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Resistance in tea plants against root rot pathogens induced by arbuscular mycorrhizal fungi and plant growth promoters

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Tea, the most important plantation crop of North Bengal and Assam is valued for the beverage obtained from it and also the foreign exchange it brings in. Tea plant (*Camellia sinensis*), being a perennial harbors a number of microorganisms in its rhizosphere, both beneficial and harmful, some of which are responsible for causing a number of root rot diseases. The present study was undertaken to explore the potential of beneficial microorganisms from the rhizosphere of tea for growth improvement and biological control of diseases. The selected microorganisms which showed positive PGPR traits *in vitro* such as phosphate solubilization, siderophore production, antagonism to pathogens and IAA production were - *Bacillus amyloliquefaciens*, *B. pumilus, B. megaterium* and *Ochrobactrum anthropi*. The16S rDNA sequencing of the bacteria was done and their phylogenetic relationships determined. *Glomus mosseae* and *G. fasciculatum,* which were the dominant arbuscular mycorrhizal fungi (AMF) colonizing tea roots were selected for mass multiplication and application in nursery grown plants. Under *in vivo* conditions, the PGPR and AMF, applied either singly, or jointly, enhanced seedling growth of tea varieties in the nursery as well as in the field. Biocontrol of root diseases of tea caused by *Sclerotium rolfsii, Phellinus noxius* and *Poria hypobrunnea* was achieved by application of PGPR and AMF. Sustainability of the applied bacteria in soil was tested by PTA-ELISA and Dot immunobinding. Localization of AMF hyphae in root cells was determined by indirect immunofluorescence. Application of the PGPR and AMF led to enhancement in activities of defense related enzymes- phenyl alanine ammonia lyase, peroxidase, chitinase and β-1,3 glucanase in tea leaves. Total phenols also increased quantitatively along with increase in isoforms of catechins. It is evident from the results of the present study that application of PGPR and AMF in the soil lead to biopriming of the plants through growth promotion, induced systemic resistance and other mechanisms

Key words: Tea, AMF, PGPR, root diseases, defense enzymes

INTRODUCTION

Tea (*Camellia sinensis* (L.) O. Kuntze) is one of the most important plantation crops in Darjeeling and Dooars regions of West Bengal, which are the tea growing regions of West Bengal. The commercial value of the tea is due to its leaves, and more specially the tender leaves which are processed to produce the beverage yielding tea. Over the years, due to excessive use of chemicals, either as fertilizers or pesticides, productivity of the plant

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is decreasing. In tea plantations, with the reduction in the permissible levels of chemicals which can be used, there is urgent need for using ecofriendly biofertilizers. The best source of such fertilizers would be the beneficial microbes already present in the rhizosphere. For this it is necessary to identify and select microbes which have the potential to control diseases and increase productivity also. Among the beneficial microorganisms, two groups are very important agriculturally- plant growth promoting rhizobacteria (PGPR) and the mycorrhizal fungi.

Plant growth promoting rhizobacteria (PGPR) include those bacteria that are able to aggressively colonize plant roots and stimulate plant growth when applied to roots, tubers and seeds. PGPR have been reported to directly enhance plant growth by a variety of mechanisms: fixation of atmospheric nitrogen that is transferred to the plant, production of siderophores that chelate iron and make it available to the plant root, solubilization of minerals such as phosphorus, and synthesis of phytohormones. 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. Some biocontrol PGPR strains protect plants by activating gene encoding defense enzymes- peroxidase,chitinase, phenylalanine-ammonia-lyase, β-1,3- glucanase and others, involved in synthesis of phytoalexin (M'Piga *et al*, 1997). Though different bacterial species have been investigated as biological control agents, the knowledge concerning the behaviour of these bacterial strains as antagonists and genetic analysis is essential for their effective use and the commercialization. Mycorrhizal fungi, which are obligate symbionts, colonize plant roots and extend the root system into the surrounding soil. The spore count, root colonization, species diversity and dominant species, vary with the region and soil nutrient conditions. The relationship is beneficial because the plant enjoys improved nutrient and water uptake, disease resistance and superior survival and growth. AM fungal association protects the plants from soil-borne diseases, detoxifies soil contaminants of certain metals. The dual activity of PGPR and AMF is generating ample evidence that their combined effects on plant growth and disease suppression surpass the individual effect (Akhtar and Siddiqui, 2008). In most of these reports, AM fungi, specially species of *Glomus* were used along with different PGPR, for disease suppression and growth enhancement of different crops.

Root rot diseases are of common occurrence in tea plants and by the time the above ground symptoms appear, most of the damage is already done. Use of fungicides for treatment is also difficult due to the perennial nature of the plants and the toxic effects of chemicals. The most suitable treatment would be exploitation of the rhizosphere for better efficient use of the beneficial microbes.

Considering the potential benefits of utilizing rhizosphere microorganisms for sustainable agriculture, the present study has been undertaken with an objective to determine whether a combination of microorganisms originally isolated from the tea rhizosphere – *Glomus fasciculatum*, *Glomus mosseae*, *Bacillus amyloliquoefaciens, B. pumilus, B. megaterium* and *Ochrobactrum anthropi* influence the growth of tea plants and induction of resistance in the host.

MATERIALS AND METHODS

Plant material

In the plains, tea plants grow best at a temperature of 28°–30°C, with a slightly acidic soil (pH 5) having an annual rain-fall of 2500 mm. Different varieties of tea (TV-18, TV-23, TV-25, TV-26, TV-29,TV-20,T-17,AV-2, T-78, UP-3, UP-26, S-449, CP-1, K1/1 and HV39) were selected for experimental purposes. The selected tea seedlings were maintained in 30 cm earthenware pots in nursery and also in the experimental field. Tea seedlings were watered regularly for proper maintenance.

Isolation of microorganisms

Initially several microorganisms were isolated from different rhizospheric soil of tea gardens and screened for *in vitro* PGPR activities. Four bacterial strains showed positive responses in *in vitro* PGPR tests. They were also preliminarily identified on the basis of morphological, microscopic and biochemical characterization and finally identity of the four strains were confirmed from the Plant Diagnostic and Identification Services, UK and also by 16S rDNA sequencing. The four strains were - *Bacillus amyloliquefaciens*, *Bacillus megaterium,*

Bacillus pumilus and *Ochrobactrum anthropi*.

Spores of arbuscular mycorrhizal fungi were isolated from rhizosphere soil of six different varieties of tea by wet sieving and decanting method and purified. Following purification, spores of *G. fasciculatum* and *G. mosseae* were used for inoculation of 7-10 day- old roots of maize plants grown in black plastic pots (30 cm) having autoclaved soil. After 45 days the presence of spores of *G. fasciculatum* and *G. mosseae* were verified and inocula were prepared by mixing the chopped roots of maize plants with the potted soil where extra radical spores of *G. fasciculatum* and *G. mosseae* were present. Approximately > 175 spores per 100 g were considered as potent inocula for application.

Molecular characterization and identification

Isolation of genomic DNA and amplification of 16S rDNA by PCR

Genomic DNA was extracted from 24 h old culture following the method of Stafford *et al*, (2005) with modifications, was quantified spectrophotometrically and the quality analyzed in 0.8% agarose gel.

For ITS-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 mMdNTPs, 1.5 μl of 100 mM reverse and forward primers and 3.5 μl of 50 ng template DNA. For amplification of the ITS region of theisolates, the primer pair-Forward Primer: 5'-AGAGTRTGATCMTYGCTWAC-3' and Reverse Primer: 5'-CGYTAMCTTWTTACGRCT-3' PCR was programmed with an initial denaturing at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 30 sec, annealing at 61°C for 30 sec and extension at 70°C for 2 min and the final extension at 72°C for 7 min in a Primus 96 advanced gradient Thermocycler. PCR product (20 μl) was mixed with loading buffer (8 μl) 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 Genie, Bangalore, India.

16 S rDNA sequence analysis

The sequenced PCR product was aligned with ex-type isolates sequences from NCBI GenBank for identification as well as for studying phylogenetic relationship. The evolutionary history was inferred using the UPGMA method. The evolutionary distances were computed using the Maximum Composite Likelihood method 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). Phylogenetic analyses were conducted in MEGA-4(Tamura *et al,* 2007). 16S rDNA of all the bacterial isolates were aligned to study the range of homology present in the conserved regions following the Clustal W algorithm (Thomson *et al,* 1994) using the Bioinformatic tool Bio Edit.

Evaluation of PGPR and antagonistic activities of bacterial isolates in vitro

Phosphate solubilization

Primary phosphate solubilizing activities of all three isolates were carried out by allowing the bacteria to grow in selective medium i.e., Pikovskaya s agar (Pikovskaya, 1948). The appearance of transparent halo zone around the bacterial colony indicated the phosphate solubilizing activity of the bacterium.

Siderophore production

Production of siderophore was detected by standard method using blue indicator chrome azurol S.

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 indole acetic acid (IAA) production by the bacteria. Production of IAA in culture supernatant was assayed by Pillet-Chollet method (Dobbelaere *et al, 1999).*

Antagonism

In vitro antagonism of bacteria to fungal pathogens- *Sclerotium rolfsii, Poria hypobrunnea* and *Phellinus noxius* obtained from the culture collection of Immuno-Phytopathology Laboratory, De-

partment of Botany, University of North Bengal, were also determined by paired culture as described by Chakraborty *et al,* (2006).

Inocula preparation of bioinoculants and in vivo application

For soil application of bacteria, bacteria were grown in Nutrient Broth medium (Himedia, M002) and aqueous suspensions were prepared of cells at the end of log phase of growth, which were then diluted as necessary to maintain the bacterial concentration at 10^8c.f.u/ml. The aqueous suspensions were then applied as a soil drench, at the rate of 100 ml per plant to the rhizosphere of tea plants 1 month after transplantation. Application was done at an interval of 1month and three applications were done. Tea roots were inoculated with *G. fasciculatum* and *G. mosseae* alone and in combination with bacteria which were applied as soil drench.

Growth promotion was studied in terms of increase in number of leaves, their biomass and number of shoots. Plants were grown under natural conditions of light and temperature (30±2°C). For each treatment 10 replicates were taken and average of the 10 replicate plants were analysed.

Inoculation of plants with fungal pathogen(s) and disease assessment

For disease assessment, cultures of *Sclerotium rolfsii*, *Phellinus noxius* and *Poria hypobrunnea* were grown separately in sand-maize meal medium (maize meal: sand: water- 1:9:1.5 w:w:v) in autoclavable plastic bags (sterilized at 121°C. pressure for 20 min) for a period of 3 weeks at 28°C until the mycelia completely covered the substrate. Rhizosphere of tea plants were inoculated by adding 100 g of previously prepared inoculum of the fungi to the rhizosphere soil. Inoculation was done 3 days after final application of *G. fasciculatum* and *B. amyloliquefaciens*, singly or jointly. *Phellinus noxius* and *Poria hypobrynnea* were also grown. Disease assessment was performed following the method of Chakraborty *et al,* (2006) after 15, 30 and 45 days of inoculation.

Biochemical analyses

All the biochemical analyses were performed from treated as well as control tea leaves after 72 hrs of treatment.

Enzyme assays

Peroxidase (POX, EC1.11.1.7.), Chitinase (CHT, EC 3.2.1.14) ,Phenyl alanine ammonia lyase (PAL, EC 4.3.1.5) and β- 1,3- glucanase (β-GLU, EC 3.2.1.39) were extracted and assayed following methods described by Chakraborty *et al,* (1993), Boller and Mauch (1998), Bhattacharya and Ward (1987) and Pan *et al,* (1991) respectively.

Phenolics

Phenols were extracted and estimated from leaf samples by the method of Mahadevan and Sridhar (1982). Quantification of total phenol was done by a standard of caffeic acid.

HPLC analysis of catechins

Extraction from tea leaf tissues was done following the method of Obanda and Owuor (1994) with slight modification. 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.

Sustainability of bacteria in soil detected by immunoformats

Polyclonal antibodies were raised against the bacteria in white, male rabbits (Alba and Devay, 1985). Sustainability of the applied bacteria in soil were tested by PTA-ELISA (Chakraborty*et al,* 1995) and Dot immunobinding assay (Lange *et al,* 1989) using polyclonal antibodies raised against the PGPR.

Localization of AMF hyphae in tea root cells by Indirect Immunofluoresecnce

Localization of AMF hyphae in tea root cells was observed in cross sections of roots using the antibody of AMF (*G. fasciculatum* or *G. mosseae*) raised within the male rabbit by Indirect Immunofluoresecnce and probing with fluorescein isothiocyanate (FITC) following the method of Chakraborty and Saha (1994). Fluorescence of the root sections were observed using Leica Leitz

biomed Microscope with fluorescence optics equipped with UV-filter set-I-3 and photograph was taken.

RESULTS AND DISCUSSION

Microscopic observation of bacteria and AM fungi

Morphological observation of *B. amyloliquefaciens*, *B. pumilus*, *B. megaterium* and *Ochrobactrum anthropi* showed that *B. amyloliquefaciens*, *B. pumilus* and *B. megaterium* were rod shaped, Gram +(ve), with wavy cell margin, rough surface and opaque nature in density. All the three isolates also produced endospores, whereas *O. anthropi* was small rod shaped, Gram -(ve). Scanning electron micrographs also confirmed the structure of bacteria.*B. amyloliquefaciens*- larger rod shaped (size-2μm), *B. pumilus* –rod shaped (size-2μm), *B. megaterium*-rod (size-2μm) and *O. anthopi*- small rod (size-1μm) (Fig 1- A-D).

Among the AM fungi, *Glomus* comprises of 60-65% followed by *Acaulospora*15-20%, *Gigaspora* 10-15%, *Scutellospora* 6-8% and *Entrophospora* 2% (Fig 2- A-K; Table 1). Out of all the AMF spores the genus *Glomus* was most frequent of which *G. fasciculatum* had the highest population followed by *G. mosseae, G. aggregatum* and *G. constrictum*. On the basis of consistent association of *G. fasciculatum* and *G. mosseae* with all the tested tea varieties, they were selected for *in vivo* tests. The spores of *G.fasciculatum* can be easily recognized by its cylindrical to slightly flared structure with prominent three wall layers.

Evaluation of PGPR activities of four isolates in vitro

Four isolates were tested for different PGPR activities as described in materials and methods. Results revealed that all four isolates could solubilize phosphate and produced siderophore. The secretion of IAA into the medium was also confirmed by quantification (Fig 1- E-J; Table 2).

16S r DNA sequence analysis for identification

The BLAST query of 16S rRNA sequence of the selected isolates against GenBank database confirmed their identity. The sequences have been deposited in NCBI, GenBank Database under the accession Nos. JN983127.1, JX 312687.1 and JQ765580.1 for *B. amyloliquefaciens, B. megaterium* and *B. pumilus* respectively.

Table 1 : AMF population in tea rhizosphere

AM fungi	AM species	AM spores in soil
Glomus	*G. aggregatum* *G. constrictum* *G. fasciculatum* *G. intraradices* *G. microaggregatum* *G. mosseae* *G. pansihalos* *G. albidum*	60-65%
Acaulospora	*A. bireticulata* *A. delicata* *A. laevis* *A. scrobiculata* *A. spinosa* *A. undulata*	15-20%
Gigaspora	*G. albida* *G. rosea* *G. gigantea* *G. margarita*	10-15%
Scutellospora	*S. rubra* *S. pellucida* *S. calospora*	6-8%
Entrophospora	*E. colombiana*	2%

In vitro antagonistic tests

Antagonism of four isolates was also tested against *Sclerotium rolfsii*, *Phellinus noxius*, *Poria hypobrunnea* in both solid and liquid medium *in vitro*. Results revealed that the bacteria inhibited test pathogens significantly (Table 3).

Growth of tea seedlings

Growth promotion was studied in terms of increase in height, number of branches, and leaf dry mass in comparison to control both in field and potted conditions. Changes in dry mass of leaves from potted plants (TV18, TV23, TV25, TV26 and T17) were determined after the treatments with *B. amyloliquefaciens* and *B. pumilus* (Table 4). Percent increase in height and no of branches in all tested varieties of tea plants were recorded in field conditions after 6 and 12 months of application of *B. amyloliquefaciens* and *B. pumilus* as a soil drench separately. Among them, *B. amyloliquefaciens* showed comparatively better response in growth promotion than *B. pumilus* in the above five tea varieties (Figs 3 and 4). *B. megaterium* and *O. anthropi* also efficiently in-

Table 2 : *In vitro* PGPR characteristics of rhizobacteria

Characteristics	*B. amyloliquefaciens*	*B. pumilus*	*B.megaterium*	*O.anthropi*
Phosphate solubilisation	+	+	+	+
Siderophore production	+	+	+	+
Protease production	+	+	+	+
Chitinase production	-	-	-	-
HCN production	-	-	-	-
Volatile production	+	+	+	+
IAA production	+	+	+	+

+= Activity present ; - = Activity absent

Table 3 : *In vitro* antagonistic tests of PGPR against root rot pathogens

Test fungi	Dia. of fungal growth (cm) after 7 days	% of inhibition
Phellinus noxius	8.2±0.23	–
P.noxius + B. megaterium	1.6±0.07	80.4±2.66
P .noxius +B.amyloliquefaciens	1.5±0.09	81.7±2.65
P. noxius +B. pumilus	1.4±0.08	82.0±2.67
P. noxius +O. anthropi	1.2±0.07	85.3±2.67
Poria hypobrunnea	8.5±0.20	–
P.hypobrunnea+B. megaterium	2.0±0.17	76.4±2.88
P.hypobrunnea +B.amyloliqu-efaciens	1.9±0.19	77.6±2.86
P.hypobrunnea +B. pumilus	2.0±0.16	76.4±2.85
P.hypobrunnea +O.anthropi	2.1±0.15	75.3±1.77
Sclerotium rolfsii	8.5±0.34	–
S.rolfsii+ B.megaterium	1.4±0.29	84.0±2.66
S.rolfsii +B.amyloliquefaciens	1.3±0.30	84.7±2.55
S.rolfsii +B. pumilus	2.8±0.31	67.0±1.90
S. rolfsii +O.anthropi	6.9±0.9	18.8±1.90

Average of 3 replicates; ± = SE

creased the rate of growth in five varieties of tea (CP-1, TV-20, UP-26, T-17 and K1/1) seedlings in relation to untreated control after 2 and 4 months (Table 5).

In case of dual application of AMF anf PGPR, *B. amyloliquefaciens* and *G. fasciculatum* were selected. Application of *G. fasciculatum* and *B. amyloliquefaciens* in the rhizosphere of tea plants (TV-18, T-17, AV-2, T-78, UP-3 and UP-26) maintained in glass house and field conditions led to an increase in the growth in terms of increase in height and number of leaves. Determination of height as well as number of leaves in seedlings, after 2 months of application, showed an increase in all varieties. Combined inoculation with both the microorganisms exhibited significant increase in height and number of leaves after 2 months of application over uninoculated control as well as single inoculation (Fig 5). Highest % increase in height in T-78, UP-3 and UP-26 varieties were observed when tea plants were treated with joint inoculation of *G. fasciculatum* and *B. amyloliquefaciens*. Statistical analysis (ANOVA) also revealed that there was no significant difference among the varieties.

Biocontrol of root rot diseases of tea by application of PGPRand AMF

An important root rot disease caused by *Poria hypobrunnea* was reduced by *B. pumilus*. Similarly, *B. megaterium* reduced brown rot in tea more significantly in comparison to *O. anthropi* when the plants (HV-39, UP-3 and T-17) were artificially inoculated with pathogen after three days of soil drenching with bacteria (Tables 6 and 7). In dual application treatments, rhizosphere of tea was inoculated by *G. fasciculatum* and *B. amyloliquefaciens* prior to challenge inoculation with *S. rolfsii*. Development of blight was determined after 15, 30 and 45 days of inoculation on the basis of disease index. Results revealed that both microorganisms could reduce sclerotial blight, but maximum suppression of disease was due to joint inoculation (Table 8).

Phenolics and catechins

Total phenol contents were estimated in tea leaves

Table 4 : Changes in biomass of tea leaves of potted tea plants

Tea varieties	Treatment	Fresh weight (g)	Dry weight taken after 7 days (g)
TV-18	Control	04.5±1.5	2.4
	B.amyloliquefaciens	15.0±1.4	7.4
	B. pumilus	14.0±2.2	6.5
TV-23	Control	09.0±1.0	4.0
	B.amyloliquefaciens	25.0±1.4	10.5
	B. pumilus	21.0±1.3	8.5
TV-25	Control	10.0±1.0	6.5
	B.amyloliquefaciens	18.0±1.2	8.8
	B. pumilus	22.0±2.0	13.0
TV-26	Control	02.0±0.5	1.3
	B.amyloliquefaciens	15.0±1.0	4.3
	B. pumilus	13.0±1.1	5.2
T-17	Control	06.0±1.0	4.3
	B.amyloliquefaciens	20.0±1.7	8.5
	B. pumilus	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; ±=SE; Results taken 2 months after application of *B. amyloliquefaciens* and *B. pumilus*

Table 5 : Effect of application of *B.megaterium* and *O.anthropi* on growth in tea seedlings

Tea varieties	Treatment	2 months after treatment		4 months after treatment	
		% increase in height	% increase in no.of leaves	% increase in height	% increase in no. of leaves
CP-1	Control	6.6±1.2	25±1.71	26.6±1.21	75.0± 1.95
	O. anthropi	56.6±1.3	100± 6.01	70.6±2.46	200.0± 1.00
	B.megaterium	60.4±1.8	120± 1.97	67.5±1.31	180.0±1.61
TV-20	Control	8.6±1.02	50± 1.92	15.7±2.33	51.0± 2.05
	O. anthropi	57.9±1.99	100± 1.64	103.5±7.59	183.3± 1.93
	B.megaterium	63.5±1.54	125± 1.64	126.9±1.55	225.0± 3.32
UP-26	Control	12.0±0.29	21.0±2.31	25.3±1.39	60.0± 2.31
	O. anthropi	45.2±1.32	38.0± 1.73	98.5±1.33	158.0± 1.16
	B.megaterium	51.3±1.04	31.0± 1.16	104.2±1.91	128.0±5.20
T-17	Control	10.4±0.87	14.3± 1.46	23.2±1.56	52.0± 1.73
	O. anthropi	58.0±0.88	33.3±0.88	110.2±0.92	127.3± 1.73
	B.megaterium	60.0±0.81	42.9± 1.21	121.3±0.92	138.6± 1.68
K1/1	Control	13.0±1.16	20.0± 1.73	26.5±0.75	60.5± 2.02
	O. anthropi	25.0±1.73	60.0± 1.16	86.6±1.91	190.0±2.61
	B.megaterium	30.0±2.31	50.0± 2.31	90.6±2.72	187.0± 5.51

10 plants/ treatment; Difference of all tests with control significant at P=0.05 as tested by Student's t test; ±=SE

of different tea varieties following application of *G. fasciculatum*, *B. amyloliquefaciens* and challenge inoculation with *S. rolfsii*. There was a significant increase in phenol contents of leaves in all treat-

Table 6 : Effect of *B. pumilus* on development of root rot disease of tea by *Poria hypobrunnea*

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

Avg. of the plant 2yr; Avg. of 10 separate inoculated plants; Rot index: 0- no symptoms; 1- small roots turn brownish and start rotting; 2- leaves start withering and 20-40% 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.

Table 7: Effect of *B. megaterium* and *O. anthropi* on development of brown rot of tea

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

[a]- Difference with control significant at P=0.05; [b]- insignificant and rest significant at P=0.01 as done by Student's t test. Rot index: 0- no symptoms; 1- small roots turn brownish and start rotting; 2- leaves start withering and 20-40% 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.

ments (*S. rolfsii, B. amyloliquefaciens + S. rolfsii, G. fasciculatum + S. rolfsii* and *G. fasciculatum + B. amyloliquefaciens + S. rolfsii*), but most significant increase was when there was challenge inoculation (Fig 6). Statistical analyses (ANOVA) revealed that there were significant differences in all treatments in case of total and ortho- phenol contents during disease development except between control and *S.rolfsii* treated plant in determination of total phenol contents. Increase in isoforms of catechins was also observed when treated with *B.pumilus* in comparison to control (Fig 7).

Defense enzymes

In case of defense enzymes- POX, CHT (Fig 8), PAL and GLU (Fig 9) also similar results were obtained. But, no significant differences was observed between control and *S. rolfsii* treated tea plants in case PAL and GLU activities.

Survival of applied bacteria in the rhizosphere and localization of AMF hyphae in tea roots

Survival of bacteria in soil following application was determined immunologically using the PAb raised against the bacteria. ELISA and DIBA were done immediately and 6 months after application to the soil. Tests revealed that the bacteria could survive and multiply in the rhizosphere (Table 9). Invasion of AMF hyphae in tea root cell was confirmed by immunofluorescence. Strong apple green fluorescence of hyhae with in the root were evident in treated leaves.

Four bacterial isolates- *Bacillus amyloliquefaciens*, *B. pumilus*, *B. megaterium* and *Ochrobactrum anthropi* showed *in vitro* characteristics of plant

Table 8 : Effect of application of *B. amyloliquefaciens* and *G. fasciculatum* on sclerotial blight disease of tea

Tea varieties	Disease index (45 days after inoculation) Pre treated with*			
	None	*G.f*	*B.a*	*G.f +B.a*
TV-18	4.1	1.3	3.2	1.9
T-17	5.9	1.9	4.4	2.7
AV-2	3.5	1.1	3.0	1.6
T-78	4.0	2.0	2.9	2.1
UP-3	4.0	1.9	2.8	2.2
UP-26	5.4	2.4	3.4	2.5
C.D.(P=0.05) (Treatments)	0.486			
(Varieties)	0.595			

10pots/treatment;**B.a*= *Bacillus amyloliquefaciens*; *G.f*= *Glomus fasciculatum*. Disease (Sclerotial Blight) Index computed on a scale of 0-6 on the basis of underground and above ground symptoms. Disease intensity was assessed as rot index on a scale of 0-6, depending on both underground and above ground symptoms as follows: Rot index: 0- no symptoms; 1- small roots turn brownish and start rotting; 2- leaves start withering and 20-40% 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

growth promoting bacteria such as phosphate solubilization, siderophore production, IAA production. They were also antagonistic to *S. rolfsii*, *P. noxius* and *P. hypobrunnea in vitro*. In our present investigation internal transcribed spacer (ITS) regions have been used successfully to confirm the identity of the bacterial isolates. The phylogenetic analysis further confirmed that the strains are phylogenetically related to the other respective ex-type strains obtained from NCBI Genbank Database High population of AM fungi such as species of *Glomus, Gigaspora, Scutellospora* and *Acaulospora* were obtained. Of all of these, *Glomus fasciculatum* showed highest percentage of occurrence followed by *G. mosseae*.

B. amyloliquefaciens showed comparatively better response in growth promotion followed by *B. pumilus* in TV18, TV23, TV25, TV26 and T17 varieties. *B. megaterium* and *O. anthropi* also efficiently increased the rate of growth in five varieties of tea (CP-1, TV-20, UP-26, T-17 and K1/1) seedlings. *G. fasciculatum* and *B. amyloliquefaciens* showed good growth promotion in tea in glass house and field conditions. Both the microorganisms alone or jointly could promote growth of the seedlings in terms of increase in height and leaf number. However, joint application gave better results. 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. Influence of *Serratia marcescens* (TRS 1) on growth promotion of tea seedlings 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). Erturk *et al.* (2012) examined the effect of

Table 9: ELISA and DIBA values of rhizosphere soil antigens reacted with PAb of *B. amyloliquefaciens*, *B. pumilus*, *B. megaterium* and *O. anthropi*

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	+	+
	B. amyloliquefaciens	1.258±0.18	1.305±0.12	++++	++++
	B. megaterium	1.400±0.63	1.36±0.04	++++	++
	B. pumilus	1.410±0.60	1.39±0.06	++++	++
	O. anthropi	1.157±0.07	1.05±0.02	++++	++
TV-23	Control	0.321±0.03	0.330±0.04	+	+
	B. amyloliquefaciens	1.432±0.04	1.532±0.18	++++	++++
	B. megaterium	1.244±0.03	1.345±0.63	++++	++++
	B. pumilus	1.200±0.05	1.350±0.61	++++	++
	O. anthropi	1.354±0.05	0.999±0.07	++++	++
TV-25	Control	0.287±0.05	0.312±0.03	+	+
	B. amyloliquefaciens	1.356±0.08	1.422±0.08	++++	++++
	B. megaterium	1.248±0.04	1.240±0.12	++++	++
	B. pumilus	1.257±0.03	1.241±0.12	++++	++
	O. anthropi	1.254±0.04	1.118±0.06	++++	++
TV-26	Control	0.341±0.03	0.368±0.08	+	+
	B. amyloliquefaciens	1.568±0.16	1.630±0.18	++++	++++
	B. megaterium	1.096±0.18	1.083±0.05	++++	++
	B. pumilus	1.095±0.19	1.088±0.03	++++	+
	O. anthropi	1.343±0.13	1.121±0.10	++++	++
T-17	Control	0.286±0.01	0.302±0.02	+	+
	B. amyloliquefaciens	1.643±0.07	1.654±0.11	++++	++++
	B. megaterium	1.132±0.02	1.308±0.01	++++	++++
	B. pumilus	1.134±0.01	1.309±0.04	++++	+++
	O. anthropi	1.096±0.08	0.889±0.07	++++	+

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.

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.

B. pumilus, *B. megaterium* and *O. anthropi* reduced the intensity of root rot disease of tea significantly. Sclerotial blight disease of tea was reduced by *B. amyloliquefaciens* and *G. fasciculatum* alone but maximum suppression of disease was due to joint inoculation. In order to determine whether the PGPR and AMF could induce systemic resistance (ISR) in tea plants, accumulation of defense related enzymes and phenolics were studied. In the present study, activities of the different enzymes were analyzed in tea following treatments with pathogen and microorganisms as follows: *S. rolfsii, B. amyloliquefaciens + S. rolfsii, G. fasciculatum + S. rolfsii, G. fasciculatum+ B. amyloliquefaciens + S. rolfsii* as well as in control. Activities of defense related enzymes and accumulation of phenolics increased significantly dur-

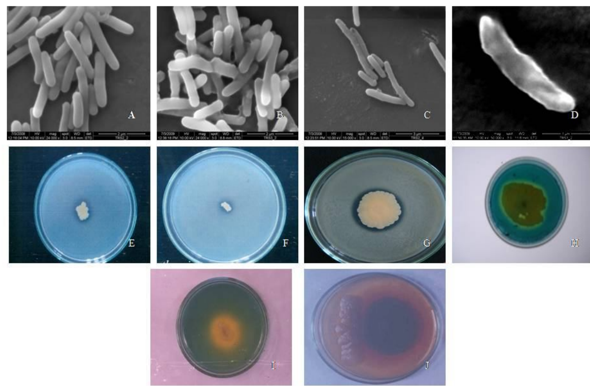


Fig. 1. Scanning electron microscopic view of *B. amyloliquefaciens* (A), *B. pumilus* (B), *B. megaterium* (C) and *O. anthopi* (D), *in vitro* PGPR tests-phosphate solubilization and siderophore production by *B. megaterium* (E&H), *O. anthropi* (F&I) and *B. pumilus* (G&J).

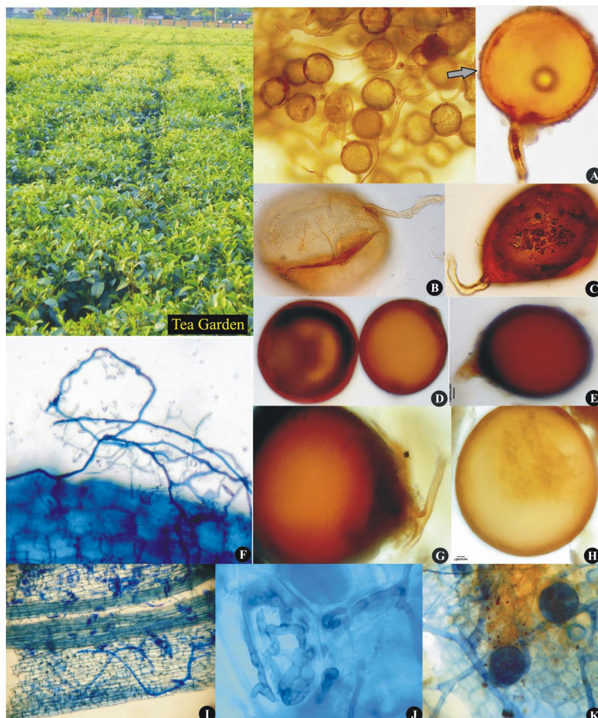
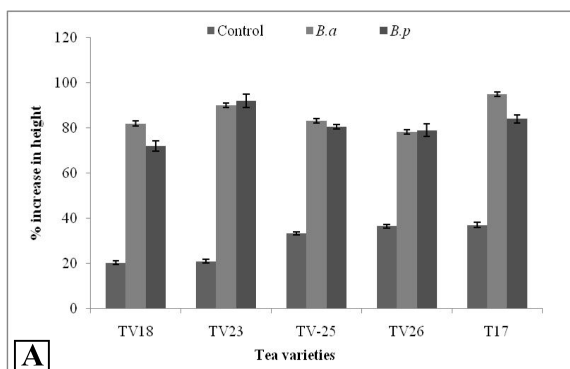


Fig. 2. *Glomus mosseae* (A&B); *Glomus drummondii* (C); *Glomus bodium* (D&E); Extradical hyphae (F); *Glomus faciculatum* (G); *Glomus* sp. (H); Intra radical hyphae (I) and Vesicles (J&K).



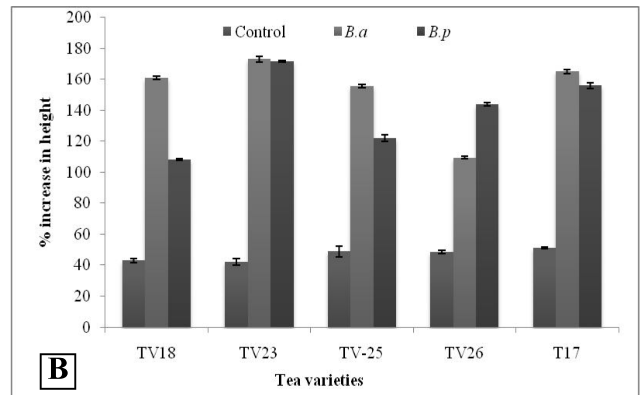
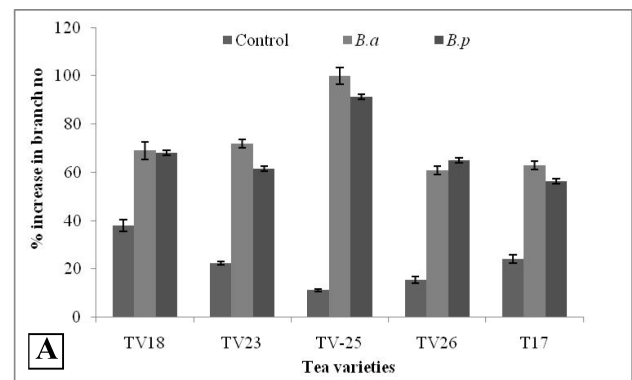


Fig.3. Effect of application of *B. amyloliquefaciens* and *B. pumilus* on growth of five varieties of tea in field in terms of % increase in height after 6 (A) and 12 (B) months. *B.a=Bacillus amyloliquefaciens* and *B.p= Bacillus pumilus*



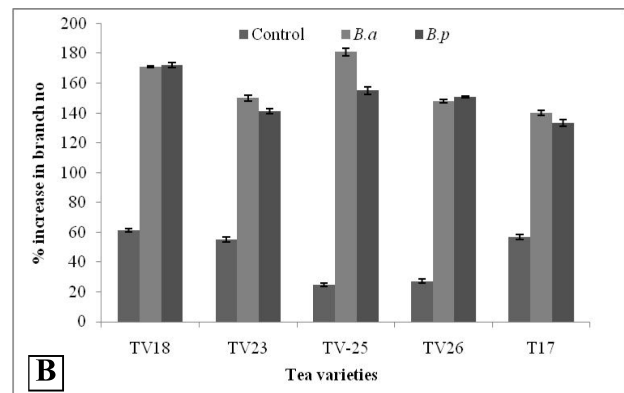


Fig. 4 : Effect of application of *B. amyloliquefaciens* and *B. pumilus* on growth of five varieties of tea in field in terms of % increase in no. of branches after 6 (A) and 12 (B) months. *B.a= Bacillus amyloliquefaciens* and *B.p= Bacillus pumilus*

ing disease suppression. Increase in isoforms of catechins was also observed when TV-29 variety was treated with joint application of *B. pumilus* and *G. fasciculatum*. It is quite evident that, in the present study in addition to other mechanisms of action reported for *B. amyloliquefaciens* involving siderophore production, IAA production, antifungal metabolites and phosphate solubilization, induction of defense mechanisms play an important

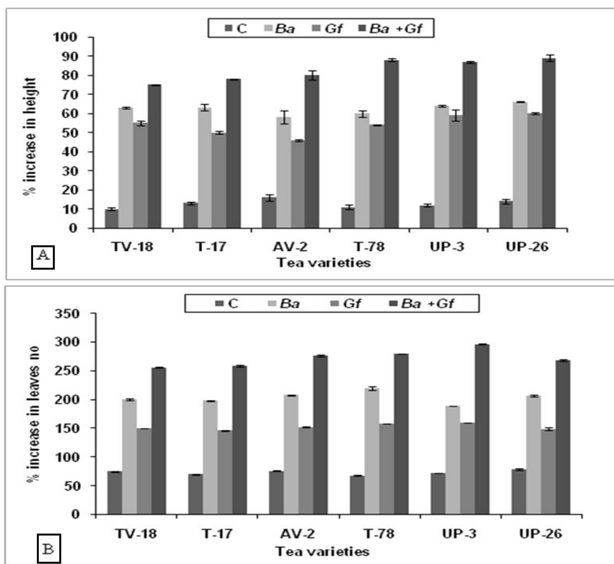


Fig 5: Effect of application of *Glomus fasciculatum* and *Bacillus amyloliquefaciens* on growth (% increase in height-**A** & no of leaves- B) of potted tea plants after 2 months.*B.a=Bacillus amyloliquefaciens*, *G.f= Glomus fasciculatum* and *B.a+G.f= Bacillus amyloliquefaciens +Glomus fasciculatum*.

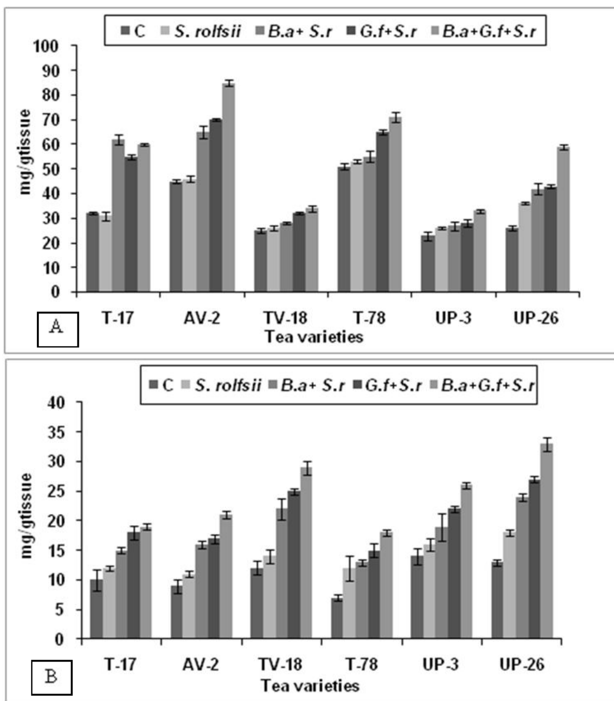


Fig. 6 : Total (A) and O-dihydroxy (B) phenols in leaves of tea varieties after 72 hr of different treatments. *S.r= Sclerotium rolfsii*, *B.a+S.r=Bacillus amyloliquefaciens + Sclerotium rolfsii*, *G.f+S.r= Glomus fasciculatum +Sclerotium rolfsii* and *B.a+G.f+S.r= Bacillus amyloliquefaciens +Glomus fasciculatum+ Sclerotium rolfsii*

role in disease control and plant growth promotion. *G.fasciculatum* was also responsible mainly for induction of resistance within the host. In green house experiments, Kumar *et al,* (2013) isolated

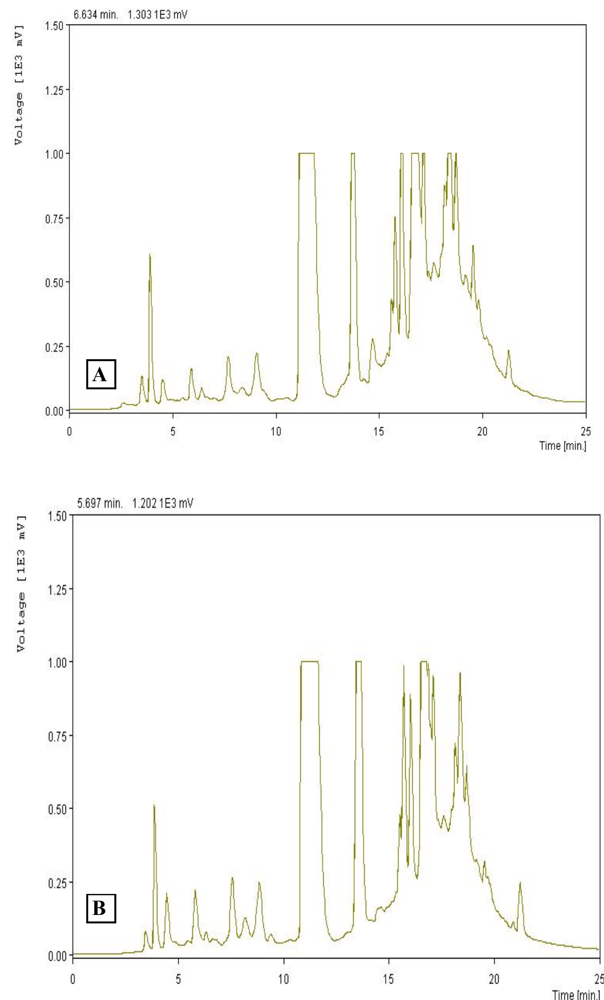


Fig. 7 : HPLC profile of catechins extracted from tea leaves (HV-39) treated with PGPR. A- Control; B- *B. pumilus* treated

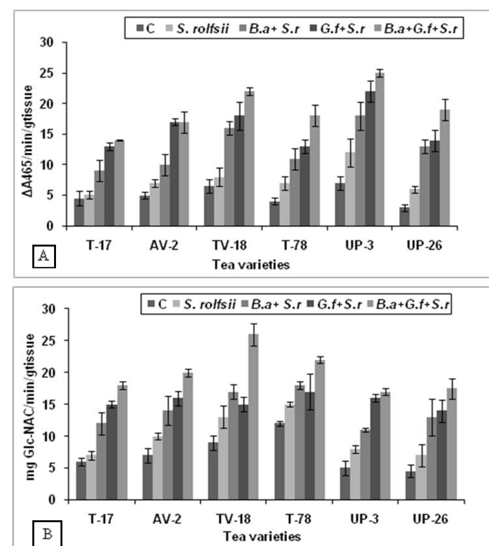


Fig. 8 : Peroxidase (A) and chitinase (B) activities in leaves of tea varieties after 72 hr of different treatments. *S.r= Sclerotium rolfsii*, *B.a+S.r=Bacillus amyloliquefaciens + Sclerotium rolfsii*, *G.f+S.r= Glomus fasciculatum +Sclerotium rolfsii* and *B.a+G.f+S.r= Bacillus amyloliquefaciens +Glomus fasciculatum+ Sclerotium rolfsii*

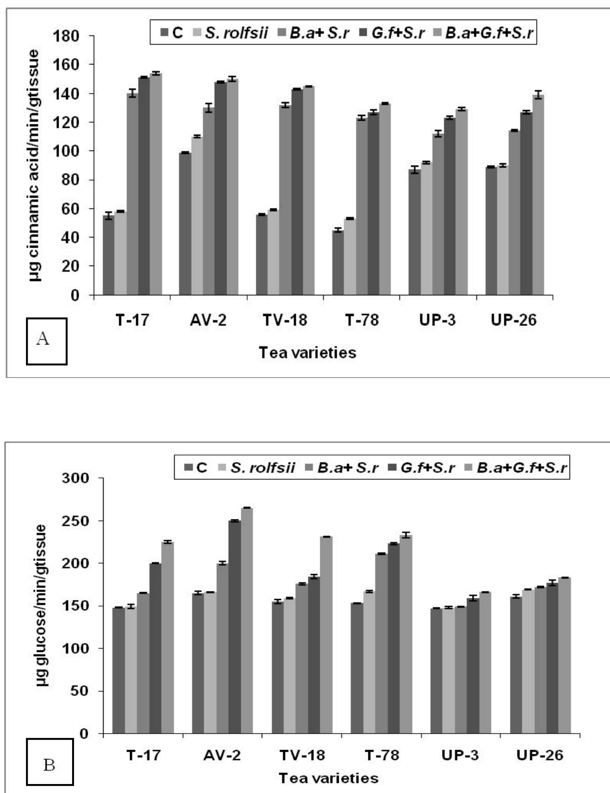


Fig. 9 : Phenyl alanine ammonia lyase(A) and â-1,3 glucanase(B) activities in leaves of tea varieties after 72 hr of different treatments. *S.r= Sclerotium rolfsii*, *B.a+S.r=Bacillus amyloliquefaciens + Sclerotium rolfsii*, *G.f+S.r= Glomus fasciculatum +Sclerotium rolfsii* and *B.a+G.f+S.r= Bacillus amyloliquefaciens +Glomus fasciculatum+ Sclerotium rolfsii.*

plant growth promoting rhizobacteria (PGPR) strains, such as *Bacillus* and *Pseudomonas*, 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.

ELISA values and intensity of dots proved that the four isolates could also survive and multiply in the rhizosphere even after 6 months of application. Localization of AMF hyphae in tea root cell was also confirmed by indirect immunofluorescence. In conclusion, it may be stated that all four isolates have shown good potential as plant growth promoters and reduced root rot and sclerotial blight disease of tea by direct and indirect mechanisms in the host. On the other hand, *G. fasciculatum* and *G. mosseae* are responsible for induction of resistance in the host. So, dual application of PGPR and AMF may lead to biopriming of the plants through growth promotion and induced systemic resistance.

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