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Influence of Indigenous Bacilli Isolated from Darjeeling Hills on Phosphate Mobilization and Induction of Resistance Against Sclerotial Blight Disease of Tea Cultivars

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Abstract—*Utilization of plant growth promoting rhizobacteria (PGPR) in agro-ecosystems significantly enhances plant–microbe interactions which positively affects ecosystem sustainability, agricultural productivity, and environmental quality. The present investigation was carried out with an objective to explore and characterize indigenous bacterial isolates of Darjeeling Hills and utilize them for growth promotion of tea saplings and manage sclerotial blight disease. Among the isolated bacteria, two isolates, BRHS/C-1 and BRHS/S-73 were found to be the most efficient in terms of phosphate solubilization, siderophores, Indole-3-acetic acid (IAA), Hydrogen Cyanide (HCN), 1-aminocyclopropane-1-carboxylate (ACC) deaminase production and inhibition of tea root fungal pathogens in laboratory conditions. 16S rDNA sequences confirmed the identity of BRHS/C-1 as Bacillus pumilus and BRHS/S-73 as B. altitudinis respectively. Both the bacilli strains could enhance growth of tea saplings (Camellia sinensis) in nursery conditions as indicated by significant increase in root and shoot length and leaf biomass. They were also found to efficiently mobilize soil phosphate by enhancing acid and alkaline phosphatase activities. Besides, B. pumilus and B. altitudinis could also successfully reduce sclerotial blight disease of tea caused by Sclerotium rolfsii. The disease incidence was reduced to 6% and the biocontrol efficacy was 80% when applied jointly. Decrease in the disease incidence was brought about by a significant increase in the key defense enzymes such as chitinase, β-1, 3-glucanase, peroxidase, phenylalanine ammonia lyase as well as phenolics in tea roots. Immuno-assays conducted using polyclonal antibodies of the pathogen showed that its population was greatly reduced in the treated rhizosphere soil. The present study shows that efficient microorganisms can be utilized to develop technologies suitable for eco-agriculture and organic framings which would promote safe and sustainable agriculture practices.*

Keywords*: Bacillus pumilus, B. altitudinis, Sclerotium rolfsii, Phosphate, Camellia sinensis.*

1. INTRODUCTION

Microorganisms in soils play important roles in many processes like decomposition of organic matter, soil structure formation, removal of toxins and the cycling of carbon, nitrogen, phosphorus, and sulphur. They are also involved in suppressing soil borne plant diseases and in promoting plant growth. These type of microorganisms typically known as Agriculturally Important Microorganisms (AIMs) are used as biocontrol and biofertilizers in different cropping systems [1]. Isolation of microorganisms, screening for desirable characters, selection of efficient strains, production of inoculum and preparation of carrier-based formulation are important steps in the use of these microbe based environment friendly and sustainable technology [2]. Sustainable agriculture is vital in today's world as it offers the potential to meet our agricultural needs without affecting soil ecosystem. This type of agriculture uses a special farming technique wherein the environmental resources can be fully utilized and at the same time ensuring that no harm is done to it. Thus the technique is environment friendly and ensures safe and healthy agricultural products [3]. Several mechanisms have been postulated to explain how the AIMs stimulates plant growth which may either be direct and/or indirect. Direct mechanisms include production of plant growth hormones that can enhance various stages of plant growth [4], mineral phosphate solubilization [5], nitrogen fixation [6] and stimulation of ion uptake [7], IAA and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production [8]. Whereas the indirect mechanisms include biochemical and physiological changes induced in the host activated by AIMs [9]. Alternative solutions to the use of pesticides include, developing new resistant crop cultivars, use of biological control agents, or the employment of novel plant activator

agrochemicals that can be used to turn on natural plant defenses [10]. Resistance elicitors, also known as plant activators or priming agents act by enhancing plant defenses against different types of stress. Most of the microbial isolates isolated from higher altitude region are capable of eliciting resistance in plantation crops against root pathogens [11,12]. Priming results in a faster and stronger induction of plant defense responses and enhanced resistance to biotic or abiotic stresses in comparison to that found in unprimed plants exposed to the same stress [13]. Given that priming provides a long-lasting, broad-spectrum resistance to stress, it has been suggested that priming of plant defense is a promising alternative approach in modern disease management [14]. Furthermore, the use of natural or synthetic resistance elicitors to induce plant immunity is now becoming commercially attractive, particularly because chemical control employing pesticides is turning out to be unsustainable and undesirable [15]. To this end, the present investigation was carried out with an objective to screen beneficial microorganisms from high altitude reasons of Darjeeling district and utilize them as efficient bio-priming agents to mobilize soil phosphate and induce resistance in tea cultivars against sclerotial blight disease.

2. MATERIALS AND METHOD

2.1. Isolation of bacteria

Soil samples from the rhizosphere of two plants *Sechium edule* and *Cryptomeria japonica* growing in Saureni and Mirik village of Darjeeling district, West Bengal were collected. Bacterial isolates from these soil samples were isolated and purified using Warcup's serial dilution technique on nutrient agar medium [16].

2.2. Plant growth promoting traits and antagonism

Plant growth promoting activities of the bacterial isolates were analyzed following stand procedures and techniques. Phosphate solubilization by the bacterial isolates was tested on Pikovskaya's (PVK) agar supplemented with 5% tricalcium phosphate [17]. Production of siderophore was detected by standard method of Schwyn and Neiland using blue indicator chromeazurol S (CAS) [18]. Production of indole acetic acid (IAA) in the culture supernatant by the bacterium was quantified specrtophotometrically by Pilet and Chollet method [19]. Hydrocyanic acid (HCN) production was tested on 35-mm petri dish containing Nutrient agar medium amended with 4.4 g glycine/l with filter paper dipped in picric acid in the upper lid and sealed with parafilm as described by Reddy and his co workers [20]. Production of chitinase was detected by standard method of Hsu and Lockwood [21]. ACC deaminmaese activity was assayed with respect to the amount of µmol of α-ketobutyrate produced upon the hydrolysis of ACC as described by Honma and Shimomura [22]. Potential bacterial isolates which showed positive test for PGP activities *in vitro* were tested for their antagonistic effect against tea root pathogens *viz Sclerotium rolfsii*, *Sphaerostilbe repens*, *Ustulina zonata* and *Fomes lamaoensis* by dual plate culture method as described by Chakraborty and his co workers [23].

2.3. Identification and phylogeny

Potential PGPR isolates were identified on the basis of 16S rDNA sequences. Genomic DNA extraction and 16S rDNA-PCR amplification were carried out following the method of Stafford and co workers, 2005. The universal bacterial 16S rDNA primer pair, forward primer: 5'-AGAGTRTGATCMTYGCTW AC-3' and reverse primer: 5'-CGYTAMCTT WTTACGRCT-3' were used [24]. The evolutionary history was inferred using the UPGMA method as described by Sneath and Sokal [25] and the Phylogenetic analyses were conducted in MEGA-4.0 as described by Tamura and co workers [26].

2.4. Growth promotion tests

A small scale nursery based trial was conducted with complete randomized block design with three replications in the experimental nursery of Immuno-phytopathology Laboratory, Department of Botany, University of North Bengal. Three treatments, uninoculated control and inoculated with the bacterial isolates singly and inoculated with bacterial isolates jointly, each treatment was carried out in three equal rows consisting 10 plants each grown in loamy-clay soil. Saplings (6-month-old) of four tea varieties, *viz.*, TV-9, TV-20, TV-25 and TV-26 were selected for experimental purpose and were grown under normal dark/light cycle of 12/12 h, irrigation was carried out at a regular interval of 24-36 h. Bacterial isolates were applied at a concentration of 10^6 cfu/ml to the rhizosphere of tea saplings @ 100 ml/plant. Three applications were done, each at an interval of 1 month. Growth promotions were studied in terms of increase root and shoot length, leaf fresh and dry biomass after one month of last application of the bacterial isolates.

2.5. Tests for phosphate mobilization

Extraction and quantitative estimation of phosphate was carried out following ammonium molybdate-ascorbic acid method as described by Knudsen and Beegle [26]. Assay of acid and alkaline phosphatase activities in the soil were expressed according to method of Tominaga and Takeshi [27].

2.6. Biocontrol experiment

Ability of bacterial isolates to suppress sclerotial blight disease of Tea saplings caused by *Sclerotium rolfsii* was tested in nursery condition with ten replicates of each treatment. Inoculation of the rhizosphere of tea saplings with the pathogen and disease assessment was done following the method of Chakraborty and co workers [28]. The experiment included four treatments: 1- Healthy; 2-Treated with bacterium but Un-inoculated with the pathogen; 3- Untreated

but inoculated with pathogen, *S. rolfsii*; 4- treated inoculated. Disease index was recorded based on the score 0-6, depending on both underground and above ground symptoms as follows: Root rot index: 0 – no symptoms; 1 – roots and collar region turn brownish and start rotting; 2 – leaves start withering and 20–30% of roots turn brown; 3 – leaves withered and 50% of the roots affected; 4 – shoot tips also starts withering; 60–70% roots affected; 5 – whole plants starts withering ; 6–Whole plant die, with upper withered leaves still remaining attached; roots fully rotted.

2.7. Biochemical Analyses

All the biochemical analyses were performed from treated as well as control tea roots after 72 h of treatment. 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 as described by various workers [29-31]. Total phenol content was estimated by Folin Ciocalteau's reagent, following the method of Mahadevan and Sridhar [32].

2.8. Detection of pathogen in the soil

The survivability of the pathogen in the soil was determined immunologically using enzyme linked immunosorbent assay (ELISA) after treatment with PGPR isolates. Sustainability of the pathogen in soil in untreated and treated soil were tested by PTA-ELISA as described by Chakraborty and co workers, 1994 and Dot immunobinding assay of Lange and co workers [33].

2.9. Statistical analysis

The efficacy of bacterial isolates in nursery grown tea saplings was undertaken under complete randomized block design. Difference between control and treated significant at P=0.01and P=0.05 in all varieties was determined by Student's'*t*' test.

3. RESULTS

3.1. Bacterial isolates and PGP activities

A total of seven bacterial isolates from rhizosphere soil of *Cryptomeria japonica* and eighteen isolates from the rhizosphere soil of *Sechuim edule* were obtained and were tested for plant growth promoting (PGP) traits *in vitro*. One isolate, BRHS/C-1 obtained from rhizosphere soil of *Cryptomeria japonica* and one isolate, BRHS/S-73 obtained from the rhizosphere soil of *Sechum edule* showed positive result in all the tests such as phosphate solubilization, IAA, HCN, Siderophore, ACC deaminase and Chitinase production (Table 1). Both the isolates could inhibit all the tested root pathogens upto 83% (data not shown). Both the isolates BRHS/C-1 and BRHS/S-73 which showed positive PGP characters were selected for *in vivo* tests. Morphological studies of both the isolates showed that they were rod shaped, Gram positive with wavy cell margin, rough surface and opaque nature in density when maintained in culture at 30ºC for 5-6 d.

3.2. Identification and phylogenetic analysis

BLAST query of 16S rRNA sequences of both the isolates against GenBank database showed isolate BRHS/C-1 to have 99% sequence homology with *Bacillus pumilus* and BRHS/S-73 to have 98% sequence homology with *Bacillus altitudinis*. Phylogenetic analysis of the sequences which showed maximum homology with our query sequences was conducted. The phylogenetic analysis shows an optimal tree with the sum of branch length = 4.55582267. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (8000 replicates) are shown next to the branches. The analysis involved 15 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding (Fig. 1).

3.3. Growth promotion of tea plants and soil phosphate mobilization

Significant growth of tea saplings were recorded in plants treated with bacterial isolates evaluated in terms of percentage increase in root and shoots length as well as leaf fresh and dry biomass over similar increase in control. Maximum increase in growth was observed in the tea variety T-20, followed by the variety TV-9.

Table 1. *In vitro* characterization of bacterial isolates for plant growth promoting activities.

Isolates	Phosphate Solubilization	Siderophores	HCN	ACC (mg/h)	IAA (mg/L)
BRHS/C-1	++	+	+	20.60	24.55
BRHS/C -2	+	+	-	-	-
BRHS/C -3	-	-	-	-	-
BRHS/C -4	-	+	-	-	-
BRHS/C -5	+	+	+	-	-
BRHS/C -6	+	+	-	-	-
BRHS/C -7	-	-	-	-	-
BRHS/S-73	+++	+	+	44.63	31.47
BRHS/S-74	+	+	+	-	-
BRHS/S-75	+	-	+	-	-
BRHS/S-76	-	+	-	-	12.36
BRHS/S-77	-	-	+	-	23.19
BRHS/S-78	-	-	-	-	-
BRHS/S-79	+	+	-	-	-
BRHS/S-80	++	-	-	-	-
BRHS/S-81	++	+	+	-	16.30
BRHS/S-82	+	-	-	-	-
BRHS/S-83	+	-	-	-	-
BRHS/S-84	+	-	+	-	-
BRHS/S-85	-	-	+	-	20.93
BRHS/S-86	+	-	-	-	18.27
BRHS/S-87	-	-	-	-	-

BRHS/S-88	-	+	+	-	-
BRHS/S-89	+	+	+	-	9.27
BRHS/S-90	++	+	+	-	14.35
- = Activity not detected; += activity moderate; ++ = activity high; Data average of three replicate experiments.					

However, differences among the different cultivars were not significant, indicating that all varieties responded to the treatments. Among the two isolates, *B. altitudinis* showed comparatively better response in growth promotion when applied singly. However, the overall growth of tea saplings were much enhanced when both the isolates were applied jointly (Table 2). In nursery conditions, both the bacterial isolates, *B. pumilus* and *B. altitudinis* were found to mobilize soil phosphate when applied as soil drench to the rhizosphere of tea saplings which was indicated by decrease in total residual phosphate in treated soil and increase in total phosphate in roots and leaves in comparison to control. Similarly the activity of both acid and alkaline phosphatase activities were also enhanced in the rhizosphere soil of all tea saplings following bacterial application. Effect on total phosphate contents and soil phosphatase activities was higher when both the bacterial isolates were applied jointly (data not shown).

3.4. Biocontrol of sclerotial blight disease and induction and resistance

Application of both the PGPR as aqueous suspension was effective in reducing sclerotial blight disease when applied three days prior to artificial inoculation of the pathogen. Disease assessment was done after 5 and 30 days of inoculation with *S. rolfsii* recorded in terms of disease index. The disease severity in the tea saplings inoculated with only *S. rolfsii* increased with time, reaching a maximum of 75-80% at the end of 30 d. When the soil was pre treated with the PGPR, the maximum disease severity was only 18-20 %. Biocontrol efficacy of both *B. pumilus* and *B. altitudinis* under nursery condition after 30 d of pathogen challenge was found to be as high as 76-85% when applied jointly. *B. pumilus* BRHS/C-1 and *B. altitudinis* BRHS/S-73 could significantly reduce sclerotial blight disease under nursery conditions (Table 3). It was observed that there was significant enhancement of key defense related enzymes- POX, PAL, CHT, GLU and phenolics in the roots of tea saplings after 5d of pathogen challenge and bacterial inoculation (Fig. 2 and 3). Pathogen population in the rhizosphere soil of tea saplings in untreated and treated soils were determined immunologically using PAbs raised against *S. rolfsii*. DIBA and ELISA were conducted after 30d of pathogen inoculation. Results revealed that application of both the PGPR in the rhizosphere significantly reduced the pathogen population in the rhizosphere soil (data not shown).

Table.2 Effect of bacterial treatment on overall growth of tea plants

Cultivars and Parameters	Treatments			
TV-9	Control	*B.p*	*B. a*	*B. p* + *B. a*
Average height (cm)	24.50 ±1.88^a	29.45 ±1.73^b	27.17±1.58^b	35.28±2.47^b
Fresh biomass (g)	8.99 ±1.66^a	12.44 ±1.17^c	10.53±1.53^b	15.50±1.82^c
TV-20				
Average height (cm)	25.50 ±2.14^a	32.43±2.17^b	30.82±2.32^b	38.38±2.75^b
Fresh biomass (g)	7.76 ±0.88^a	11.43±1.16^b	9.75 ±1.27^c	13.73±1.73^c
TV-25				
Average height (cm)	20.25 ±1.33^a	24.36±2.18^c	28.15±2.13^b	32.74±2.74^b
Fresh biomass (g)	8.55 ±0.85^a	15.14±1.18^b	14.15±1.12^c	17.44±1.43^c
TV-26				
Average height (cm)	25.50 ±1.12^a	28.17±1.73^b	27.33±1.38^b	33.28±2.27^b
Fresh biomass (g)	9.74 ±0.74^a	14.36±1.22^b	13.18±1.16^b	16.44±1.58^b

Values are average of three replicate sets (10 plants each). ±= Standard error. Different letters indicate significant differences between control and treated (ab, P =0.01; ac, P=0.05). Similar letters indicate insignificant.

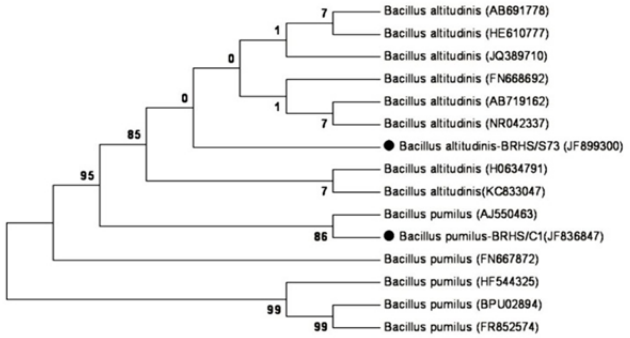


Fig. 1: Phylogenetic analyses of *B. pumilus* BRHS/C-1 and *B. altitudinis* BRHS/S-73 with other ex-type strains obtained from NCBI Gene Bank database. The optimal tree with the sum of branch length =0.528336689) is shown.

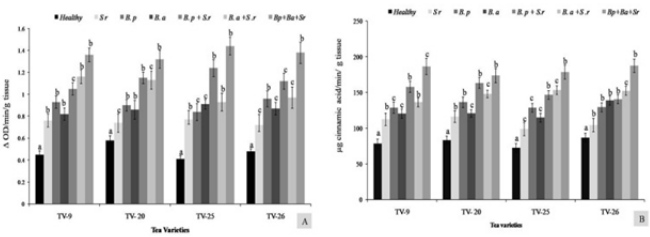


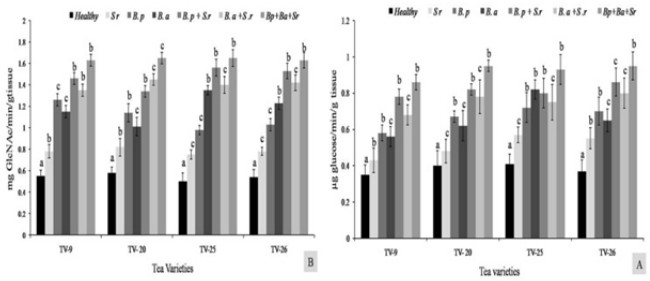
Fig. 2: (A& B) – Peroxidase (A) and Phenylalanine ammonia lyase (B) activities in the roots of different varieties of tea sapling following bacterial treatment and pathogen challenge. Values are average of three replicate sets (10 plants each). Data of three replicate experiments and bars indicate SE. Bars with different letters indicate significant differences between control and treated (ab, P =0.01; ac, P=0.05). Similar letters indicate insignificant.

Fig. 3. (A& B) – Glucanase (A) and Chitinase (B) activities in the roots of different varieties of tea sapling following bacterial treatment and pathogen challenge. Values are average of three replicate sets (10 plants each). Data of three replicate experiments and bars indicate SE. Bars with different letters indicate significant differences between control and treated (ab, P =0.01; ac, P=0.05). Similar letters indicate insignificant.

Table 3. Sclerotial blight incidence following application of bacterial isolates and inoculation with *S. rolfsii*

Cultivars	Treatments	Days after inoculation	
		5d	30d
TV-9	UI (*S. r*)	1.15±0.06	5.63±0.45
	B. p	0.51±0.03	2.15±0.05
	B. a	0.52±0.04	2.10±0.07
	B. p + B. a	0.25±0.03	1.25±0.08
TV-20	UI (*S. r*)	1.13±0.08	5.75±0.76
	B. p	0.50±0.02	1.75±0.04
	B. a	0.50±0.06	2.15±0.06
	B. p + B. a	0.26±0.01	1.15±0.08
TV-25	UI (*S. r*)	1.16±0.07	5.75±0.54
	B. p	0.54±0.02	2.15±0.06
	B. a	0.50±0.01	2.35±0.07
	B. p + B. a	0.35±0.03	1.28±0.08
TV-26	UI (*S. r*)	1.23±0.07	5.56±0.32
	B. p	0.53±0.04	2.00±0.07
	B. a	0.52±0.03	2.15±0.05
	B. p + B. a	0.35±0.01	1.38±0.06

Disease intensity was assessed as rot index on a scale of 0-6, 0 – no symptoms; 1 – roots and collar region turn brownish and start rotting; 2 – leaves start withering and 20–30% of roots turn brown; 3 – leaves withered and 50% of the roots affected; 4 – shoot tips also starts withering; 60–70% roots affected;5– Whole plant starts withering; 6– entire plants die, with upper withered leaves still remaining attached; roots fully rotted. Values are average of three replicate sets (10 plants each). ± = Standard Error. Difference between untreated inoculated and treated significant at P=0.01 in all varieties as determined by Student's*t*' test;
S.r = Sclerotium rolfsii; B. p = B. pumilus; B. a= B. altitudinis



4. DISCUSSION

In the present investigation, two bacterial isolates identified as *B. pumilus* (BRHS/C-1) and *B. altitudinis* (BRHS/S-73) isolated from the rhizosphere of *Cryptomeria japonica* and *Sechuim edule* of Darjeeling Hills showing *in vitro* PGP characteristics like phosphate solubilization, chitinase, siderophore, HCN, IAA and ACC deaminase production were selected. All these characters are considered to be the most important PGP traits [34]. Therefore microorganisms with simultaneous phosphate solubilizing and biocontrol potential are the best bioinoculants which can be used as potential plant growth promoters and biocontrol agents. Combined treatment with *B. pumilus* and *B. altitudinis* showed significant enhancement of growth of tea saplings. PGPR may increase plant growth through a number of mechanisms – both direct and indirect. It has been reported that IAA is capable of inducing root growth [34], and that ACC deaminase reduces ethylene production and thereby stimulates plant growth [35]. The enhancement in growth of the tea saplings was also found to be significantly correlated with increased soil acid and alkaline phosphatase activities. In our study, total phosphate content of the treated soils were significantly reduced while root and leaf phosphate increased in comparison to control. The results are in conformity with previous workers who have reported the ability of rhizospheric microorganisms to promote growth and have suggested phosphate solubilization to be one of the mechanisms involved in plant growth promotion [36]. Therefore the results clearly confirms that *B. pumilus* and *B. altitudinis* have the ability to efficiently mobilize phosphate in the soil. Combined application of *B. pumilus and B. altitudinis* were also found to efficiently suppress sclerotial blight disease of tea saplings caused by *S. rolfsii.* Bacterial treatment were seen in activities of enzymes- chitinase (CHT), β-1, 3-glucanase (GLU), Phenyl alanine ammonia lyase (PAL) and Peroxidase (POX) along with phenolics which increased significantly. Our results are in agreement with the earlier reports where enhancement of defense enzymes has been shown to be key mechanisms in suppressing root diseases and induction of resistance by bacterial isolates [37,38]. Increase in these key defense enzymes during plant growth promotion and disease suppression of tea by several PGPR was reported in the earlier studies by Chakraborty and his co workers, where PGPR like *Bacillus megaterium, B. pumilus, Ochrobactrum anthropi* and *Serratia marcescens* were successfully utilized to promote growth as well as to overcome

several root diseases of tea under nursery, pot and field conditions [39,40]. Combined effect of both the PGPR strains could trigger plant responses which led to growth and resistance against *S. rolfsii*. Moreover co-inoculation with microorganisms with different functions is now being recognized as a cheap and innovative technique [41].

In our present investigation two bacterial isolates exhibiting multiple plant growth promoting traits obtained from the rhizosphere of two plants grown in Darjeeling hills were utilized to promote growth in tea saplings in nursery conditions wherein they were able to mobilize soil phosphate. On the other hand these bacterial isolates were also able to induce resistance against sclerotial blight disease in these plants. It has therefore been demonstrated that biopriming of tea saplings at an early stage of growth and development in nursery condition by combination of efficient bacilli like *B. pumilus* and *B. altitudinis* could efficiently enhance growth as well as induce resistance against *S. rolfsii*.

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