.5	4±1.0	09±1.7	12±1.1	56
	B.megaterium +acephate	14±1.2	53±1.3	62±1.7	5±0.9	11±1.7	14±0.8	19
TV-23	Control	13±1.0	21±1.4	30±1.0	2±0.4	03±0.9	08±0.9	53
	Control+acephate	14±1.1	23±2.1	32±0.8	2±0.6	04±1.1	09±0.8	18
	B.megaterium	12±1.2	33±2.2	35±0.9	3±0.5	05±1.2	12±0.8	58
	B.megaterium +acephate	14±1.0	32±2.1	40±0.6	2±0.3	06±1.2	15±0.9	17
TV-25	Control	12±0.7	29±1.7	31±0.8	2±0.6	03±0.6	07±1.0	48
	Control+acephate	10±0.5	31±1.6	35±0.6	3±0.4	04±0.5	07±1.2	18
	B.megaterium	09±0.5	79±1.7	80±0.7	3±0.7	08±0.4	13±1.1	51
	B.megaterium +acephate	11±0.4	81±1.5	84±1.1	3±0.3	10±0.4	16±1.0	16
TV-26	Control	14±1.0	68±2.3	69±2.9	5±1.0	08±1.1	10±1.0	47
	Control+acephate	18±0.5	70±2.4	75±2.7	6±0.9	07±1.3	09±1.3	22
	B.megaterium	17±0.4	90±2.2	92±2.8	4±0.8	08±1.4	14±1.2	44
	B.megaterium +acephate	16±0.5	91±2.1	95±2.2	6±0.9	11±1.0	19±0.9	16
T-17	Control	13±1.0	67±2.2	71±1.9	4±0.6	04±0.9	09±1.1	46
	Control+acephate	16±0.4	66±2.7	72±1.4	3±0.3	03±0.6	08±	16
	B.megaterium	15±0.5	76±2.8	81±1.6	4±0.4	05±0.8	1.7	45
	B.megaterium +acephate	14±1.1	75±2.1	79±1.0	3±0.5	06±0.7	16±1.8 17±0.9	14
CD (P=0.05) (Treatments)		2.01	14.65	14.22	0.99	1.67	1.76	-
(Varieties)		2.25	16.38	15.90	1.11	1.87	1.96	-

Table 29 a: ANOVA of data presented in Table 29 (Initial leaves no.)

Source of Variation	SS	df	MS	F
Rows	4.55	3	1.516667	0.708171
Columns	81.5	4	20.375	9.513619
Error	25.7	12	2.141667	
Total	111.75	19		

Table 29b: ANOVA of data presented in Table 29 (leaves no. after 6 mo)

Source of Variation	SS	df	MS	F
Rows	1828.55	3	609.5167	5.387199
Columns	6616.7	4	1654.175	14.62039
Error	1357.7	12	113.1417	
Total	9802.95	19		

Table 29c: ANOVA of data presented in Table 29 (leaves no. after 12 mo)

Source of Variation	SS	df	MS	F
Rows	1855.75	3	618.5833	5.806023
Columns	5752.3	4	1438.075	13.49777
Error	1278.5	12	106.5417	
Total	8886.55	19		

Table 29d: ANOVA of data presented in Table 29 (Initial branch no.)

Source of Variation	SS	df	MS	F
Rows	0.95	3	0.316667	0.603175
Columns	21.7	4	5.425	10.33333
Error	6.3	12	0.525	
Total	28.95	19		

Table 29e: ANOVA of data presented in Table 29 (Branch no. after 6 mo)

Source of Variation	SS	df	MS	F
Rows	62.95	3	20.98333	14.14607
Columns	51	4	12.75	8.595506
Error	17.8	12	1.483333	
Total	131.75	19		

Table 29f : ANOVA of data presented in Table 29 (Branch no. after 12 mo)

Source of Variation	SS	df	MS	F
Rows	218.15	3	72.71667	44.52041
Columns	16.8	4	4.2	2.571429
Error	19.6	12	1.633333	
Total	254.55	19		

Table 30: Effect of combined application of *S. marcescens* and low dose of acephate (1:2000-0.5mg/ml) on number of leaves, branches and insect attack of tea plants

Tea varieties	Treatment	No. of leaves			No. of branches			Insect attack (%)
		Initial	(6 mo)	(12mo)	Initial	(6mo)	(12 mo)	
TV-18	Control	11±0.8	47±1.4	51±1.6	3±0.5	04±0.5	08±1.2	57
	Control+acephate	10±0.7	48±1.4	52±1.4	4±0.9	06±1.5	10±1.5	21
	S.marcescens	14±0.8	56±1.2	68±1.3	5±0.9	11±1.9	14±1.6	54
	S.marcescens +acephate	17±1.5	57±1.3	70±1.7	6±0.9	12±1.7	16±0.8	21
TV-23	Control	13±1.0	21±1.4	30±1.0	2±0.4	03±0.9	08±0.9	53
	Control+acephate	14±1.1	23±2.1	32±0.8	2±0.6	04±1.1	09±0.8	18
	S.marcescens	15±1.0	35±2.2	38±0.9	4±0.5	06±1.2	15±0.6	56
	S.marcescens+acephate	18±1.7	34±2.1	42±0.9	3±0.7	05±1.5	18±1.0	15
TV-25	Control	12±0.7	29±1.7	31±0.8	2±0.6	03±0.6	07±1.0	48
	Control+acephate	10±0.5	31±1.6	35±0.6	3±0.4	04±0.5	07±1.2	18
	S.marcescens	11±0.3	67±1.2	74±0.7	4±0.2	10±0.4	16±1.2	52
	S.marcescens+acephate	13±0.6	72±1.4	80±1.2	4±0.4	13±0.6	19±1.0	14
TV-26	Control	14±1.0	68±2.3	69±2.9	5±1.0	08±1.1	10±1.0	47
	Control+acephate	18±0.5	70±2.4	75±2.7	6±0.9	07±1.3	09±1.3	22
	S.marcescens	18±0.9	88±2.6	93±3.0	5±0.5	10±1.4	15±1.5	47
	S.marcescens+acephate	20±0.5	90±2.1	96±2.2	7±0.8	15±1.2	20±0.8	19
T-17	Control	13±1.0	67±2.2	71±1.9	4±0.6	04±0.9	09±1.1	46
	Control+acephate	16±0.4	66±2.7	72±1.4	3±0.3	03±0.6	08±1.7	16
	S.marcescens	16±0.4	72±2.8	79±1.8	3±0.5	07±0.8	16±1.8	49
	S.marcescens+acephate	15±1.2	73±2.5	77±1.0	4±0.4	08±0.9	18±1.3	16
CD (P=0.05)								
	(Treatments)	2.16	10.76	12.05	1.04	2.36	1.62	-
	(Varieties)	2.41	12.03	13.48	1.16	2.63	1.81	-

Table 30a: ANOVA of data presented in Table 30 (Initial leaves no.)

Source of Variation	SS	df	MS	F
Rows	44.4	3	14.8	6
Columns	82.8	4	20.7	8.391892
Error	29.6	12	2.466667	
Total	156.8	19		

Table 30b: ANOVA of data presented in Table 30 (leaves no. after 6 mo)

Source of Variation	SS	df	MS	F
Rows	1523.8	3	507.9333	8.318821
Columns	6143.7	4	1535.925	25.15504
Error	732.7	12	61.05833	
Total	8400.2	19		

Table 30c: ANOVA of data presented in Table 30 (leaves no. after 12 mo)

Source of Variation	SS	df	MS	F
Rows	2016.55	3	672.1833	8.780015
Columns	5472.5	4	1368.125	17.87036
Error	918.7	12	76.55833	
Total	8407.75	19		

Table 30d: ANOVA of data presented in Table 30 (Initial branch no.)

Source of Variation	SS	df	MS	F
Rows	7.35	3	2.45	4.26087
Columns	22.7	4	5.675	9.869565
Error	6.9	12	0.575	
Total	36.95	19		

Table 30e: ANOVA of data presented in Table 30 (Branch no. after 6 mo)

Source of Variation	SS	df	MS	F
Rows	138.55	3	46.18333	15.74432
Columns	76.8	4	19.2	6.545455
Error	35.2	12	2.933333	
Total	250.55	19		

Table 30f: ANOVA of data presented in Table 30 (Branch no. after 12 mo)

Source of Variation	SS	df	MS	F
Rows	358.8	3	119.6	85.94012
Columns	5.3	4	1.325	0.952096
Error	16.7	12	1.391667	
Total	380.8	19		

Table 31: Effect of combined application of *B. megaterium*, *S. marcescens* and acephate (1:2000-0.5mg/ml) on number of leaves, branches and insect attack of tea plants

Tea varieties	Treatment	No. of leaves			No. of branches			Insect attack (%)
		Initial	(6 mo)	(12 mo)	Initial	(6 mo)	(12 mo)	
TV-18	Control	11±0.8	47±1.4	51±1.6	3±0.5	04±0.5	08±1.2	57
	Control+acephate	10±0.7	48±1.4	52±1.4	4±0.9	06±1.5	10±1.5	21
	B.m+S.m	15±0.5	59±1.2	72±1.5	6±1.0	13±1.6	16±1.5	55
	B.m+S.m+acephate	19±1.4	58±1.5	74±1.9	7±0.8	14±1.7	17±0.6	18
TV-23	Control	13±1.0	21±1.4	30±1.0	2±0.4	03±0.9	08±0.9	53
	Control+acephate	14±1.1	23±2.1	32±0.8	2±0.6	04±1.1	09±0.8	18
	B.m+S.m	16±0.5	37±2.0	40±0.9	4±0.2	07±1.2	16±0.7	57
	B.m+S.m+acephate	19±1.6	36±1.7	43±0.7	5±0.8	06±1.4	19±1.0	14
TV-25	Control	12±0.7	29±1.7	31±0.8	2±0.6	03±0.6	07±1.0	48
	Control+acephate	10±0.5	31±1.6	35±0.6	3±0.4	04±0.5	07±1.2	18
	B.m+S.m	13±0.1	68±1.2	76±0.3	5±0.2	11±0.6	17±1.1	53
	B.m+S.m+acephate	14±0.7	74±1.9	83±1.6	6±0.3	13±0.9	18±1.3	16
TV-26	Control	14±1.0	68±2.3	69±2.9	5±1.0	08±1.1	10±1.0	47
	Control+acephate	18±0.5	70±2.4	75±2.7	6±0.9	07±1.3	09±1.3	22
	B.m+S.m	19±0.8	89±2.5	94±3.3	5±0.4	12±1.2	16±1.4	48
	B.m+S.m+acephate	21±0.7	91±2.2	95±2.1	8±0.3	17±1.7	19±0.6	20
T-17	Control	13±1.0	67±2.2	71±1.9	4±0.6	04±0.9	09±1.1	46
	Control+acephate	16±0.4	66±2.7	72±1.4	3±0.3	03±0.6	08±1.7	16
	B.m+S.m	17±0.5	74±2.9	80±1.9	4±0.9	09±0.8	17±1.9	51
	B.m+S.m+acephate	16±1.1	72±2.4	78±0.8	5±0.5	10±0.7	19±1.4	17
CD (P=0.05)								
	(Treatments)	2.27	10.91	12.41	1.08	2.32	1.32	-
	(Varieties)	2.54	12.20	13.87	1.20	2.58	1.48	-

Table 31a: ANOVA of data presented in Table 31 (Initial leaves no.)

Source of Variation	SS	df	MS	F
Rows	82.8	3	27.6	10.12844
Columns	74.5	4	18.625	6.834862
Error	32.7	12	2.725	
Total	190	19		

Table 31b: ANOVA of data presented in Table 31 (leaves no. after 6 mo)

Source of Variation	SS	Df	MS	F
Rows	1772.4	3	590.8	9.413889
Columns	5981.3	4	1495.325	23.82672
Error	753.1	12	62.75833	
Total	8506.8	19		

Table 31c: ANOVA of data presented in Table 31 (leaves no. after 12 mo)

Source of Variation	SS	df	MS	F
Rows	2386.15	3	795.3833	9.80341
Columns	5284.8	4	1321.2	16.28431
Error	973.6	12	81.13333	
Total	8644.55	19		

Table 31d: ANOVA of data presented in Table 31 (Initial branch no.)

Source of Variation	SS	df	MS	F
Rows	27.35	3	9.116667	14.78378
Columns	18.2	4	4.55	7.378378
Error	7.4	12	0.616667	
Total	52.95	19		

Table 31e: ANOVA of data presented in Table 31 (Branch no. after 6 mo)

Source of Variation	SS	df	MS	F
Rows	224.6	3	74.86667	26.50147
Columns	87.3	4	21.825	7.725664
Error	33.9	12	2.825	
Total	345.8	19		

Table 31f: ANOVA of data presented in Table 31 (Branch no. after 12 mo)

Source of Variation	SS	Df	MS	F
Rows	406.15	3	135.3833	146.3604
Columns	3.7	4	0.925	1
Error	11.1	12	0.925	
Total	420.95	19		

4.12. Influence of *B.megaterium* and *S.marcescens* on sclerotial blight

Since the bacteria showed antagonistic activity *in vitro*, experiments were further conducted to determine whether these could also control diseases caused by *Sclerotium rolfsii*.

4.12.1. Disease development

Effect of the two bacteria on development of sclerotial blight of tea, caused by *S. rolfsii* was determined (Fig. 50; A-H). Inoculation was done with the PGPR and pathogen and disease assessment was done in five varieties of tea after 15,30 and 45 days of inoculation. It was observed that both the bacteria reduced sclerotial blight of tea caused by *Sclerotium rolfsii* in all five tested varieties when observations were made 45 days after inoculation with the pathogen. *B. megaterium* reduced sclerotial blight more significantly in comparison to *S. marcscens,* when the plants were artificially inoculated with pathogen after three days of soil drenching with bacteria (Table 32).

Table 32: Sclerotial blight development in tea roots in presence and absence of bacteria

Tea varieties	Disease index (45 days after inoculation)			
	S.r.	***S.r.+ S.m***	***S.r.+ B.m***	***S.r.+ S.m+ B.m***
TV-18	4.1±0.57	3.2±1.15	1.3±0.83	1.9±0.69
TV-23	5.9±0.83	4.4±1.73	1.9±0.80	2.7±0.38
TV-25	3.5±0.80	3.0±0.57	1.1±0.58	1.6±0.63
TV-26	4.0±1.21	2.9±0.58	2.0±1.15	2.1±1.15
T-17	4.0±1.15	2.8±1.15	1.9±1.44	2.2±0.63

10 pots/treatment; *S.r.= Sclerotium rolfsii*; *S.m= Serratia marcescens*; *B.m=Bacillus megaterium*. Disease (Sclerotial Blight) Index computed on a scale of 0-6 on the basis of underground and above ground symptoms. 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% of roots affected; 4- shoot tips also start withering; 60-70% roots affected; 5- shoots withered with defoliation of lower withered leaves, 80% roots affected; 6- whole plants die, with upper withered leaves still remaining attached; roots fully rotted.

4.12.2. Biochemical changes

Application of *B. megaterium* and *S. marcescens* to soil was found to affect the biochemical responses of plants. Disease establishment is also known to cause biochemical changes in the host. Hence, in another series of experiments, biochemical responses of tea following application of bacteria and challenge inoculated with the pathogen- *Sclerotium rolfsii* were determined. The biochemical responses tested included defense enzyme activities and phenolics.

4.12.2.1. Defense enzymes

In all cases, young leaves were selected from tea plants of all five varieties (TV-18, TV23,TV25, TV26 and T17) and analyses were performed immediately after 72 h of bacterial inoculation to the rhizosphere.

4.12.2.1.1. Peroxidase and Chitinase

There was a significant increase in peroxidase and chitinase activities of tea leaves in pathogen inoculated as well as in *B. megaterium*, *S. marcescens* or *B. megaterium+S. marcescens* treated plants challenge inoculated with *S. rolfsii*. Relatively higher activity was shown by plants inoculated with *B. megaterium+S. marcescens* and challenge inoculated with pathogen followed by plants inoculated with pathogen alone. Least enzyme activity was recorded in untreated control tea plants. Isozyme analysis of peroxidase showed that maximum intensity of bands was noticed in the TV-26 variety after the treatment by *S. rolfsii* (7 bands with Rm values of 0.93, 0.79, 0.726, 0.562, 0.520, 0.452 and 0.383) in comparison to control (6 bands with Rm values of 0.79, 0.726, 0.562, 0.520, 0.452 and 0.383) (Table 33, Fig. 51 A & Fig. 32 C).

Table 33: Peroxidase activities of leaves of tea varieties following treatment with aqueous suspensions of *B.megaterium*, *S.marcescens* followed by *S. rolfsii*.

Treatment	**POX activity** (Δ A_{465}/min/gtissue)				
	TV-18	TV-23	TV-25	TV-26	T-17
Control	3.6±0.23	4.0±0.62	5.0±0.09	3.0±0.22	4.5±0.37
S. rolfsii	3.4±0.69	9.0±0.83	8.0±0.72	6.0±1.20	5.1±0.61
B.megaterium	5.2±0.33	11.0±0.66	12.0±0.55	12.0±0.59	5.2±0.56
S. marcescens	6.0±1.26	10.0±0.59	14.0±0.78	10.0±0.68	6.0±0.14
B.megaterium+S.marcescens	7.0±0.59	12.0±0.44	16.0±0.49	11.0±0.22	7.3±0.23
B.megaterium+S.rolfsii	8.2±0.62	15.0±0.69	22.0±0.82	17.0±0.38	13.0±0.48
S.marcescens+S.rolfsii	9.5±0.87	18.0±0.57	24.0±1.21	19.0±0.69	14.0±2.15
B.megaterium+ S.marcescens+S.rolfsii	10.1±0.79	21.0±0.67	25.0±0.65	20.0±0.77	16.5±0.49
CD (P=0.05) (Treatments)	2.73				
(Varieties)	2.16				

Table 33a: ANOVA of data presented in Table 33 (Peroxidase activities in tea leaves followed by the treatments)

Source of Variation	**SS**	**df**	**MS**	**F**
Rows	925.823	7	132.2604	29.59508
Columns	395.896	4	98.974	22.14679
Error	125.132	28	4.469	
Total	1446.851	39		

Table 34: Phenyl alanine ammonia lyase activities of leaves of tea varieties following treatment with aqueous suspensions *B.megaterium* and *S.marcescens* followed by *S.rolfsii*.

Treatment	**PAL activity** (µg cinnamic acid/gtissue/min)				
	TV-18	TV-23	TV-25	TV-26	T-17
Control	55±2.15	47±0.59	87±1.21	88±1.10	59±2.33
S. rolfsii	59±1.75	54±1.35	91±0.41	92±2.72	62±0.47
B.megaterium	61±3.30	62±0.55	93±2.34	97±0.58	69±1.13
S. marcescens	63±1.18	71±1.76	96±1.17	98±1.73	72±3.15
B.megaterium+S.marcescens	66±0.23	75±1.12	101±1.64	114±0.44	81±2.45
B.megaterium+S.rolfsii	73±1.16	123±0.59	112±0.59	127±1.14	131±0.65
S.marcescens+S.rolfsii	122±0.77	124±1.30	125±3.41	139±0.59	135±1.72
B.megaterium+ S.marcescens+S.rolfsii	132±1.25	134±1.45	131±1.70	144±1.23	212±2.31
CD (P=0.05) (Treatments)	21.45				
(Varieties)	16.96				

Table 34a: ANOVA of data presented in Table 34 (PAL activities in tea leaves followed by the treatments)

Source of Variation	**SS**	**df**	**MS**	**F**
Rows	32490.38	7	4641.482	16.92262
Columns	6146.65	4	1536.663	5.602598
Error	7679.75	28	274.2768	
Total	46316.78	39		

4.12.2.1.2. β-1,3 glucanase and Phenyl alanine ammonia lyase

Higher activities of β- 1,3 glucanase were observed in *S. rolfsii* and *B. megaterium* and *S. marcescens* treated tea plants (Fig. 51 B). Activities of PAL increased significantly about two times in *B. megaterium+S. marcescens* inoculated plants challenged with pathogen *S. rolfsii* followed by single application of bacteria, pathogen and untreated control plant (Table 34).

4.12.2.2. Phenolics

Total and O-phenol contents were estimated in tea leaves of the different tea varieties following single as well as dual application of *S. marcescens*, *B. megaterium* and challenge inoculation with *S. rolfsii*. Phenol contents were even more significantly increased when challenged with pathogen. Maximum phenol content was obtained in the presence of both *B. megaterium+S. marcescens* and *S. rolfsii*. Similar trend was also observed in O-phenol content (Fig. 52 A&B).

4.13. Determination of sustainability of *B. megaterium* and *S. marcescens* in soil

Survival of *S. marcescens* and *B. megaterium* in soil following application was determined immunologically using PAb raised against the bacteria. ELISA and DIBA were performed immediately and six months after application to the soil.

4.13.1. Immunotechniques

4.13.1.1. ELISA

The results revealed that bacteria could successfully survive and multiply in the tea rhizosphere of all five varieties even after six months of inoculation. Maximum A_{405} values were obtained from the soils collected soon after application of bacteria. The values reduced to some extent with time, though even after six months, these were still detectable at fairly high concentrations (Table 35).

Table 35: ELISA and DIBA values of rhizosphere soil antigens reacted with PAb of *S.marcescens* and *B. megaterium*

Antigens from rhizosphere of	Treatment	ELISA A_{405} values		DIBA Colour intensity of dots*	
		0 days	**180 days**	**0 days**	**180 days**
TV-18	Control	0.254±0.02	0.245±0.07	+	+
	S.marcescens	1.258±0.18	1.305±0.12	++++	++++
	B. megaterium	1.400±0.63	1.36±0.04	++++	++
TV-23	Control	0.321±0.03	0.330±0.04	+	+
	S.marcescens	1.432±0.04	1.532±0.18	++++	++++
	B. megaterium	1.244±0.03	1.345±0.63	++++	++++
TV-25	Control	0.287±0.05	0.312±0.03	+	+
	S.marcescens	1.356±0.08	1.422±0.08	++++	++++
	B. megaterium	1.248±0.04	1.240±0.12	++++	++
TV-26	Control	0.341±0.03	0.368±0.08	+	+
	S.marcescens	1.568±0.16	1.630±0.18	++++	++++
	B. megaterium	1.096±0.18	1.083±0.05	++++	++
T-17	Control	0.286±0.01	0.302±0.02	+	+
	S.marcescens	1.643±0.07	1.654±0.11	++++	++++
	B. megaterium	1.132±0.02	1.308±0.01	++++	++++

Average of 3 replicates; PAb dilution: 1:1000; Alakaline phosphatase dilution:1:10,000. ± = SE;* + =Light pink; ++ = Pink; +++ = Bright pink; ++++ = Pinkish red. Difference between ELISA values of control and treated significant at P=0.01 (Student's t test) in all cases.

4.13.1.2. Dot-blot

Dot immunobloting technique is rapid and sensitive method for detection of survival of bacteria in soil. The presence of *B. megaterium* and *S. marcescens* in the soil 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. megaterium or S. marcescens*, colour intensity was highest when soil antigens were prepared soon after bacterial application. With time, the intensity decreased, though not significantly (Table 35; Figs. 53 & 54, B,I&J).

4.13.1.3. Western blot

Western blot analysis was performed by *B. megaterium* and *S. marcescens* antigens against 1st to 4th bleeds. Differences in band intensities and expression of bands were observed more in homologous antigens of both bacteria when probed with 3rd and 4th bleeds in comparison to 1st and 2nd bleeds (Figs. 53 & 54, D-G). Western blotting using PAb of *B. megaterium* revealed that the homologous antigens showed 9 bands ranging from 14.3 to 97 kDa in 4th bleed, 8 bands ranging from 14.3 to 68 KDa in 3rd bleed, 5 bands in 2nd bleed (14.3 to 97 kDa) and (16 to 97) KDa for 1st bleed. Similarly, Western blotting using PAb of *S. marcescens* revealed that the homologous antigens showed 5 bands ranging from 15 to 97 kDa in 4th bleed, 5 bands ranging from 15 to 52 KDa in 3rd bleed, 3 bands in 2nd bleed (20 to 52 kDa) and 4 bands (15 to 52) KDa for 1st bleed.

4.13.1.4. Colony blot transfer

By this method, specific bacteria could be directly detected on nitrocellulose membrane. Soil suspensions were prepared by using bacteria inoculated rhizosphere soil and applied on the surface of solidified NA medium in sterile petriplates. The colonies that appeared after 24 h incubation were transferred to NCM and probed with respective PAb. Violet dots were observed in NCM which showed the survival of bacteria in the soil (Figs. 53 & 54, H&K).

4.13.2. Antibiotic resistance test

Antibiotic sensitivity test were performed to know the tolerance of rhizobacteria against particular antibiotic. This was also felt necessary because it would help in determining the sustainability of the applied bacteria in the field. To determine the antibiotic resistance of the two isolates, they were tested by agar cup bioassay, against the following antibiotics: Ampicillin (A^{2} mcg/disc); (A^{10} mcg/disc); (A^{25} mcg/disc), Chloramphenicol (C^{10} mcg/disc); (C^{25} mcg/disc), Kanamycin (K^{30} mcg/disc), Metronidazole (Mt^{5} mcg/disc), Rifampicin capsule (450 mg), Tetracycline capsule (500 mg), Benzylpenicillin and Streptomycin. Results revealed that *B. megaterium* was resistant to 450µg/ml concentration of rifampicin and tested three

concentrations of ampicillin. Similarly, *S. marcescens* was found to be resistant to Mt^5, C^{10} and C^{25} but sensitive to others (Table 36; Fig. 55, A-D).

4.14. Detection of pathogen in soil by immunotechniques

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.

4.14.1. ELISA

S. rolfsii in the soil was detected by ELISA using specific PAbs of *S. rolfsii*. Antigens were prepared from treated and untreated soil and tested by ELISA, using PAbs of specific pathgens. The results showed that population of *S. rolfsii* were reduced when treated with PGPR. It was also showed that when soil inoculated with bacteria and pathogen lower A_{405} values were obtained as compared to antigens from soil treated only with *S. rolfsii* (Table 37).

Table 36: *In vitro* antibiotic resistance tests

Antibiotics	*B. megaterium*	*S.marcescens*
Ampicillin (A^2 mcg/disc)	Resistant	Sensitive
Ampicillin (A^{10} mcg/disc)	Resistant	Sensitive
Ampicillin (A^{25} mcg/disc)	Resistant	Sensitive
Chloramphenicol (C^{10} mcg/disc)	Sensitive	Resistant
Chloramphenicol (C^{25} mcg/disc)	Sensitive	Resistant
Kanamycin (K^{30} mcg/disc)	Sensitive	Sensitive
Metronidazole (Mt^5 mcg/disc)	Sensitive	Resistant
Rifampicin capsule (450 mg)	Resistant	Sensitive
Tetracycline capsule (500 mg)	Sensitive	Sensitive
Benzylpenicillin	Sensitive	Sensitive
Streptomycin	Sensitive	Sensitive

Table 37: ELISA and Dot blot values of soil antigens from treatments after reaction with PAb of *S.rolfsii*.

Soil antigen *	ELISA A 405 values	Dot Blot Colour intensity**
Uninfested soil	0.308±0.09	
Treatments		
S. rolfsii	1.057±0.04	++
B. megaterium	0.006±0.00	-
S. marcecsens	0.008±0.00	-
S. rolfsii + *B. megaterium*	0.440±0.002	-
S. rolfsii + *S. marcescens*	0.459±0.001	-

± =S.E; Average of 3 replicates: PAb dilution: 1:500; *Sample collected 30 days after inoculation with pathogen; ** colour intensity- Pinkish red: ++++; Bright pink: +++; Pink: ++; Light pink: +; no colour:-

4.14.2. Dot-blot

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 *S. rolfsii*. Violet coloured permanent dots were visible in pathogen alone treated samples indicating the survival of the *S. rolfsii* in the rhizosphere of the plant whereas the samples from both PGPR and pathogen treated showed no colour indicating the inability of pathogen to survive in presence of PGPR (Table 37).

4.15. Localization of pathogen in tea root tissues

4.15.1. Light microscopy

Cross sections of healthy as well as *S. rolfsii* infected tea roots were cut and light microscopic view revealed the establishment of pathogen within the tea root tissues showing the invasion of hyphae of *S. rolfsii* in root tissues (Fig. 50, I-J).

4.16. Immunodetection of chitinase in tea leaves

4.16.1. Western Blot

Western blotting using antibodies of chitinase revealed that in *B. megaterium* and *S. marcescens* treated tea leaves chitinase was expressed over control leaves. Expression of chitinase was observed in appearance of bands. Intensity of bands was higher and total three bands with molecular weights of 15,20 and 29 KDa in TV-25, 14.3, 15 and 29 KDa in T-17 varieties were appeared in *B. megaterium* and *S. marcescens* treated leaves in comparison to control (Fig. 56, A-D).

4.16.2. Dot blot

Dot-blot was also used for detection of expression of chitinase. Violet coloured permanent dots were visible in *B. megaterium* and *S. marcescens* treated samples whereas the samples from control leaves showed less intensity in colour indicating that the chitinase was expressed more in treatment over control (Fig. 56, E).

4.16.3. Immunofluorescence

Immunolocalization of chitinase in treated as well as control tea leaves were observed by immunofluorescence study. *B. megaterium* and *S. marcescens* treated along with control tea leaves were treated with chitinase antibody and FITC labeled Con A. Strong apple green fluorescence were evident in treated leaves (Fig. 56, F-N).

Figure 50: A-J: Sclerotial blight disease development by *Sclerotium rolfsii.* A-C: Inocula preparation in sand maize meal media (A); pure culture of *S. rolfsii* in PDA medium; (B) and prepared inocula of *S. rolfsii* for field application (c); D-H: application of inoculum in root region and effects on plant; D: inocula application; E&F: appearance of sclerotia in the surrounding regions of roots after application of 2 weeks; G&H: tea plants infected by *S. rolfsii*; I-J: tea root sections, I: T.S of healthy tea root; J: T.S of infected tea roots showing the invasion of hyphae of *S. rolfsii.*

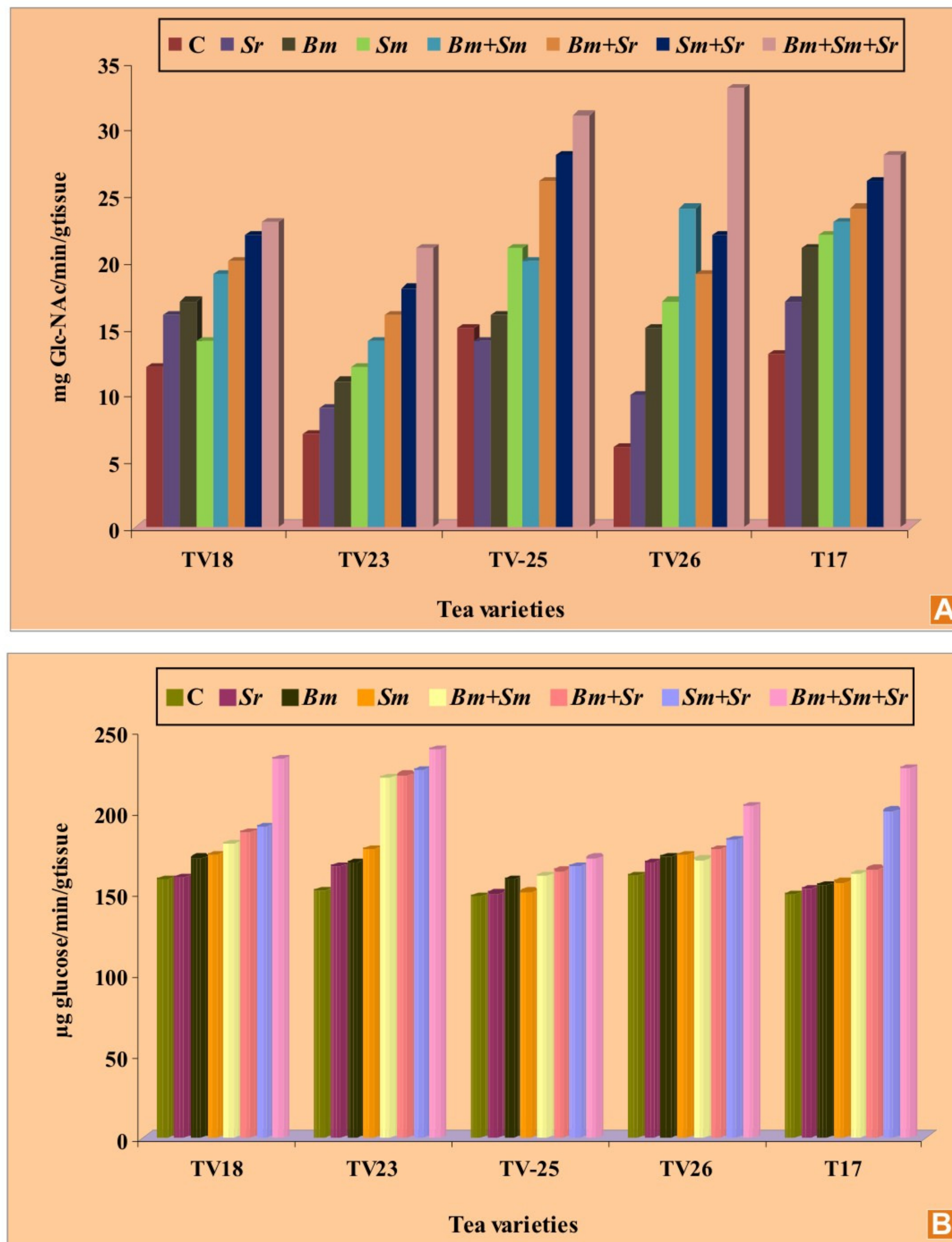


Figure 51: Effect of application of *B. megaterium*, *S. marcescens* along with *Sclerotium rolfsii* on chitinase (A) and β-1,3 glucanase (B) activities of five different varieties of tea plants.

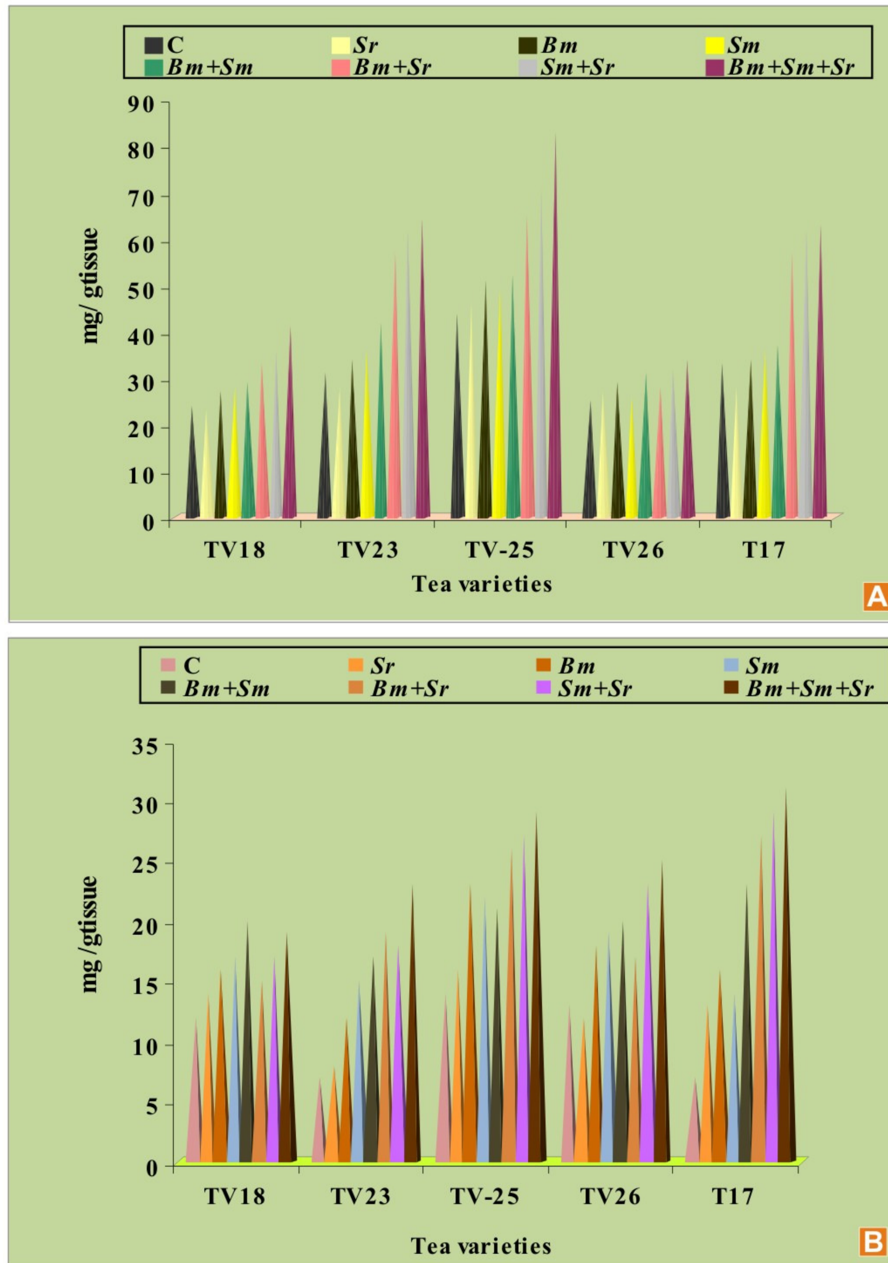


Figure 52: Changes in total (A) and ortho-dihydroxy (B) phenol contents in leaves of five different varieties of tea plants by application of *B. megaterium* and *S. marcescens* along with *Sclerotium rolfsii*.

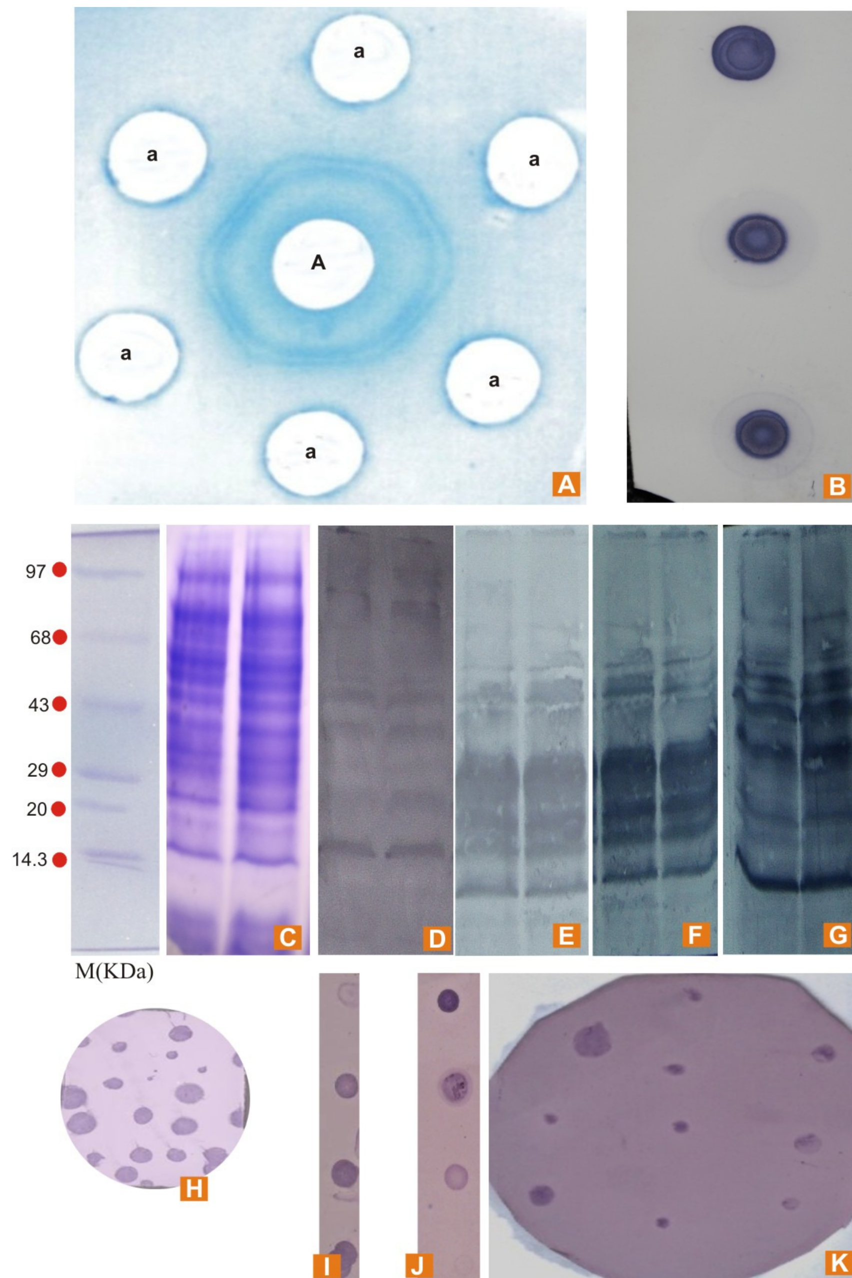


Figure 53: A-K: Immunological studies of *B. megaterium;* A: agar gel double diffusion of PAbs raised against *B. megaterium* with homologous antigens; B: Dot blot of homologous antigens probed with PAbs of *B. megaterium*; C: SDS of proteins of *B.megaterium.* Western blot analysis of *B. megaterium* antigens against Pabs from 1st bleed (D); 2nd bleed (E); 3rd bleed (F) and 4th bleed (G); H: colony transfer of bacteria in NCM probed with PAb of *B. megaterium*; K: soil bacteria transferred and probed with PAbs. Dot blot of homologous(I) as well as soil antigens (J) probed with PAbs of *B. megaterium*.

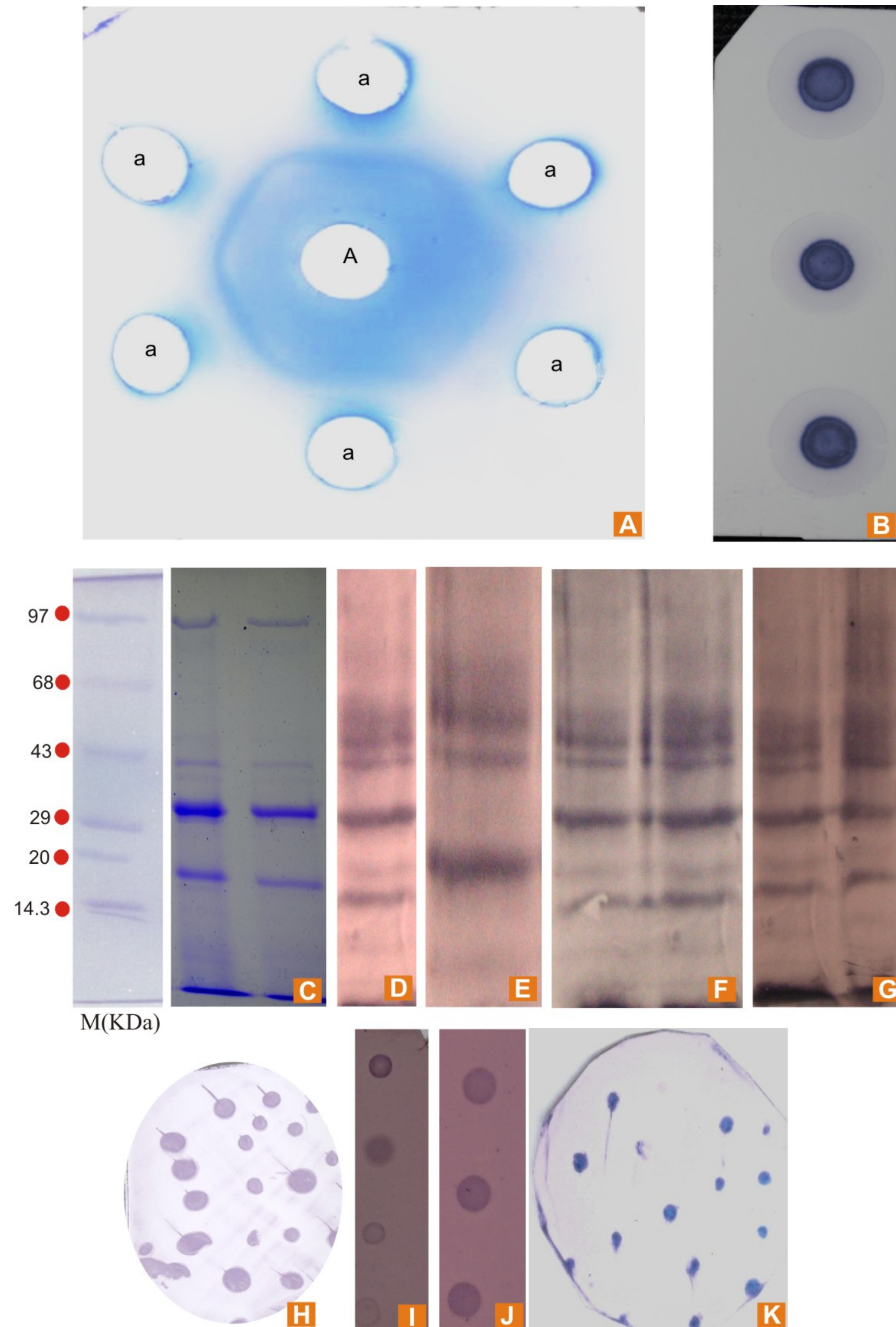


Figure 54: A-K: Immunological studies of *S. marcescens;* A: agar gel double diffusion of PAbs raised against *S. marcescens* with homologous antigens; B: Dot blot of homologous antigens probed with PAbs of *S. marcescens*; C: SDS of proteins of *S. marcescens*. Western blot analysis of *S. marcescens* antigens against PAbs from 1st bleed (D); 2nd bleed (E); 3rd bleed (F) and 4th bleed (G); H: colony transfer of bacteria in NCM probed with PAbs of *S. marcescens;* K: soil bacteria transferred and probed with PAb. Dot blot of homologous(I) as well as soil antigens (J) probed with PAbs of *S. marcescens*.

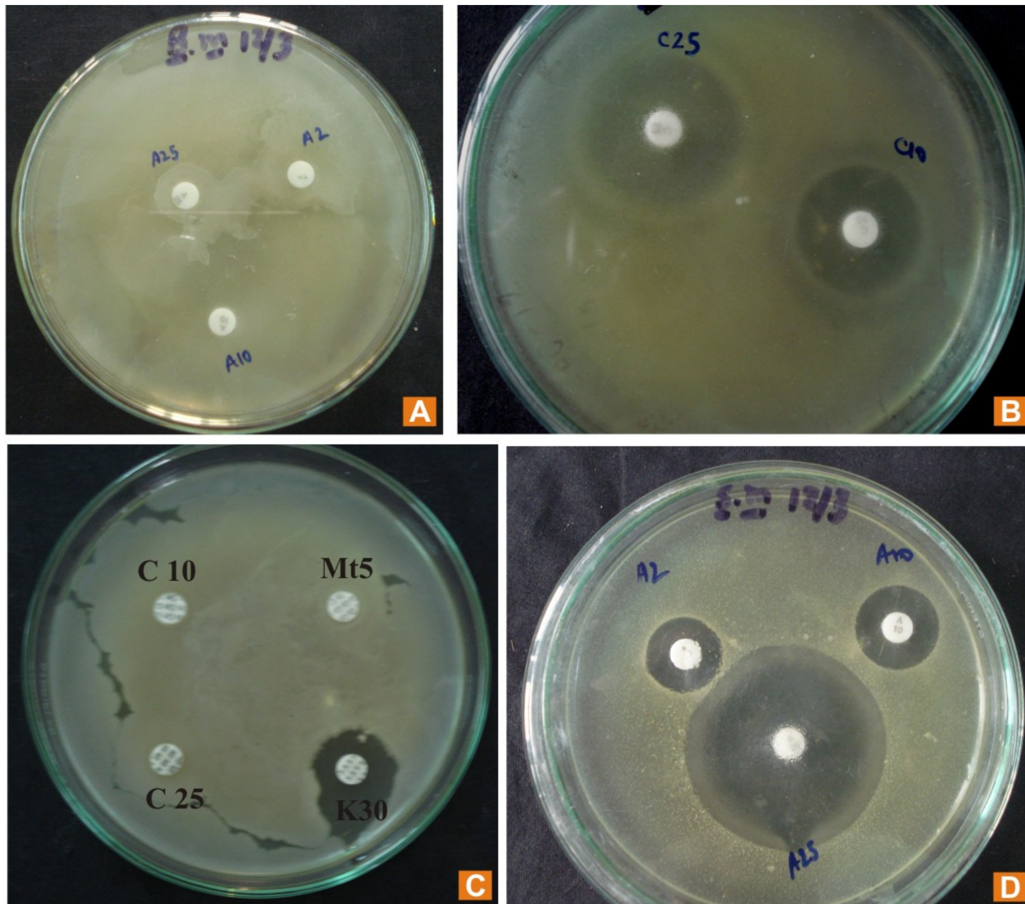
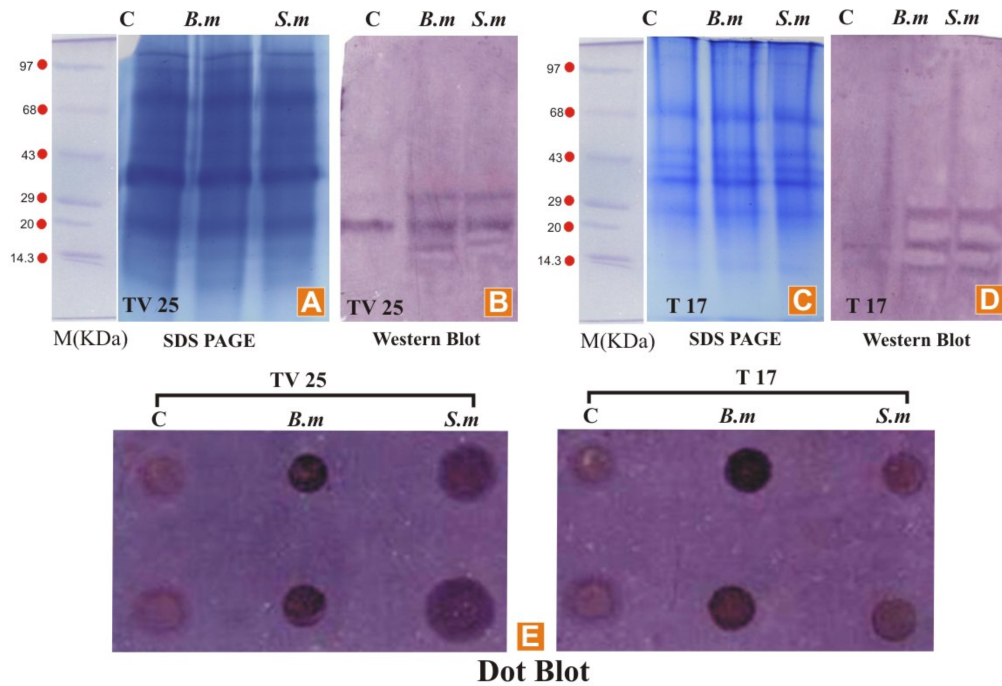


Figure 55: A-D: *In vitro* antibiotic resistance tests of *B. megaterium* and *S. marcescens* by disc method. A&B: Response of *B. megaterium* towards different concentrations of ampicilllin 2,10,25 mcg/disc (A) and chloramphenicol 10 and 25 mcg/disc (B); C&D: Resistance tests of *S. marcescens* against Cm^{10}, Cm^{25}, K^{30} and Mt^{5} (C) and A^{2}, A^{10} and A^{25} (D).



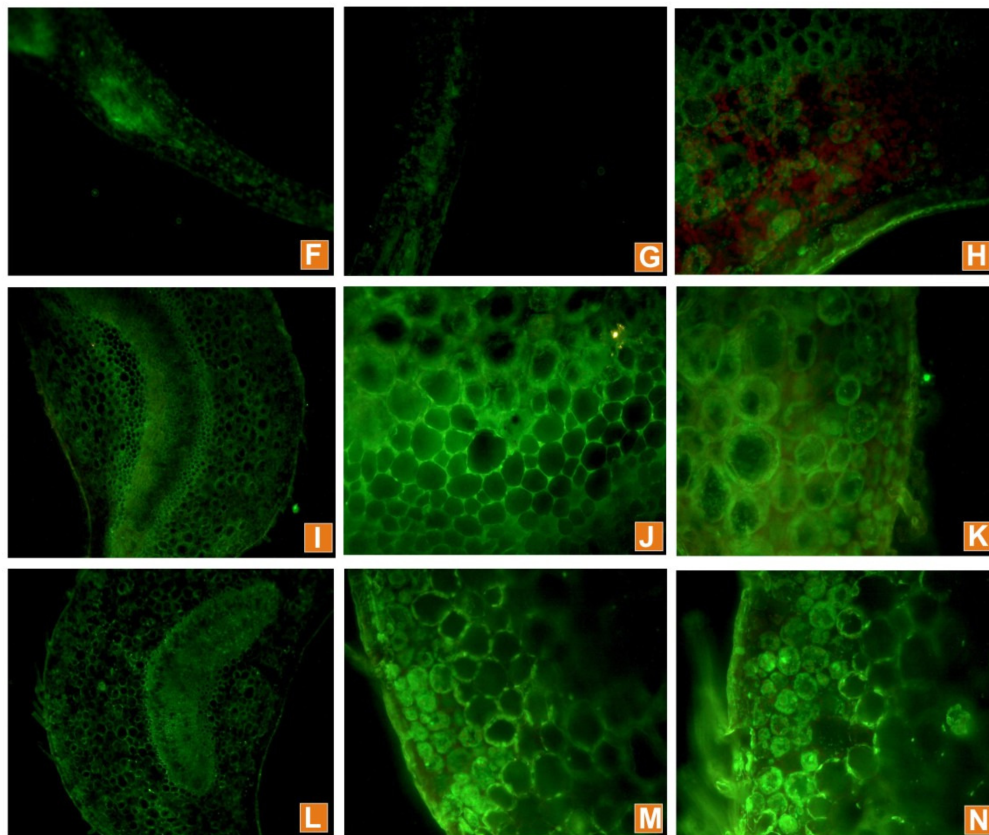


Figure 56: A&B: SDS PAGE and western blot analysis of chitinase in *B. megaterium* and *S. marcescens* treated tea leaves (TV25); C&D: SDS PAGE and western blot analysis of chitinase in *B. megaterium* and *S. marcescens* treated tea leaves (T17); E: Dot blot of chitinase and F-N:Immunolocalization of chitinase in treated(I-K- *B. megaterium* ; L-N- *S. marcescens*) as well as control (F-H) leaves by immunofluorescence.

DISCUSSION

Plant growth in agricultural soils is influenced by many abiotic and biotic factors. There is a thin layer of soil immediately surrounding plant roots that is an extremely important and active area for root activity and metabolism which is known as rhizosphere. A large number of microorganisms such as bacteria, fungi, protozoa and algae coexist in the rhizosphere. Bacteria are the most abundant among them. Plants select those bacteria contributing most to their fitness by releasing organic compounds through exudates creating a very selective environment. Since bacteria are the most abundant microorganisms in the rhizosphere, it is highly probable that they influence the plant's physiology to a greater extent, especially considering their competitiveness in root colonization (Barriuso *et al.* 2008). Rhizosphere bacteria can have a profound effect on plant health. Since rhizosphere colonization is important not only as the first step in pathogenesis of soil borne micro-organisms, but also is crucial in the application of microorganisms for beneficial purposes (Lugtenberg *et al.* 2001). Colonising microorganisms can be detected attached to the roots, as free organisms in the rhizosphere or as endophytes. The interactions between plants and microorganisms are immensely complex and very little is known about the sum of factors that lead to reliable biocontrol and biofertiliser applications. Plant growth promoting rhizobacteria (PGPR) are a common group of bacteria that can actively colonize plant roots and increase plant growth (Kloepper and Schroth 1978). These PGPR can prevent the deleterious effects of phytopathogenic organisms from the environment. The mechanisms by which PGPR can influence plant growth may differ from species to species as well as from strain to strain. Growth promotion mechanism may be direct i.e. production of growth hormones, phosphate solubilization, nitrogen fixation or indirect viz, suppression of deleterious microorganisms by siderophore production or secretion of antifungal metabolites (Kloepper 1993). But, in field conditions, the above traits may not be sufficient to account for the observed growth promotion. The biochemical or physiological changes induced in the host that are activated by the PGPR, also lead to plant growth promotion and develop resistance capacity in the host against pathogens. Thus, though hundreds of candidate PGPR strains have been screened and evaluated in laboratory, greenhouse and field studies across the world, implementation of this technology has been hindered by the lack of

consistency and variation in responses that are obtained in field trials from site to site, year to year, for different crops (Martinez-Viveros *et al.* 2010). Successful establishment of the introducing bacteria depends on proper selection that must tailored to the soil and crop combination. Besides, understanding of the interactions between PGPR and their plant hosts and the resident microflora are still limited, and there is also paucity of information on how environmental factors influence processes that contribute to plant growth promotion.

Keeping the above in mind, the present study was undertaken to study the influence of two bacteria isolated from tea rhizosphere on growth and yield of tea (*Camellia sinensis*). It was felt that since the bacteria were originally isolated from tea rhizosphere, their inoculation into tea rhizosphere would not hinder the ecological balance and their sustainability would also be better.

The two selected bacteria were morphologically, biochemically characterized and finally identified as *Bacillus megaterium* (TRS 7) and *Serratia marcescens* (TRS 1). The BLAST query of 16S r DNA sequence of the isolates against GenBank database confirmed their identity. The sequences have been deposited in NCBI, GenBank database under the accession Nos. JX 312687.1 and JN 020963.1 for *B.megaterium* and *S. marcescens* respectively. In the last decade, 16S rDNA sequencing has played a pivotal role in the accurate identification of bacterial isolates and the discovery of novel bacteria. Chakraborty *et al.* (2011) demonstrated that analysis of aligned rDNA sequences was a reliable clustering strategy for identification purposes in a variety of taxonomic groups and systemic level. The study showed that it was also applicable in analyzing much shorter DNA sequences from a single gene, which is going to be the fundamental block in the massive rDNA database. The sequenced PCR products of *Bacillus megaterium* (TRS 7) and *Serratia marcescens* (TRS 1) were aligned with extype isolate sequences from NCBI GenBank for identification as well as for studying phylogenetic relationship with other ex-type sequences. The phylogenetic analysis was conducted using the UPGMA method among the isolates of *S. marcescens* with other ex-type strains obtained from NCBI GeneBank database by MEGA4.1 software. *S. marcescens* (TRS 1-JN020963.1) is closely related to the extype- *S. marcescens* (DQ 866838.1) which are placed nearer to each other in the phylogenetic tree. The evolutionary history was also inferred using the UPGMA method among the isolates of *Bacillus megaterium*. *B.*

megaterium (TRS7- JX312687.1) is distantly related to *B. megaterium* (JQ 790514.1) and *B. megaterium* (HQ 238636.1) in the phylogenetic tree. 16S rDNA sequence alignments of *S. marcescens* (TRS 1- JN020963.1) with extypes isolates and *B. megaterium* (TRS7- JX312687.1) with extypes isolates were carried out. Presence of regions with similar sequences indicated relationships among the isolates of *B. megaterium* and the isolates of *S. marcescens* separately. Comparative analysis of the combinations and percentage of occurrence of different nucleotides in the entire sequences of the isolates further confirms their genetic relatedness among each other. Different pattern in the nucleotide compositions in the same type of gene for individual isolates proves the uniqueness of the conserved sites designated to each species. *Bacillus megaterium* from soil of different agro climatic zones of Karnataka were isolated and identified by Reddy *et al.* (2010). Molecular characterization of the *Bacillus megaterium* isolates was done using RAPD technique. The cluster analysis was based on 20 RAPD bands. The dendrogram clearly depicted that all the 10 *B.megaterium* isolates formed two major clusters. Among the two major groups, isolates from zone 4 (CDP), zone 5 (EDP), and zone 6 (SDP) formed the first group and the isolate from zone 1 (NETP), zone 2 (NEDP), zone 3 (NDP), zone 7 (STP), zone 8 (NTP), zone 9 (HP) and zone 10 (CP) formed the second group. The dissimilarity matrix for *B.megaterium* isolates revealed that within the *B.megaterium* isolates used in the present investigation, the highest dissimilarity was observed between isolate of zone 1 (NETP) and isolate of zone 4(CDP) followed by zone 4(CDP) and zone 9 (HP) isolates. Least dissimilarity was observed between zone 2 (NEDP) and zone 3 (NDP) isolates, followed by zone 3 (NDP) and zone 10 (CP) isolates. To visualize the genetic relatedness among the *B.megaterium* isolates in detail principal component analysis (PCA) was done for 81 RAPD bands generated by 10 decamer random primers. The polymerase chain reaction (PCR)-based procedures of randomly amplified polymorphic DNA (RAPD) and repetitive element (RE)- based PCR were used to amplify total DNA prepared from each of 62 clinical *Serratia marcescens* isolates. Three different random primers, designated 1060, 1254 and 1283, were used individually in RAPD-PCR. PCR-based approaches were a valid means of discriminating strain differences among isolates of *S. marcescens* and the amount of differentiation depend on the primer used. These techniques should prove useful for routine surveillance or in examining outbreaks of *S. marcescens* in clinical settings (Patton *et al.* 2001).

Genetic diversity among the isolates of *B. megaterium* (**JX 312687**, MTCC 428, MTCC 1684 and MTCC 2949) and *S. marcescens* (**JN 020963**, MTCC 86, MTCC 97, MTCC 2645, MTCC 3124, MTCC 4301, MTCC 4822 and MTCC 7298) obtained from tea rhizosphere were studied on the basis of DGGE analysis of 16 S rDNA sequences. The banding pattern among the four isolates of *B. megaterium* and the eight isolates of *S. marcescens* were identical to that of the reference isolates used for conformation. In a study by Zhao *et al.* (2012), they showed that the bacterial communities in the soils from tea orchards and their adjacent wasteland in Anhui Province, China, analysed by nested PCR-DGGE technique combined with sequencing. The DGGE patterns of different soils were similar to each other and the most intense bands appeared in all lanes. The bacterial genetic diversity index of tea orchard soils was lower than that of wasteland. For the tea orchard soils, Shannon's diversity index decreased in the order: 45-year old tea orchard>25-year-old tea orchard>7-year-old tea orchard > 70-year-old tea orchard. The analysis of 16S rRNA gene sequences indicated that the fragments belong to Proteobacteria, Acidobacteria, TM7, Cyanobacteria and Firmicutes. A comprehensive analysis of the bacterial community structure in the tea orchard soils indicated the bacterial community was dominantly composed of Acidobacteria, followed by Proteobacteria (Gamma and Alpha), Firmicutes, Cyanobacteria and candidate division TM7.

Following identification and phylogenetic studies, the optimum conditions of the growth of the selected bacteria were determined. *B. megaterium* and *S. marcescens* grew best at pH 6.0 with log cfu values of 22.8 log cfu/ml and 23.07 log cfu/ml. Both the bacteria grew well within 20-50ºC ranges of temperature but grew best at around 35ºC. 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 40 ºC and 50°C respectively. Umamaheswari *et al.* (2003) also 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.

B. megaterium and *S. marcescens* were tested *in vitro* for properties related to plant growth promotion and disease suppressing mechanism prior to their use *in vivo.* Results revealed that both the bacteria were able to produce IAA, volatiles, siderophores and solubilised phosphates *in vitro* but did not produce HCN and *B. megaterium* was non- chitinase producing strain. 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). Siderophore production has also long been considered as one of the mechanisms of suppression of fungal growth in the rhizosphere. Jagadeesh and Kulkarni (2003) reported that of 38 rhizobacterial strains isolated from tomato which showed antagonism to *Alstonia solanacearum,* 23 were siderophore producers. Hydrogen cyanide (HCN) production by different bacterial species including *P. aeruginosa*, *P. fluorescens* and *Chromobacterium violaceum* was observed (Siddiqui *et al.* 2003). Khalid *et al.* (2004) also 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. Huang *et al*. (2010) reported that volatiles produced from *Bacillus megaterium* YFM3.25 were characterized and had nematicidal activity against *Meloidogyne incognita.* It has also been reported by Ortiz-Castro *et al.* (2008) that plant growth promotion by *Bacillus megaterium* involves cytokinin signaling in *A. thaliana* and *P. vulgaris* seedlings. The role of cytokinin signaling in mediating the plant responses to bacterial inoculation was investigated using *A. thaliana* mutants lacking one, two or three of the putative cytokinin receptors CRE1, AHK2, AHK3 and RPN12- a gene involved in cytokinin signaling. Cytokinin receptors play a complimentary role in plant growth promotion by *B. megaterium.* Arkhipova *et al.* (2005) isolated many PGPR from the rhizosphere that could produce cytokinins that exerted a pronounced growth stimulatory effect in different crop plants.

Series of *in vivo* experiments were next carried out with the two selected bacteria to determine their plant growth promoting activity in the field and potted conditions. *S. marcescens* TRS-1 and *B. megaterium* TRS 7 were applied as aqueous suspensions to the rhizosphere of eighteen- month old plants of five varieties of tea in the field, tea bushes and also to potted tea plants where the soil was non-sterile, with the natural rhizosphere micro flora. Application of the bacteria resulted in significant increase in growth, measured in terms of height, leaf number, number of branches and dry mass of leaves. In order to confirm the result in tea estate, bacteria were applied as soil drench to the leaves of T17/154 in tea bushes of Hansqua tea estate. Significant increases in leaf biomass were obtained under this condition also. 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. 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. Besides, all the eight isolates of *S. marcescens* along with *S. marcescens* (TRS 1) showed significant increase in height and leaf numbers in potted tea plants but *S. marcescens* (TRS 1) comparatively promoted better plant growth in comparison to other isolates. Since insecticides or fungicides are applied in tea gardens, the ability of the bacteria to grow in their presence was also investigated. Results revealed that *B. megaterium* could tolerate acephate concentration of 1250mg/ml, confidor, ethion 50EC ,calixin concentrations of 1000 µl/ml and contaf 5E conc. of 500 µl/ml; whereas *S. marcescens* could tolerate acephate concentration of 625mg/ml, confidor, ethion 50EC ,calixin concentrations of 250, 1000 and 500 µl/ml and contaf 5E concentrations of 125 µl/ml. Combined application of *B. megaterium* and low dose of acephate (1:2000-0.5mg/ml), commonly used in tea gardens was tried in order to reduce insect attack and induce growth promotion by *B. megaterium*. Results revealed that significant growth promotion was achieved in both cases, but in case of combined application, leaves were healthier with a significant reduction in insect attack (% of insect attack reduced to 16-22% after foliar spray of acephate). Similarly, significant increase in leaf, branch numbers and reduction in % of insect attack (14-21% from 47-56% after foliar spray of acephate with aqueous suspensions of *S. marcescens*; 14-20% from 48-57% after foliar spray of acephate with aqueous suspensions of *S. marcescens* and *B. megaterium*) were also observed.

Earlier studies with other isolates of the two bacteria have shown them to possess plant growth promoting and or biocontrol activities. Dashti *et al*. (1997) observed that application of plant growth-promoting rhizobacteria- *Bradyrhizobium japonicum*, *Serratia liquefaciens* 2-68 or *Serratia proteamaculans* 1-102 to soybean (*Glycine max* [L.] Merr.) increased protein and dry matter yield under short-season conditions. Javed *et al*. (1998) selected 11 isolates of plant growth promoting rhizobacteria and reported that four of these improved the growth of maize and could be used as biofertilizers. Shankaraiah *et al*. (2000) reported that *B.megaterium* alone, or in combination with *Agrobacterium awamori* could enhance phosphate solubilization and increase yield in sugarcane. Previous studies have shown that *Serratia proteamaculans* 1-102 (1-102) promotes soybean- bradyrhizobia nodulation and growth, but the mechanism is unknown (Bai 2002). *S. marcescens* strain B2 was

reported to be particularly effective in inhibiting both hyphal growth and sclerotial germination of *R. solani* (Someya *et al*. 2000). 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. aeruginosa.* In an early study, *Serratia marcescens* NBR11213 was reported to induce plant growth promotion and biological control of foot and root rot of betelvine caused by *Phytophthora nicotianeae* (Lavania *et al*. 2006). Tilak *et al.* (2006) observed that dual inoculation of pigeon pea with PGPR including *P. fluorescens* and *B. cereus* along with *Rhizobium* sp. increased growth nodulation and nitrogenous activity by various degree. Trivedi and Pandey (2008) reported plant growth promotion abilities of *Bacillus megaterium* strain B 388 (MTCC6521) isolated from a temperate Himalayan location. Herman *et al.* (2008) also reported the effect of plant growth promoting rhizobacteria- a mixture of two species of *Bacillus* PGPR (*Bacillus subtilis* and *Bacillus amyloliquefaciens*) on bell pepper production and green peach aphid infestations in New York. Fruit yield in the *Bacillus* spp. treatment was significantly greater than yield in the control treatment. Seed treatment of *Bacillus licheniformis* MML2501 in groundnut showed a significant increase in seed germination, other growth parameters and yield parameters under potted plant experiments (Prashanth and Mathivanan 2010). Plant growth-promoting activities of fluorescent pseudomonads, isolated from the Iranian soils were reported by Abbas-Zadeh *et al*. (2010). Influence of *Serratia marcescens* (TRS 1) on growth promotion of tea plants as evidenced by increase in height, emergence of new leaves and branches, as well as increase in leaf biomass was also observed by Chakraborty *et al*. (2010).Yadegari *et al.* (2010) reported that the PGPR strains *Pseudomonas fluorescens* P-93 and *Azospirillum lipoferum* S-21 as well as two highly effective *Rhizobium* strains significantly increased nodule number, dry weight, shoot dry weight, amount of nitrogen fixed, seed yield and protein content in *Phaseolus vulgaris*. Abbasi *et al.* (2011) isolated plant growth promoting rhizobacteria from wheat rhizosphere and reported their effect on improving growth, yield and nutrient uptake of plants. Strains- WPR-32, WPR-42 and WPR-51 were identified as PGPR. Application of PGPR significantly increased plant height, shoot fresh weight and shoot dry weight by 25, 45 and 86%, respectively, while increase in root length, root fresh and dry weight was 27, 102 and 76%, respectively, over the un-inoculated

control. PGPR also increased number of tillers per plant, 1000-grain weight and grain yield by 23, 48 and 59% over the control. Uptake of N and P by plant shoot was increased by three-fold, while K uptake was increased by 58% with PGPR application. The rate of increase in growth, yield and nutrient accumulation was higher in treatments receiving combined application of PGPR and N-fertilizer. Erturk *et al.* (2012) examined the effect of inoculation of plant growth promoting rhizobacteria (PGPR) on phenological data, total yield and fruit quality characteristics of strawberry. RC19 (*Bacillus simplex*), RC05 (*Paenibacillus polymyxa*), and RC23 (*Bacillus* spp.) also increased the yield and growth of strawberries. All bacterial root inoculations significantly increased yield per plant (1.98–20.85%), average fruit weight (3.05–19.26%) and quality fruit ratio (10.30–32.05%) compared to control, whereas the bacterial inoculations did not affect leaf area, first flowering and harvest dates in strawberry. Soil samples were collected from six different rhizospheric soils of bean plants from different locations of Shimla and Solan in H.P (India) by Kumar *et al.* (2012). A total of thirty bacteria were isolated and *in vitro* screening was done for different plant growth promotion activities i.e. phosphate solublization, IAA production, ammonia production, ACC deaminase activity, HCN production and catalase. Twelve bacterial isolates were positive for phosphate solublization. IAA production was shown by almost all the bacterial isolates. Three isolates were positive for ammonia production. ACC deaminase activity was shown by nine isolates. Two isolates were positive for HCN production and all the isolates were found to be catalase positive. Seven isolates showed maximum plant growth promotion activities and further identified on the basis of colony morphology, Gram staining and biochemical tests. These isolates were identified as *Acinetobacter* sp., *Bacillus* sp., *Enterobacter* sp., *Micrococcus* sp. and *Pseudomonas* sp. as potent PGPR. A bacterial strain-H10, was isolated from the rhizosphere at Laixi in Shandong Province, China and was identified as *Ochrobactrum haematophilum* based on API 20 NE tests and 16S rRNA gene sequence analysis. The plant growth-promoting characteristics of the strain were further characterized and the results showed that strain H10 produced siderophore, indol-3-acetic (IAA) and solubilized phosphate. Inoculation with the strain was found to significantly increase ($p < 0.05$) the growth of cucumber in pot experiments. Strain H10 was assessed *in vitro* for antagonism against several pathogenic fungi and showed high antifungal activity (Zhao *et al.* 2012). Jha *et al.* (2012) reported the effect of single and dual phosphate-

solubilizing bacterial strain (*Pseudomonas fluorescens* BAM-4, *Burkholderia cepacia* BAM-6, *B. cepacia* BAM-12 and *Aeromonas vaga* BAM-77) inoculations on overall growth of mung bean plants. Among the four strains studied, *A. vaga* and *P. fluorescens* were found to be more valuable as single inoculants in terms of plant growth. Kadyan *et al.* (2013) isolated fifty two aerobic and endospore forming bacilli (AEFB) strains from rhizospheric soil of *Phyllanthus amarus*. Morphological, biochemical and molecular characterization by 16S rDNA gene sequencing showed that these bacterial strains belong to six different genera of AEFB i.e. *Bacillus*, *Brevibacillus*, *Lysinibacillus*, *Paenibacillus*, *Terribacillus* and *Jeotgalibacillus*. Analysis of their PGP activities showed that 92.30 % strains produced indole acetic acid hormone, 86.53 % of the strains solubilized phosphate and 44.23 % strains produced siderophore. Chitinase production activity was shown by 42.30 % of the strains and 21.15 % of the strains produced 1-amino cyclopropane-1-carboxylate (ACC) deaminase. 46.15 % of isolates showed antagonistic activity against common fungal pathogen of the plant i.e. *Corynespora cassiicola*. Among all of the isolated strains *B. cereus* JP44SK22 and JP44SK42 showed all of the six plant growth promoting traits tested. *B. megaterium* strains (JP44SK18 and JP44SK35), *Lysinibacillus sphaericus* strains (JP44SK3 and JP44SK4) and *Brevibacillus laterosporus* strain JP44SK51 showed multiple PGP activities except ACC deaminase production activity. Bacterial strain belonging to genera *Jeotgalibacillus* sp. JP44SK37 was reported first time as a member of rhizospheric soil habitat and also showed PGP activities. George *et al.* (2013) isolated two plant growth promoting bacteria designated as KiSII and RNF 267, from the rhizosphere of coconut plants were identified as *Serratia marcescens* and *Enterobacter* sp. based on their phenotypic features, BIOLOG studies and 16S rRNA gene sequence analysis. Both bacteria exhibited phosphate solubilization, ammonification, and production of indole acetic acid, β-1, 3 glucanase activities and 1-aminocyclopropane-1-carboxylate-deaminase activity. *S. marcescens* KiSII exhibited N- fixation potential, chitinase activity, siderophore production and antibiotics production. Seed bacterization with these bacteria increased the growth parameters of test plants such as paddy and cowpea over uninoculated control in green house assay. In coconut seedlings, significant increase in growth and nutrient uptake accompanied with higher populations of plant beneficial microorganisms in their rhizospheres were recorded on inoculation with both the PGPR. 336 bacterial strains were isolated representing

31 different bacterial genera. Strains belonging to the genera *Agrobacterium*, *Burkholderia, Enterobacter*, and *Pseudomonas* were the most prominent isolates. Siderophore and indolic compounds producers were widely found among isolates, but 101 isolates were able to solubilize phosphate. Under gnotobiotic conditions, eight isolates were able to stimulate the growth of rice plants. Five of these eight isolates were also field tested in rice plants subjected to different nitrogen fertilization rates. The condition of half-fertilization plus separate inoculation with the isolates AC32 *Herbaspirillum* sp.), AG15 (*Burkholderia* sp.), CA21 (*Pseudacidovorax* sp.), and UR51 (*Azospirillum* sp.) achieved rice growth similar to those achieved by full-fertilization without inoculation, thus highlighting the potential of these strains for formulating new bioinoculants for rice crops (Souza *et al.* 2013). Thus it is clear that the potential of various isolates of *Bacillus megaterium* and *Serratia marcescens* as plant growth promoters and biocontrol agents have also been recorded previously.

In vivo phosphate solubilizing abilities of *S.marcescens* and *B. megaterium* were confirmed. Soil P content decreased following application of both bacteria, while root and leaf phosphate contents showed an significant increase both in treated tea plants in potted as well as field conditions in comparison to control. Maximum phosphate content was obtained in the leaf tissues. It was reported that *Bacillus megaterium*, the most effective phosphate-solubilizing microorganism, could release P from organic phosphates, but did not solubilize mineral phosphates in field experiments (Kucey *et al.* 1989). Some RP-solubilizing bacteria such as *Serratia marcescens* EB 67 and *Pseudomonas sp.* CDB 35 were reported to be able to use root exudates or a broad range of carbon substrates in soil and supply P to plants in the rhizosphere (Hameeda *et al*. 2006a). Reports in the literature mentioned *Serratia marcescens* EB 67, *Pseudomonas sp.* CDB 35 (Hameeda *et al*. 2006b), *Enterobacter sp*. and *Bacillus subtilis* (Toro *et al*. 1997) as contributing to an improvement of the plant growth as rock phosphate-solubilizing bacteria. The application of biofertilizers containing the phosphate-solubilizing bacterium *B. megaterium* significantly increased the growth of Zea mays (Wu *et al.* 2005) and promoted growth of eggplant (Han and Lee 2005) pepper and cucumber (Han *et al*. 2006). Abou El- Yazeid *et al.* (2007) noticed that use of bio dissolving phosphor bacteria *B*. *megaterium* (Bio phos-phor) combined with boron foliar spray enhanced growth, productivity and quality of squash plants. Phosphatases remove the phosphate from organic compounds and convert it in soluble form to the plants. Some PGPR biofertilizers also influence the

availability of phosphate by secreting phosphatases for mineralization of organic phosphates (Rodriguez and Fraga 1999). Both acid and alkaline phosphatase activities in rhizosphere soil of all five varieties were enhanced following application of the bacteria. Results of the present study, therefore clearly revealed *S. marcescens* and *B. megaterium* to have the ability of phosphate solubilization in the soil which would be one of the mechanisms of observed plant growth promotion by both bacteria.

The isolated bacteria were tested against pathogens- *Sclerotium rolfsii, Rhizoctonia solani, Fusarium graminearum and F. oxysporum* for determining antagonistic activity. All the tested fungi were inhibited to some degree, the percentage inhibition varying between 57-84% by *B. megaterium* where as 27-75% by *S. marcescens*. Several biocontrol bacteria, including *Pseudomonas spp*., *Serratia marcescens*, *Bacillus sp.* and *Streptomyces sp*. were reported for controlling *Fusarium* wilt diseases (Scher and Baker 1982; Van Peer *et al*. 1991; Liu *et al*. 1995; Raajmakers *et al*. 1995). Someya *et al.* (1997, 2000) reported that *S. marcescens* strain B2 produced lytic enzymes- chitinases, siderophores and the antibiotic pigment prodigiosin as an antifungal factors. *Lysobacter* strains inhibited a wide range of fungal pathogens: *Pythium spp*. (Folman *et al*. 2003), *R. solani* (Giesler and Yuen 1998; Zhang *et al*. 2001), *Bipolaris sorokiniana* (Zhang and Yuen 2000), and *Uromyces appendiculatus* (Yuen *et al*. 2001). Chitinases from *Chromobacterium sp*. strain C-61 and *L. enzymogenes* played an important role in the inhibition of *R. solani* (Park *et al*. 1995) and *B. sorokiniana* (Zhang and Yuen 2000) respectively. PGPR strains *Bacillus pumilus* SE34 and *Pseudomonas fluorescens* 89B61, elicited systemic protection against the blight on tomato and reduced disease (Yan *et al*. 2002). Treatment with *Ochrobactrum anthropi* also decreased brown root rot of tea, caused by *Phellinus noxius* (Chakraborty *et al*. 2009).

It is apparent from the present study as well as studies by a large number of previous workers that PGPR have the ability to promote growth in plants, which in many cases is associated with pathogen suppression in the soil. These PGPR secrete one or more metabolites in the soil which then elicit the observed response in the host. Whether it is growth promotion or disease suppression, the ultimate expression is in the host. Thus, these microorganisms or their products have the ability to elicit responses at molecular level which would include activation of a number of metabolic pathways in the host, the end product of which is finally expressed as

increased growth of plant or reduced disease. Induced resistance has emerged as a potential tool in crop protection practices based on biological control. Research over the past years has demonstrated that ISR (induced systemic resistance) can be a potential mechanism by which PGPR demonstrate biological disease control (Kloepper *et al*. 1996). ISR is dependent on colonization of the root system by sufficient numbers of PGPR. Induced resistance can be defined as the phenomenon by which plants exhibit increased levels of resistance to a broad spectrum of pathogens by the prior activation of genetically programmed defence pathways. The most extensively studied type of induced resistance is systemic acquired resistance (SAR) (Durrant and Dong 2004). SAR is expressed locally and systemically after a localised infection by a necrotising pathogen or the application of some chemicals such as benzothiadiazole (BTH) (Conrath *et al.* 2006) and is characterised by the accumulation of salicylic acid (SA) and pathogenesis-related (PR) proteins. The colonisation of roots with selected PGPR (De Vleesschauwer *et al.* 2006) can also lead to a type of systemic resistance, commonly denoted as ISR. Hence, in order to get a proper insight into the plant growth promotion and induced systemic resistance, analysis of the biochemical changes especially those known to be involved in these mechanisms are essential. Besides growth promotion, in order to determine whether the bacteria could induce systemic resistance in tea plants, accumulation of defense related enzymes and phenolics were studied. Results revealed that both the bacteria enhanced activities of defense related enzymes- peroxidase, chitinase, phenylalanine ammonia lyase and β- 1,3- glucanase as well as total and O-dihydroxy phenols significantly. Since the applications of *S.marcescens* and *B. megaterium* were in the soil and increased accumulation of defense related biochemical components were observed in the leaves, it is quite apparent that both bacteria induce systemic response. Both POX and CHT are PR proteins and their increased activity would indicate activation of such PR proteins during defense. No significant changes in either protein content or protein profile was observed by treatments with the two bacteria. Chlorophyll content however increased significantly 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. New isoforms and increase of isomers were observed in *B. megaterium* treated plants where as few were lost or there was suppression of few isomers by the treatment of *S. marcescens*. Similar trends were observed in TV26, TV18, TV25 and T17. But in T-

17/154 some new isomers were enhanced by the treatment of *S. marcescens*. Accumulation of higher levels of phenolics in plants resistant to various stresses was reported by several authors (Daayf *et al.* 1997). Induction of defense related enzymes by *P. fluorescens* in black pepper and *Phytophthora capsici* pathosystem was reported by Paul and Sharma, (2003). Radjacommare *et al.*(2005) reported the induction of defense enzymes, phenols and lignin in rice by *P. fluorescens* against *R. solani*. Ability of *B.megaterium* to promote growth in *Lactuca sativa* alone, or in combination with arbuscular mycorrhiza was reported by Marulanda-Aguirre *et al.*(2008). They also reported increased accumulation of chlorophylls and carotenoids.

Since the bacteria showed antagonistic activity *in vitro*, experiments were further conducted to determine whether these could also control sclerotial blight disease caused by *Sclerotium rolfsii*. At the onset, roots of tea plants of selected five varieties were infected with cultures of *S. rolfsii* and then bacterial suspensions were applied as soil drench in order to control the disease. Cross sections of healthy as well as *S. rolfsii* infected tea roots were cut and light microscopic view revealed the establishment of pathogen within the tea root tissues showing the invasion of hyphae of *S. rolfsii* in root tissues. *B. megaterium* reduced sclerotial blight more significantly in comparison to *S. marcscens*. Punja (1985) suggested the possibility of controlling *Sclerotium rolfsii* infection by preventing establishment of its infection through enhancing the levels of phenolic compounds in host tissues. PGPR are also known to induce host resistance through activation of the phenylpropanoid pathway (Van Peer *et al.* 1991). Antagonism and disease suppression by *P. fluorescens* against *S. rolfsii* were also reported by Rangeshwaran and Prasad (2000). *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). Guo *et al.* (2004) also reported the ability of PGPR *Serratia* sp., *Pseudomonas* sp. and *Bacillus* sp. to reduce wilt of tomato. In another series of experiments, biochemical responses of tea following application of bacteria and challenge inoculated with the pathogen- *Sclerotium rolfsii* were determined. There was a significant increase in peroxidase and chitinase activities of tea leaves in pathogen inoculated as well as in *B. megaterium*, *S. marcescens* or *B. megaterium+S. marcescens* treated plants challenge inoculated with *S. rolfsii*. Relatively higher activity was shown by plants inoculated with *B. megaterium+S. marcescens* and

challenge inoculated with pathogen followed by plants inoculated with pathogen alone. Similar trends were observed in β-1,3 glucanase and Phenyl alanine ammonia lyase activities. Maximum total and O- phenol contents were also obtained in the presence of both *B. megaterium+S. marcescens* and *S. rolfsii*. The higher POX activity was noticed in cucumber roots treated with *Pseudomonas corrugata* challenged with *Pythium aphanidermatum* (Chen *et al*. 2000). Two isolates of *B. pumilus* were reported to be best plant growth promoters and biocontrol agents against downy mildew disease in pearl millet (Niranjan *et al.* 2003). They also reported increased activities of PAL, PO and β-1,3-GLU but not of CHT activity. Geetha *et al*. (2005) quoted that PAL activity was increased in alteration of host phenolic compounds and lignin upon inoculation with the pathogen. Lavania *et al.* (2006) observed enhanced accumulation of phenolics and defense enzymes in betelvine treated with *S.marcescens* NBR11213 and challenge inoculated with *Phytophthora nicotineae.* Chakraborty *et al.* (2006) obtained 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.* Kim *et al*. (2008) selected three chitinolytic bacteria, *Serratia plymuthica* strain C-1, strongly antagonistic to *P. capsici*, *Chromobacterium sp*. strain C-61, strongly antagonistic to *R. solani*, and *Lysobacter enzymogenes* strain C-3, antagonistic to *R. solani* and *Fusarium spp*. to develop a field-effective biocontrol strategy against *Phytophthora* blight of pepper. Mohamed *et al*. (2009) mentioned the potentiality of *Serratia marcescens* and *Pseudomonas fluorescens* as biocontrol agents in controlling root-knot nematodes in Egypt. Fluorescent pseudomonad mixtures were used for disease resistance in rice plants against sheath rot (*Sarocladium oryzae*) disease by Saravanakumar *et al.* (2009). Chakraborty *et al*. (2010) also reported that *Serratia marcescens* (TRS 1) showed antagonism to a number of fungal pathogens *in vitro*. It also reduced brown root rot of tea caused by *Fomes lamaoensis*. Significant increase in phenolics, as well as peroxidise, chitinase, β-1,3-glucanase and phenylalanine ammonia-lyase, were observed in tea plants on application of *S. marcescens* alone or followed by *F. lamaoensis*. Jogaiah *et al.* (2010) reported that two strains of *Pseudomonas* spp., UOM ISR 17 and UOM ISR 23, were capable of protecting pearl millet against downy mildew significantly. *Pseudomonas* UOM ISR 17 and UOM ISR 23 were able to offer 56.3 and 47.5%, respectively against downy mildew disease. Solano *et al.* (2010) isolated siderophore and chitinase producing isolates from the rhizosphere of

Nicotiana glauca which showed induce systemic resistance in *Solanum lycopersicum* L. The six PGPR strains induced systemic resistance against the leaf pathogen *Xanthomonas campestris* in tomato. Five of them effectively reduced disease symptoms (up to 50%). The six strains were identified by 16s rDNA sequencing resulting in 3 *Pseudomonas*, 1 *Bacillus* and 2 *Stenotrophomonas*. Solanki *et al.* (2012) reported the antagonistic potential of *Bacillus* spp. associated to the rhizosphere of tomato for the management of *Rhizoctonia solani*. *Bacillus amyloliquefaciens* MB101 and *Bacillus subtilis* MB14 showed drastic reduction in disease index by 55.7 and 41.74% with significant elevation in fruit yield up to 220 and 184 qha^{-1}, respectively. Antibiotic potential of plant growth promoting rhizobacteria (PGPR)- *Pseudomonas aeruginosa*, *Pseudomonas fluorescens* 4 and *Pseudomonas* sp. against *Sclerotium rolfsii* was reported by Singh *et al.* (2012). *P. aeruginosa* had nine phenolic acids in which ferulic acid (14.52 mg/ml) was maximum followed by other phenolic acids. However, the culture filtrates of *P. fluorescens* 4 had six phenolic acids with maximum ferulic acid (20.54 mg/ml) followed by indole acetic acid (IAA), caffeic, salicylic, o-coumeric acid and cinnamic acids. *Pseudomonas* sp. also showed eight phenolic acids where caffeic acid (2.75 mg) was maximum followed by trace amounts of ferulic, salicylic, IAA, vanillic, cinnamic, o-coumeric and tannic acids. A bio-organic fertilizer (BIO) fortified with an antagonistic strain of *Bacillus subtilis* Y-IVI was used to control *Fusarium* wilt disease in muskmelon. BIO significantly reduced the disease incidence. Population of *F. oxysporum* in plant shoots of the BIO treatment were about 1000-fold lower than the control. Population of Y-IVI remained high in muskmelon rhizosphere of the BIO treatment during the experiment (Zhao *et al.* 2013). Sowndhararajan *et al.* (2013) isolated a total of 316 morphologically different phylloplane bacteria. Among the antagonists, the isolates designated as BMO- 075, BMO-111 and BMO-147 exhibited maximum inhibitory activity against blister blight disease of tea. Foliar application of 36-h-old culture of BMO-111 (1x 10^8 colony-forming units ml^{-1}) significantly reduced the blister blight disease incidence. The isolate BMO-111 was identified as *Ochrobactrum anthropi* based on the morphological and 16S rDNA sequence analyses. The efficacy of *Bacillus subtilis* OTPB1 and *Trichoderma harzianum* OTPB3 were evaluated for *in vitro* antibiosis to *Alternaria solani* and *Phytophthora infestans*, growth stimulation, and induction of systemic resistance in tomato seedlings against early and late blight by Chowdappa *et al.* (2013). Both isolates

inhibited mycelial growth of *A. solani* and *P. infestans* under *in vitro* conditions. OTPB1 and OTPB3 also enhanced systemic resistance in tomato seedlings through induction of growth hormones and defense enzymes. Four bacterial strains of *Bacillus* spp. which were antagonistic to the mango anthracnose pathogen were isolated and screened by Zheng *et al.* (2013). Among them, TB09 and TB72 were identified by 16S rDNA sequence as *Bacillus pumilus* and *Bacillus thuringiensis*, respectively. *In vitro*, the anthracnose fungus showed 88.87% and 80.07% of mycelia growth inhibitions in presence of *B. pumilus* and *B. thuringiensis*, respectively and *in vivo*, the inhibitions of the disease were 94.28% and 87.06%, respectively. *Trichoderma harzianum* Tr6 and *Pseudomonas* sp. Ps14, isolated from the rhizosphere of cucumber, were tested as a single application and in combination for their abilities to elicit induced resistance in cucumber against *Fusarium oxysporum* f. sp. *radicis cucumerinum* and in *A. thaliana* against *Botrytis cinerea* (Alizadeh *et al.* 2013). In Arabidopsis both Ps14 and Tr6 triggered ISR against *B. cinerea* but their combination did not show enhanced effects.

Since both *B. megaterium* and *S. marcescens* applied either as soil drench or foliar spray could promote growth in all the tested plants, it was decided to determine whether these could be applied as suitable formulations in the rhizosphere. This is because, for easy handling of such bacteria, it is necessary to pack such bacteria in inert materials which can also be packaged and stored. Initially, it is essential to determine whether the bacteria can survive in the bioformulations for a reasonable period of time and whether they can induce similar effects to those observed by live bacterial cells. Keeping this in mind, three bioformulations- saw dust, rice husk and tea waste of *S. marcescens* and *B. megaterium* were prepared and initially their survivability was determined. Results revealed that *B. megaterium* could survive in the range of $6.1\times\log_{10}$ cfu/ml in bioformulations of saw dust, rice husk and $6.98\times\log_{10}$ cfu/ml in tea waste respectively where as *S. marcescens* could survive in the range of 7.12, 7.11 and $7.2\times\log_{10}$ cfu/ml in saw dust, rice husk and tea waste formulations respectively up to nine months.

Further, besides aqueous suspensions, the single and joint effects of *S. marcescens* and *B. megaterium* in various bioformulations were also tested on growth promotion of potted tea plants. Observations were recorded after 2 months of application which revealed that significant growth promotion was accorded by

application. The observed increase was similar to that observed by application of aqueous suspensions of the bacteria. Statistical analysis (ANOVA) revealed that there was no significant difference among the aqueous suspension or different bioformulations although all of them were significantly higher than control. The increase in growth in the bacterial bioformulations was not due to any effect of saw dust, rice husk or tea waste, as these are neutral substances with no known plant growth promoting activities. Sharathchandra *et al.* (2004) reported that a bioformulation of *Bacillus* was able to induce plant growth promotion and induce resistance in pearl millet. Bora *et al*. (2004) mentioned that wettable powder formulations of the two strains of *Pseudomonas putida* were used for biological control of *Fusarium oxysporum f. sp. melonis*. Trivedi and Pandey (2005) reported that carrier-based preparations of two plant growth-promoting rhizobacteria (PGPR) viz. *Bacillus subtilis* and *Pseudomonas corrugata*, developed in five formulations were evaluated for their growth promotion, rhizosphere colonization, and viability under storage. The effect of these formulations as fresh preparations, and after 6 months of storage at 4ºC and room temperature, was also determined. The bacterial inoculants in all the formulations were found to enhance the growth parameters of the test plant species; best results were obtained in case of alginate-based formulations. New formulations of *B. subtilis* for management of tomato damping off caused by *Pythium aphanidermatum* were also 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. Previous reports are also available where *Bacillus* bioformulations could survive upto one year or more in several bioformulations (El-Hassan and Gowen 2006). Talc formulations of *Pseudomonas* spp. improved the sugarcane vegetative germination and sugarcane growth under field conditions. Optimal talc formulations were assessed for their effect on induction of systemic resistance against the pathogen in the canes under artificial inoculation. All the four isolates CHAO, EP1, KKM1 and VPT4 were effective in inducing systemic resistance against *C. falcatum* in two seasons. The bacterial formulations were also assessed to induce resistance in sugarcane in a sick plot situation (Viswanathan and Samiyappan 2008). Plant growth promotion was noted and was found to be at par with the live cells, showing no significant difference. Biochemical changes in terms

of activities of defense enzymes and phenolics in tea leaves of five different varieties were also noticed following the application of aqueous suspensions as well as different bioformulations of *S. marcescens* and *B. megaterium*. There was significant increase in activities of POX, CHT, PAL, β-1,3 GLU and phenolics following the treatment of the rhizosphere with various bioformulations of bacteria. In an earlier study, Chakraborty *et al*. (2009) reported that talc formulation of *Ochrobactrum anthopi* was as effective as aqueous suspensions in both plant growth promotion and disease suppression. Mejri *et al.* (2012) developed a semolinakaolin granular (Pesta) and talc-kaolin powder formulation of *Pseudomonas trivialis* X33d. The formulation in Pesta increased the growth of wheat and reduced brome growth. This formulation could be used for biocontrol of brome (*Bromus diandrus*) in durum wheat. A carrier based PGPR consortium with four selected strains *viz*., *Azospirillum lipoferum* VAZS-18, *Azotobacter chroococcum* VAZB-6, *Bacillus megaterium* VBA-2 and *Pseudomonas fluorescens* VPS-19 was prepared and the shelf life for each inoculant was studied up to six months of storage by Sangeetha and Stella (2012). The surviving population in the lignite based consortium was 1.64×10^{8} cfu g^{-1} for *Azospirillum lipoferum* VAZS-18, 1.46×10^{8} cfu g^{-1} for *Azotobacter chrococcum* VAZB-6, 1.22×10^{8} cfu g^{-1} for *Bacillus megaterium* VBA-2 and 2.01×10^{8} cfu g^{-1} for *Pseudomonas fluorescens* VPS-19 after six month of storage. The population in vermiculite based consortium was 4.32×10^{8} cfu g^{-1} for *Azospirillum lipoferum* VAZS-18, 1.98×10^{8} cfu g^{-1} for *Azotobacter chroococcum* VAZB-6, 1.14×10^{8} cfu g^{-1} for *Bacillus megaterium* VBA-2 and 3.32×10^{8} cfu g^{-1} for *Pseudomonas fluorescens* VPS-19 after six months of storage. In the alginate bead based consortium, *Azospirillum lipoferum* VAZS-18 survived at the rate of 64.61×10^{8} cfu g^{-1}, *Azotobacter chroococcum* VAZB-65 at 6.81×10^{8} cfu g^{-1}, *Bacillus megaterium* VBA-2 at 47.83×10^{8} cfu g^{-1} and *Pseudomonas fluorescens* VPS-19 at 63.89×10^{8} cfu g^{-1} after six months of storage. In the pressmud based consortium, the population was 3.25×10^{8} cfu g^{-1} for *Azospirillum lipoferum* VAZS-18, 3.00×10^{8} cfu g^{-1} for *Azotobacter chroococcum* VAZB-6, 2.14×10^{8} cfu g^{-1} for *Bacillus megaterium* VBA-2 and 3.42×10^{8} cfu g^{-1} for *Pseudomonas fluorescens* VPS-19 after six months of storage.

In the present study, it was felt necessary to determine the sustainability of *B. megaterium* and *S. marcescens* in the soil as this would be important in the field. Hence the survival of the bacteria applied as aqueous solution in the soil was

determined. Determination of bacterial survival in soil was done by immunological techniques using antibodies raised against the two bacteria. These techniques i.e. ELISA, Dot blot and Colony blot transfer 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 both bacteria could successfully survive and multiply in the tea rhizosphere of all five varieties even after six months of inoculation. Maximum A_{405} values were obtained from the soils collected soon after application of bacteria. The values reduced to some extent with time, though even after six months, these were still detectable at fairly high concentrations. Besides immunological techniques, antibiotic sensitivity tests of *B. megaterium* and *S. marcescens* were also performed keeping in mind that it would help in determining the sustainability of the applied bacteria in the field.

Results revealed that *B. megaterium* was resistant to 450µg/ml concentration of rifampicin and ampicillin (A^2, A^{10} and A^{25} mcg/disc) where as *S. marcescens* was found to be resistant to Mt^5, C^{10} and C^{25}. Results revealed that *B. megaterium* could successfully survive and multiply in tea rhizosphere even after three months of application. Populations of *S. rolfsii* were determined in the soil using dot using PAbs raised against *S.rolfsii* the causal agent of sclerotial blight of tea. It was shown that the population of the pathogen reduced significantly in *B. megaterium* and *S. marcescens* 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. Immunofluorescence technique was also used for immunodetection of chitinase in tea leaves. *B. megaterium* and *S. marcescens* treated along with control tea leaves were treated with chitinase antibody and FITC labeled Con A. Strong apple green fluorescence were evident in treated leaves. Western blotting using antibodies of chitinase revealed that in *B. megaterium* and *S. marcescens* treated tea leaves chitinase was expressed over control leaves. Dot-blot was also used for detection of expression of chitinase. Violet coloured permanent dots were visible in *B. megaterium* and *S. marcescens* treated samples whereas the samples from control leaves showed less intensity in colour indicating that the chitinase was expressed more in treatment over control.

The overall results of the present study have shown that two rhizobacteria isolated from tea rhizosphere, *B. megaterium* (TRS-7) and *S. marcescens* (TRS-1)

have shown good potential as a plant growth promoter by its ability to increase growth of tea plants in experimental plot, potted conditions as well as in commercial tea plantation with the reduction of sclerotial blight disease of tea. Although foliar spray of bacterial suspension alone induces growth and emergence of new leaves but still insect attack is a common problem in tea plantation that damages that newly emerged leaves. Hence combined application of bacterial and insecticide (low dose) spray was tried in order to control insect attack as well as to promote growth of plants. This integrated approach gave good results. Though both soil drench and foliar spray gave experimentally good result, soil drench is preferable mode of application. This is because tea being cultivated for its beverage produced from its leaves and soil drench induced systemic response transmitted to the leaves, foliar treatment of leaves can be avoided. Increase in growth was associated with phosphate solubilisation, increase in activities of phosphatases, defense enzymes as well as increased accumulation of phenolics, chlorophyll and catechins. All the elements commonly known to be involved in the induced systemic resistance have been enhanced. Regarding the mechanism of action of the bacteria it seems probable that these bacteria act through a combination of methods. It is difficult to predict the actual happening in the soil environment but probably the PGPR secrete metabolites into the soil which in turn elicit responses in the host. The relative importance of the metabolites in inducing plant growth promotion, as well as disease suppression is not yet clear. Application of both bacteria in bioformulations was equally effective as aqueous suspensions. ELISA values and intensity of dots proved that both bacteria could survive and multiply in the rhizosphere even after 6 months of application. *B. megaterium* (TRS-7) showed better response as a plant growth promoter and biocontrol agent in comparison to *S marcescens* (TRS 1). Though both bacteria could survive in various bioformulations but use of *B. megaterium* in bioformulation product will be preferable than *S. marcescens* due to formation of endospores that give longer viability of *Bacillus* than other bacteria.

Thus *Bacillus megaterium* (TRS 7) could be used in suitable formulations commercially which would benefit the tea industry where use of biological products to replace or supplement chemical use is the need of the hour.

CONCLUSION

In order to effectively reduce the excessive use of chemicals in agriculture, currently, much emphasis is being laid on use of eco friendly biological materials for use in sustainable agriculture. One of the worst affected crops is tea, where, in order to overcome any loss of productivity, fungicides and insecticides, along with chemical fertilizers have been regularly applied. Tea plantations of North Bengal region, including Darjeeling hills have been facing this problem and keeping this in mind, the present study has been undertaken. The major aim of this study was to study whether rhizobacteria, isolated from tea rhizosphere, could be used for increasing crop productivity and determine their mechanism of action. Two bacteria, identified as *Bacillus megaterium* (TRS 7) and *Serratia marcescens* (TRS 1) were used throughout this study. The major conclusions drawn from this study have been enumerated below:

- ❖ The two selected bacteria were morphologically, biochemically characterized and finally identified as *Bacillus megaterium* (TRS 7) and *Serratia marcescens* (TRS 1). The sequences were deposited in NCBI, GenBank database under the accession Nos. JX 312687.1 and JN020963.1 for *B.megaterium* and *S. marcescens* respectively. Genetic diversity among the isolates of *B. megaterium* (JX 312687, MTCC 428, MTCC 1684 and MTCC 2949) and *S. marcescens* (JN 020963, MTCC 86, MTCC 97, MTCC 2645, MTCC 3124, MTCC 4301, MTCC 4822 and MTCC 7298) were analyzed on the basis of DGGE analysis of 16 S rDNA sequences. The identity of the isolates of *B. megaterium* (JX312687) and *S. marcescens* (JN 020963) were further confirmed by DGGE.
- ❖ Both the bacteria were tested against pathogens- *Sclerotium rolfsii, Rhizoctonia solani, Fusarium graminearum and F. oxysporum* for determining antagonistic activity. All the tested fungi were inhibited to some degree, the percentage inhibition varying between 57-84% by *B. megaterium* and 27-75% by *S. marcescens*. Sclerotial germination of *S. rolfsii* with cell free culture filtrates of *B. megaterium* and *S. marcescens* showed about 90-95% inhibition in comparison to control.
- ❖ These two bacteria were characterized *in vitro* for their plant growth promoting activities. Both the bacteria were able to produce IAA, volatiles, siderophores and

solubilised phosphates *in vitro* but did not produce HCN and *B. megaterium* was non- chitinase producing strain. *B. megaterium* and *S. marcescens* grew best at pH 6.0 with log cfu values of 22.8 log cfu/ml and 23.07 log cfu/ml. Both the bacteria grew best at around 35ºC.

- The plant growth promoting abilities of *B.megaterium* and *S. marcescens* were evaluated in field, pot and tea estates using bacterization methods such as soil drenching in five tea varieties- TV-18, TV-23, TV-25, TV-26 and T-17. The bacteria were applied singly or in dual combination.
- The growth promotion of five varieties of tea plants in field and potted conditions and T-17/154 in Hansqua Tea Estate field was observed in terms of growth parameters such as increase in height, leaf number, number of branches and dry mass of leaves. It was observed that in relation to control, tea plants subjected to single or dual application showed increased growth. Both *B.megaterium* and *S. marcescens* significantly promoted the growth of the plants. All the eight isolates of *S. marcescens* (MTCC 86, MTCC 97, MTCC 3124, MTCC 4301, MTCC 7298, MTCC 2645, MTCC 4822 and MTCC 7103) along with *S. marcescens* (TRS 1) showed significant increase in height and leaf numbers in potted tea plants but *S. marcescens* (TRS 1) comparatively promoted better plant growth in comparison to other isolates.
- Since insecticides or fungicides are applied in tea gardens, the ability of the bacteria to grow in their presence was also investigated. *B. megaterium* could tolerate acephate concentration of 1250mg/ml, confidor, ethion 50EC ,calixin concentrations of 1000 µl/ml and contaf 5E conc. of 500 µl/ml; whereas *S. marcescens* could tolerate acephate concentration of 625mg/ml, confidor, ethion 50EC ,calixin concentrations of 250, 1000 and 500 µl/ml and contaf 5E concentrations of 125 µl/ml.
- *In vivo* phosphate solubilizing abilities of *S.marcescens* and *B. megaterium* were confirmed. Soil P content decreased following application of both bacteria, while root and leaf phosphate contents showed a significant increase both in treated tea plants in potted as well as field conditions in comparison to control. Maximum phosphate content was obtained in the leaf tissues. Acid and alkaline phosphatase activities in rhizosphere soil of all five varieties were enhanced following application of bacteria.

- In addition to growth promotion, biochemical changes such as enzyme activities (Chitinase, Phenyl alanine ammonia lyase, Peroxidase and β-1,3 Glucanase), protein, phenol and chlorophyll content associated with PGPR application were also studied. Higher activities of CHT, PO, PAL, β-1,3 GLU and accumulation of higher phenolic compound in *S.marcescens* and *B. megaterium* treated tea varieties were observed.
- Native PAGE analysis of peroxidase revealed that all the isozymes were constitutively present in untreated control plants and in treated plants. However, maximum intensities of bands were noticed in the treated plants with Rm (Relative mobility) values of 0.883, 0.58, 0.348 in T-17 and 0.85, 0.574, 0.404, 0.255 in TV-25 varieties. Soluble proteins extracted from control and PGPR treated tea plants were analysed by SDS-PAGE. No significant changes in either protein content or protein profile was observed by treatments with the two bacteria; however, the level of expressions of some proteins were higher in bacteria treated plants.
- HPLC analysis was performed with the catechins extracted from tea leaves treated with *S.marcescens* and *B. megaterium*. New isoforms and increase of isomers were observed in *B. megaterium* treated plants where as few were lost or there was suppression of few isomers by the treatment of *S. marcescens*. Similar trends were observed in TV26, TV18, TV25 and T17. But in T-17/154 some new isomers were enhanced by the treatment of *S. marcescens*.
- *B. megaterium* reduced sclerotial blight more significantly in comparison to *S. marcscens*. Disease development was significantly reduced in PGPR treated plants compared to control plants.
- The biochemical response of plants such as defense related enzyme activities (PAL, CHT, PO and β-1,3 GLU) and phenols were determined where *B. megaterium* or *S.marcescens* as well as *Sclerotium rolfsii* were inoculated in the soil. Increase in the activity of various defense related enzymes indicated the induction of systemic resistance against pathogen infection. Isozyme analysis of peroxidase showed that maximum intensity of bands was noticed in the TV-26 variety after the treatment by *S. rolfsii* (7 bands with Rm values of 0.93, 0.79, 0.726, 0.562, 0.520, 0.452 and 0.383) in comparison to control (6 bands with Rm values of 0.79, 0.726, 0.562, 0.520, 0.452 and 0.383).

- Three bioformulations- saw dust, rice husk and tea waste of *S. marcescens* and *B. megaterium* were prepared and initially their survivability was determined. *B. megaterium* could survive in the range of $6.1\times\log_{10}$ cfu/ml in bioformulations of saw dust, rice husk and $6.98\times\log_{10}$ cfu/ml in tea waste respectively where as *S. marcescens* could survive in the range of 7.12, 7.11 and $7.2\times\log_{10}$ cfu/ml in saw dust, rice husk and tea waste formulations respectively up to nine months. The single and joint effects of *S. marcescens* and *B. megaterium* in three bioformulations were also tested on growth promotion of potted tea plants. All treatments with formulation showed enhancement in growth over untreated controls.

- Biochemical changes in terms of activities of defense enzymes and phenolics in tea leaves of five different varieties were noticed following the application of different bioformulations of *S. marcescens* and *B. megaterium.* Significant increase in activities of PO and CHT was observed. β-1,3-glucanase and phenyl alanine ammonia lyase activities and phenolic contents increased due to single and joint application of bacteria as aqueous suspensions or formulations of saw dust, rice husk and tea waste.

- Polyclonal antibodies (PAbs) were raised against antigens prepared from *B. megaterium* and *S. marcescens*. The IgG obtained in each case was used for immunodiffusion, Dot-blot, Western blot and ELISA.
- Agar gel double diffusion tests were performed using crude antibody. Strong precipitins were obtained in both the bacteria.
- The bacterial sustainability in the soil was evaluated by ELISA and Dot blot. This was confirmed by colony blot transfer. *B. megaterium* and *S. marcescens* could successfully survive and multiply in tea rhizosphere even after three months of application.
- Western blot analysis was performed by *B. megaterium* and *S. marcescens* antigens against 1st to 4th bleeds. Differences in band intensities and expression of bands were observed more in homologous antigens of both bacteria when probed with 3rd and 4th bleeds in comparison to 1st and 2nd bleeds. Western blotting using PAb of *B. megaterium* revealed that the homologous antigens showed 9 bands ranging from 14.3 to 97 kDa in 4th bleed, 8 bands ranging from 14.3 to 68 KDa in 3rd bleed, 5 bands in 2nd bleed (14.3 to 97 kDa) and (16 to 97) KDa for 1st bleed.

Similarly, Western blotting using PAb of *S. marcescens* revealed that the homologous antigens showed 5 bands ranging from 15 to 97 kDa in 4th bleed, 5 bands ranging from 15 to 52 KDa in 3rd bleed, 3 bands in 2nd bleed (20 to 52 kDa) and 4 bands (15 to 52) KDa for 1st bleed.

- Besides immunological techniques, antibiotic sensitivity tests of *B.megaterium* and *S. marcescens* were also performed. *B. megaterium* was resistant to 450µg/ml concentration of rifampicin and ampicillin (A^2, A^{10} and A^{25} mcg/disc) where as *S. marcescens* was found to be resistant to Mt^5, C^{10} and C^{25}.
- Populations of *S. rolfsii* were determined in the soil by using PAbs raised against *S.rolfsii*. It was shown that the population of the pathogen reduced significantly in *B. megaterium* and *S. marcescens* treated soil, as detected on the analysis by ELISA and Dot blot.
- Immunofluorescence technique was also used for immunodetection of chitinase in tea leaves. *B. megaterium* and *S. marcescens* treated along with control tea leaves were treated with chitinase antibody and FITC labeled Con A. Strong apple green fluorescence were evident in treated leaves.
- Western blotting using antibodies of chitinase revealed that in *B. megaterium* and *S. marcescens* treated tea leaves chitinase was expressed over control leaves. Intensity of bands was higher and total three bands with molecular weights of 15,20 and 29 KDa in TV-25, 14.3, 15 and 29 KDa in T-17 varieties were appeared in *B. megaterium* and *S. marcescens* treated leaves in comparison to control.
- Dot-blot was also used for detection of expression of chitinase. Violet coloured permanent dots were visible in *B. megaterium* and *S. marcescens* treated samples whereas the samples from control leaves showed less intensity in colour indicating that the chitinase was expressed more in treatment over control.
- Statistical analyses- ANOVA, t-test were performed.
- *B.megaterium* (TRS-7) showed better response as a plant growth promoter and biocontrol agent in comparison to *S marcescens* (TRS 1). Though both bacteria could survive in various bioformulations but use of *B. megaterium* in bioformulation product will be preferable than *S. marcescens* due to formation of endospores that give longer viability of *Bacillus* than other bacteria.
- Thus *Bacillus megaterium* (TRS 7) could be used in suitable formulations commercially which would benefit the tea industry where use of biological products to replace or supplement chemical use is the need of the hour.

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APPENDICES

APPENDIX A: List of thesis related publications

In Journals:

1. Chakraborty U, Chakraborty BN, Chakraborty AP, Sunar K and Dey PL. Plant growth promoting rhizobacteria mediated improvement of health status of tea plants. *Ind. J. Biotechnol.* **12:** 20-31, 2013.
2. Chakraborty U, Chakraborty BN and Chakraborty AP. Induction of plant growth promotion in *Camellia sinensis* by *Bacillus megaterium* and its bioformulations. *World. J. Agr. Sci*. **8(1):** 104-112, 2012.
3. Chakraborty U, Chakraborty BN and Chakraborty AP. Evaluation of *Bacillus megaterium* and *Serratia marcescens* and their bioformulations for promoting growth of *Camellia sinensis*. *Int. J. Tea. Sci.* **8(1):** 69-80, 2012.
4. Chakraborty AP, Chakraborty BN and Chakraborty U. Protection of tea plants against pest and pathogen through combined application of pesticide and plant growth promoting rhizobacterium. *J. Mycol. Pl. Pathol.* **40(4):** 519-531, 2010.
5. Chakraborty U, Chakraborty BN and Chakraborty AP. Influence of *Serratia marcescens* TRS-1 on growth promotion and induction of resistance in *Camellia sinensis* against *Fomes lamaoensis*. *J. Plant. Interact*. **5(4):** 261-272, 2010.

In Proceeding volume:

Chakraborty AP, Chakraborty BN and Chakraborty U. Application of bioformulations of PGPRs for plant growth promotion in tea seedlings. *Microbial Resources for Crop Improvement* (Eds. Bishwanath Chakraborty and Usha Chakraborty), Satish Publishing House, 101-110, 2013.

APPENDIX B: List of Abbreviations

ACC- 1-amino-cyclopropane-1-carboxylic acid hydrochloride
APS- Ammonium per sulphate
BLAST- Basic local alignment search tool
BSA- Bovine serum albumin
BSS-2- Biclonal seed stock- 2
$Ca_3(PO_4)_2$ - Tri-calcium phosphate
$CaHPO_4$ - Calcium phosphate
CAS- Chrome azurol S
CAT- Catalase
CDA- Chitinase detection agar
cfu- Colony forming unit
CHT- Chitinase
CMC- Carboxy methyl cellulose
$CuSO_4$ - Copper sulphate
DAC-ELISA- Direct antigen coating- Enzyme linked immune-sorbent assay
DAPG- 2,4-Diacetylphloroglucinol
dATP- Deoxy adenosine tri-phosphate
dCTP- Deoxy cytosine tri-phosphate
DEAE cellulose- diethyl aminoethyl cellulose
DGGE- Denaturing Gradient Gel Electrophoresis
dGTP- Deoxy guanosine tri-phosphate
DMAB- Di methyl amino benzaldehyde
DNA- Deoxyribonucleic acid
dNTPs- Deoxy nucleotide tri-phosphates
DNSA- Dinitro salicylic acid
dTTP- Deoxy thymidine tri- phosphate
EDTA- Ethylene diamine tetra acetic acid
ELISA- Enzyme linked immune- sorbent assay
ER- Root endosphere
$FeCl_3$- Ferric chloride
FITC- Fluorescein isothiocyanate
g- gram
µl- Micro litre
Gfp- Green fluorescent protein
GlcNAc- N-acetyl glucosamine
H_2O_2 - Hydrogen peroxide
H_2So_4- Sulphuric acid
HCl- Hydrochloric acid
HCN- Hydrocyanic acid
HDTMA- Hexa-decytrimethyl ammonium bromide
HPLC- High performance liquid chromatography
IAA- Indole acetic acid
ISR- Induced systemic resistance
KCl- Potassium chloride
KH_2PO_4 – Potassium dihydrogen phosphate
MEGA 4- Molecular Evolutionary Genetics Analysis 4
mg- Mili gram

$MgCl_2$- Magnesium chloride
ml- Mili litre
Na_2CO_3- Sodium carbonate
Na_2HPO_4- Di sodium hydrogen phosphate
Na_2MoO_4 - Sodium molybdate
NaCl- Sodium chloride
NaN_3- Sodium azide
$NaNO_2$- Sodium nitrite
NaOH- Sodium Hydroxide
NB- Nutrient Broth
NBT/BCIP substrate- Nitro blue tetrazolium/ (5-bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate substrate
NCBI- National Center for Biotechnology Information
NCM- Nitrocellulose membrane
NH_4Cl- Ammonium chloride
PAL- Phenylalanine ammonia lyase
PBS-Tween- Phosphate buffer saline- Tween
PCA- Phenazine-1-carboxylic acid
PCI- Water saturated phenol: Chloroform: Isoamyl alcohol
PCR- Polymerase chain reaction
PCR–RFLP- Polymerase chain reaction- Restriction fragment length polymorphism
PGPR- Plant Growth Promoting Rhizobacteria
PGPTs- Plant growth promoting traits
pNPP- p- nitrophenyl phosphate
POX- Peroxidase
PPO- Polyphenol oxidase
PR proteins- Pathogenesis related proteins
PRN- Pyrrolnitrin
PSB- Phosphate solubilising bacteria
PVK- Pikovskaya's agar
PVP- Poly vinyl- pyrollidone
RAPD- Random amplified polymorphic DNA
RNA- Ribonucleic acid
RP- Rock phosphate
RS- Rhizosphere soil
SA- Salicylic acid
SAR- Systemic acquired resistance
SDS- Sodium dodecyl sulphate
SDS-PAGE- Sodium dodecyl sulphate- Poly-acrylamide gel electrophoresis
SKM- Skim milk agar
T-17- Teenali-17
TAE buffer- Tris Acetic Acid and EDTA buffer
TE buffer- Tris-EDTA buffer
TEMED- N,N,N',N'-Tetramethylethylenediamine
Tris Hcl- Tris hydrochloric acid
TV- Toklai variety
UP- UPASI variety
UPGMA- Unweighted Pair Group Method with Arithmetic Mean
yr- Year
β-1,3-GLU- β-1,3 glucanase

APPENDIX C: List of Chemicals

Ammonium chloride
Ammonium per sulphate
Bovine serum albumin
Calcium phosphate
Carboxy methyl cellulose
Chrome azurol S
Colloidal chitin
Copper sulphate
Deoxy nucleotide tri-phosphates
Di methyl amino benzaldehyde
Di sodium hydrogen phosphate
Diethyl aminoethyl cellulose
Dinitro salicylic acid
Ethylene diamine tetra acetic acid
Ferric chloride
Fluorescein isothiocyanate
Helicase (3%)
Hexa-decytrimethyl ammonium bromide
Hydrochloric acid
Hydrocyanic acid
Hydrogen peroxide
Indole acetic acid
Magnesium chloride
N,N,N',N'-Tetramethylethylenediamine
N-acetyl glucosamine
Nitro blue tetrazolium/ (5-bromo-4-chloro-1H-indol-3-yl) dihydrogen phosphate substrate
O-dianisidine (5 mg/ml methanol)
p- nitrophenyl phosphate
Phosphate buffer saline- Tween
Poly vinyl- pyrollidone
Potassium chloride
Potassium dihydrogen phosphate
Sodium azide
Sodium carbonate
Sodium chloride
Sodium dodecyl sulphate
Sodium Hydroxide
Sodium molybdate
Sodium nitrite
Sulphuric acid
Tri-calcium phosphate
Tris Acetic Acid and EDTA buffer
Tris hydrochloric acid
Tris-EDTA buffer
Water saturated phenol: Chloroform: Isoamyl alcohol
0.05(M) sodium phosphate buffer (pH 6.8)
0.1(M) sodium acetate buffer (pH 5.0)

0.2M Na-phosphate buffer (pH 5.4)
0.3mM borate buffer (pH 8.0)
1 M Potassium phosphate buffer (pH 7.1)
1-amino-cyclopropane-1-carboxylic acid hydrochloride
1M Na-acetate buffer (pH 4)
Sodium borate buffer (pH 8.8)
2 mM β- mercaptoethanol
1 M Na-borate buffer (pH 9.8)
2% L-phenylalanine
2,4-Diacetylphloroglucinol
4 mM H_2O_2.
4% laminarin