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Bacillus altitudinis and *Bacilllus pumilus* induce resistance in *Brassica juncea* L. against *Thanatephorus cucumeris* causing Seedling Blight

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Plant Growth Promoting Rhizobacteria (PGPR) isolated from natural undisturbed environment of Darjeeling Hills showed plant growth promoting activities *in vitro* which included phosphate solubiliztion, IAA, siderophore production, HCN production, ACC deaminase activity as well as inhibition of *Thanatephorus cucumeris*, the causal organism of seedling blight of *Brassica juncea* L. the common Indian Mustard. Versatality of the PGPR isolates were tested for their ability to induce resistance in *B. juncea* seedlings towards the seedling blight disease. Seeds were first bioprimed with the test PGPR isolates and were aslo added to the rhizosphere as soil drench to the pathogen infested soil. Among all the tested PGPR, *Bacillus pumilus* and *B. altitudinis* were found to be the most efficient isolates and could reduce the disease incidence upto 77.90 %. Biocontrol Efficacy (BE%) of both the isolates were found to be to be 64% and 72% respectively which were the highest among all the PGPR tested. Biopriming and PGPR application led to enhanced activities of PR proteins such as chitinase, β-1, 3-glucanase and peroxidase. Enhanced accumulation of enzymes like phenylalanine ammonia lyase was also noticed after 36 h of PGPR treatment. Increment in the concentration of PR proteins and PAL activities were significantly higher in seedlings treated with *B. altitudinis* and *B. pumilus*. Direct effects of both the PGPR were also seen when pathogen population in the rhizosphere were significantly reduced. The current investigation suggests that both *B. altitudinis* and *B. pumilus* are versatile in function and exhibited multifaceted mode of action for disease suppression. PGPR isolates with versatile attributes are ideal microorganisms for developing effective and eco-friendly solutions for sustainable agriculture.

Key words: *Brassica juncea,* PGPR, *Bacillus altitudinis*, *B. pumilus,* ISR

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 agents and biofertilizers in different cropping systems. 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. 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 (Singh *et al*. 2011). Several mechanisms have been postulated to explain how the AIMs stimulate 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, mineral phosphate solubilization, nitrogen fixation (Bashan and Bashan, 2013) and stimulation of ion uptake IAA and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase production (Laslo *et al.* 2012,). Whereas the indirect mechanisms include biochemical and physiological changes induced

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in the host activated by AIMs (Salomon *et al.* 2017). Further, these root-associated mutualistic microbes, besides impacting on plant nutrition and growth, can further boost plant defenses, rendering the entire plant more resistant to pathogens and pests (Romera *et al.* 2019).

Pseudomonas and *Bacillus* species are the predominant plant growth-promoting bacteria. The spore-forming ability of *Bacillus* is distinguished from that of *Pseudomonas*. Members of this genus also survive for a long time under unfavorable environmental conditions. *Bacillus* spp. secrete several metabolites that trigger plant growth and prevent pathogen infection. *Bacillus* spp. can be successfully established in the soil and root rhizospheres without any lasting effects on other bacterial populations (Chowdhury *et al.* 2015). The application of *Bacillus*-based fertilizers to soil can enhance the plant-available forms of nutrients in rhizospheres, control disease-causing pathogenic microbial growth and induce pest defense systems (Garcia-Fraile *et al*. 2015; Kang *et al*. 2015). In the Indian context, *Bacillus* spp. isolated from higher altitude region are capable of eliciting resistance in plantation crops against root pathogens (Sunar *et al.* 2014, 2015, 2017).

Biopriming is now an emerging approach for application of PGPR in agriculture. 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 (Conrath, 2011; Mahmood *et al.* 2016). 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 (Bruce *et al.* 2017). Furthermore, the use of natural 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 (Roberts and Taylor, 2016). To this end, the present investigation was carried out with an objective to isolate beneficial microorganisms form sub-Himalayan region of Darjeeling hills with multiple plant growth promoting traits. Further *in planta* experiments were conducted to utilize selected beneficial microorganisms for induction of resistance against root rot disease of *Brassica juncea* caused by *Thanatephorus cucumeris.*

MATERIALS AND METHODS

Isolation and identification of bacterial isolates

Soil samples were collected from different geographical locations of Darjeeling hills representing forest soil, agricultural soil and riverine soil. The locations of soil samples were recorded through GIS mapping tool (Garmin). Soil samples were collected from the three subdivisions of Darjeeling District Kalimpong, Kurseong and Mirik which includes the area between 24 40’28’’ N to 27 13' N Latitudes and 87 45'50'' to 89 54’35'' E Longitudes. Physical conditions and pH of the samples were noted. Microorganisms from these soil samples were isolated using soil plate and direct soil plating methods (Warcup, 1950). Pure cultures of bacterial isolates were streaked on NA plates for colony development. The individual colonies were examined for shape, size, structure of colonies and pigmentation. The Gram reactions of all the isolated bacteria were recorded according to Buchanan and Gibbson (1974) and Gram positive/ negative reactions, shape of cells observed were recorded.

Identification of PGPR using ribosomal DNA sequences 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 *et al.* (2005). The universal bacterial 16S rDNA primer pair, forward primer: 5'-AGAGTRTGATCMTYGCTW AC-3' and reverse primer: 5'-CGYTAMCTT WTTACGRCT-3' were used (Sukumar and Ghosh, 2010). The PCR products were sent for sequencing to Genie, Bangalore, India and rDNA sequences were deposited to NCBI GenBank through BankIt sequence submission procedure and approved as the ITS sequence after complete annotation and accession numbers for respective sequences have been provided. The bioinformatics analysis was performed using NCBI BLAST (http://ncbi.nlm.nih.gov/blast) for identifying the microorganism using NCBI reference sequence genomic database with 100 % coverage of the query sequence and with a degree of identity 95%. 16S rRNA gene sequence of isolate BRHS/S-73 was aligned with 10 ex-type sequences obtained

from NCBI genbank database which showed more than 96% identity with our reference sequence. Phylogenitic analysis was conducted using 16S rDNA sequences with the help of bioinformatic tool MEGA as described by Tamura *et al.* (2007).

In vitro assays for plant growth promoting traits

Plant growth promoting activities of the bacterial isolates were analyzed following standard procedures and techniques. Phosphate solubilization by the bacterial isolates was tested on Pikovskaya's (PVK) agar supplemented with 5% tricalcium phosphate (Pikovskaya, 1948). Production of siderophore was detected using blue indicator chromeazurol S (CAS) as described by Schwyn and Neiland (1987). Production of indole acetic acid (IAA) in the culture supernatant by the bacterium was quantified specrtophotometrically as described by Pilet and Chollet (1970). 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 *et al.* (2008). Production of chitinase was detected by standard method of Hsu and Lockwood (1975). ACC deaminase activity was assayed with respect to the amount of *µ*mol of á-ketobutyrate produced upon the hydrolysis of ACC as described by Honma and Shimomura (1978). Potential bacterial isolates which showed positive test for PGP activities *in vitro* were tested for their antagonistic effect against fungal root pathogen *Thanatephorus cucumeris* by dual plate culture method as described by Chakraborty *et al.* (2006). *T. cucumeris* causes seedling blight of *Brassica juncea* and under severe conditions, can cause crop loss of up to 50-80%. This pathogen was obtained from the culture collection maintained at Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal. Results were expressed as mean of percentage of inhibition of the growth of the pathogen in presence of the bacterial isolate. For each test three replicate plates were used.

In planta assays for biocontrol of seedling blight disease caused by T. cucumeris

Ability of seven selected PGPR in reducing seedling blight of *B. juncea* caused by *T. cucumeris* was tested in small scale glass house based pot experiment with complete randomized block design with three replications at Immunophytopathology Laboratory, Department of Botany, University of North Bengal. Seeds of local varieties of *B. juncea* were surface sterilized with sodium hypochlorite and rinsed in distilled water after which seeds were dried under sterile air stream. Bacterial suspension containing $3x10^6$ cfu ml^{-1} was taken in 500 ml glass beakers. The seeds were soaked in bacterial suspension using 0.2% sterilized carboxymethyl cellulose as an adhesive (Seed Biopriming). The seeds soaked in sterile distilled water served as control. 10 cm diameter plastic pots were filled with 500 g of sterilized soil and infested with the pathogen propagules (0.5g/ pot) three days prior to seed sowing. 100 ml of bacterial suspension per pot containing $3x10^6$ cfu ml^{-1} were once again applied as drench treatment after the emergence of shoots (5 days old plant). Each treatment was carried out in three replications with at least 50 plants in each replicate under same physical and environmental conditions. The experiment included four treatments: 1- Healthy; 2-Treated with bacteria but un-inoculated with the pathogen; 3- Untreated but inoculated with pathogen ; 4- treated inoculated. Disease assessment was done after 5, 10, 15, 20, 25 and 30 days of drench application of PGPR. Disease index was recorded based on the score 0-6, depending on both underground and above ground symptoms as follows: Seedling blight 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 – entire plant starts withering ; 6–Whole plant die, with upper withered leaves still remaining attached; roots fully rotted. Disease Incidence and Biocontrol efficiency were calculated as described by Xue *et al.* (2009), Disease assessment was done after 5, 10, 15, 20, 25 and 30 days of inoculation.

ISR related enzymes

Peroxidase (EC.1.11.1.7) was extracted following the method as described by Chakraborty *et al.* (1993) with modifications. Peroxidase activity was assayed spectrophotometrically at 460 nm by monitoring the oxidation of O- dianisidine in presence of H_2O_2. Activity was expressed as the increase in absorbance at 460 nm / g tissue/min. Extraction of phenylalanine ammonia lyase (PAL,

EC 4.3.1.5) was done following the method described by Chakraborty *et al.* (1993). Enzyme activity was determined by measuring the production of cinnamic acid from L-phenylalanine spectrophotometrically. The enzyme activity was expressed as µg cinnamic acid produced in 1 min/ g fresh weight of tissue. Extraction and assay of â-1, 3- Glucanase (â-GLU, EC 3.2.1.38) was done using laminarin dinitrosalicylate method as described by Pan *et al.* (1991). The enzyme activity was expressed in terms of µg glucose released/min/g fresh tissue. Extraction and assay of chitinase (CHT, EC 3.2.1.14) was done by following the method described by Boller and Mauch, (1988) with modifications, using N-acetyl glucosamine (GlcNAc) as standard. The enzyme activity was expressed in terms of µg GLcNAc released/ min/g fresh tissue.

Detection of pathogen in soil using Immuno-formats

Polyclonal antibody prepared against *T. cucumeris* in white male rabbit was obtained from the antiserum collection of Immuno-Phytopathology Laboratory, Department of Botany, University of North Bengal. Sustainability of the pathogen in soil in untreated and treated pots were tested by PTA-ELISA (Chakraborty *et al.* 1993) and Dot immunobinding assay (Lange *et al.* 1989).

Statistical analysis

The efficacy of bacterial isolates in field, pot and nursery conditions were undertaken under complete randomized block design. Standard error of mean and Student's '*t*' test were used in various experiments.

RESULTS

Plant growth promoting traits and inhibition of fungal pathogen

A total of 135 rhizospheric soil bacteria were obtained from soil samples collected from different regions of Darjeeling district of North Bengal. All the bacterial isolates were tested for different plant growth promoting traits *in vitro*. A total of 48 isolates were found to solubilize phosphate on initial screening but all of these isolates did not show positive results for all the tested PGP traits. Seven isolates designated as BRHS/C-1, BRHS/P-22, BRHS/R-71, BRHS/R-72, BRHS/S-73, BRHS/P-91 and BRHS/B-104 exhibited all the tested PGP traits *in vitro* conditions which included production of IAA, HCN, Siderophores and ACC deaminase activity (Table 1). These seven isolates were further tested for antifungal activity against *T. cucumeris.* Antagonistic tests against the pathogen showed that all the seven isolates had the ability to inhibit the fungal growth. However two isolates, BRHS/ C-1 and BRHS/S-73 were found to be the most efficient in inhibiting the test pathogen up to 77.90 and 75.50 percentages respectively (Table 2).

16S rDNA sequence analysis for identification of PGPR isolates and Phylogeny

Seven bacterial isolates designated as BRHS/C-1, BRHS/P-22, BRHS/R-71, BRHS/R-72, BRHS/S-73, BRHS/P-91 and BRHS/B-104 were found to be the most promising PGPR isolates based on their *in vitro* characterization. The identity of each individual PGPR isolate was confirmed on the basis of 16S rDNA sequences. The ITS region of the bacterial isolates were amplified with the help of universal primer pair. After direct sequencing of the PCR product 16S rRNA gene sequence of approximately 800 to 1,400 base pairs were obtained. The obtained sequences were further used to query against NCBI Genbank Database through BLAST. The analysis revealed that the isolate BRHS/C-1 had 99 % similarity with *Bacillus pumilus*, BRHS/R-71 showed 98 % similarity with *Enterobacter cloacae*, BRHS/R-72 had 99 % similarity with *Paenibacillus polymyxa*, BRHS/S-73 had 99% similarity with *Bacillus altitudinis,* BRHS/ P-91 had 99 % similarity with *Bacillus methylotrophicus*, BRHS/P-92 had 99 % similarity with *Burkholderia sp*. and BRHS/B-104 had 99 % similarity with *Bacillus aerophylus.* The sequences were approved as 16S rRNA gene sequence by NCBI after complete annotation (base pair after annotation= 1,321). All these PGPR isolates have been deposited in the National Agriculturally Important Microbial Culture Collection (NAIMCC) of National Bureau of Agriculturally Important Microorganisms (NBAIM), ICAR and have been provided accession numbers. The NCBI Accession numbers and NAIMCC culture codes for each isolate has been provided in Table 3.

For phylogenetic analysis, 16S rRNA gene sequence of all the seven isolates were compared with ex-type sequences of the representative

Table 1: Quantification of Phosphate solubilizing, IAA production and ACC deaminase production of bacterial isolates in modified liquid broth medium

Isolate	ACC Deaminase*	IAA (mg/L)	Phosphate solubilized (µg/ml) TCP**	RP* **	Isolate	ACC Deaminase*	IAA (mg/L)	Phosphate solubilized (µg/ml) TCP**	RP***
BRHS/C-1	20.60	24.55±2.4	765.66±11.5	434.33±10.8	BRS/T-58	-	-	481.55±12.3	329.68±12.6
BRHS/C-2	-	16.22±1.4	612.44±09.4	212.37±07.4	BRS/T-60	-	-	375.25±12.7	298.55±14.4
BRHS/C-5	-	-	429.66±10.8	317.33±10.4	BRHS/R-71	12.22	10.42±1.1	355.12±12.8	287.36±11.2
BRHS/C-6	-	-	404.66±11.0	278.33±08.4	BRHS/R-72	16.42	8.43±1.7	370.55±11.3	278.55±11.3
BRHS/M-10	-	-	416.44±12.4	308.42±07.4	BRHS/S-73	40.63	31.47±3.7	837.33±9.4	411.67±4.7
BRHS/M-17	-	18.43±1.3	630.33±12.6	383.33±12.5	BRHS/S-74	-	-	601.46±7.8	328.33±8.5
BRHS/M-18	-	12.66±1.1	655.66±13.8	354.66±12.6	BRHS/S-75	-	-	421.32±6.5	213.53±9.9
BRHS/M-19	-	-	428.22±11.6	310.44±12.5	BRHS/S-79	-	-	518.25±4.6	283.16±5.3
BRHS/M -21	-	-	513.88±10.5	203.44±10.4	BRHS/S-80	-	-	602.18±4.3	349.24±9.0
BRHS/P-22	-	24.22±1.5	506.29±11.4	316.98±10.8	BRHS/S-81	-	16.3±1.3	657.68±5.9	308.32±7.5
BRHS/P-25	-	16.43±1.5	605.66±12.9	426.33±13.3	BRHS/S-82	-	-	322.72±8.4	121.81±06.3
BFS/M-26	-	18.22±1.2	488.55±13.0	297.66±12.4	BRHS/S-83	-	-	259.33±6.8	125.47±04.1
BFS/M-28	-	11.43±1.1	346.33±12.1	290.33±12.8	BRHS/S-84	-	-	513.44±5.2	172.74±08.6
BFS/M-29	-	-	422.33±17.5	279.66±11.6	BRHS/S-86	-	18.27±3.2	518.25±4.7	283.16±05.3
BFS/S-34	-	-	362.66±17.8	272.33±10.7	BRHS/S-89	-	9.27±0.3	563.12±6.8	218.18±05.8
BFS/S-35	-	-	474.33±14.9	269.66±11.2	BRHS/S-90	-	14.35±1.4	606.66±5.2	324.27±07.4
BRS/Mr-37	-	-	440.58±15.3	280.33±13.4	BRHS/P-91	15.83	13.44±1.5	702.36±11.8	384.56±07.4
BRS/Mr-38	-	-	363.66±11.4	298.33±14.8	BRHS/P-92	18.62	-	714.66±12.4	345.75±08.9
BRS/Mr-39	-	-	518.22±11.3	281.55±13.7	BRHS/B-98	-	-	511.28±11.6	212.44±06.
BRS/Mr-40	-	-	445.78±10.4	235.88±11.5	BRHS/B-99	-	-	487.0±10.1	215.43±08.6
BRS/Mr-42	-	7.82±1.2	368.67±11.5	272.45±14.3	BRHS/B-104	17.14	23.44±1.5	782.33±11.4	387.65±07.6
BFS/Md-46	-	12.44±1.6	345.23±10.7	248.55±14.8	BRS/R-119	-	10.46±1.7	564.20±10.5	213.45±08.3
BFS/Md-55	-	-	312.85±13.8	326.22±13.3	BRS/R-121	-	-	463.22±10.4	255.15±06.5
BFS/Md-56	-	-	452.77±11.5	348.55±12.7	BFS/C-133	-	-	212.43±10.1	352.18±08.2

*ACC deaminase activity expressed as -α-ketobutyrate/ mg/h, **TCP=Tricalcium Phosphate (Total P-920 mg/L) ***RP=Rock Phosphate (Total P-592 mg/L); - = Activity not detected; Data average of three replicate experiments ±= SE

Table 2: *In vitro* pairing of bacterial isolates with phytopathogenic test fungus

	Diameter of fungal colony after 7 days growth(cm)	% of Inhibition
Thanatephorus cucumeris (Control)	8.60±0.12	-
T. cucumeris + B. pumilus (BRHS/C-1)	1.90±0.15	77.90±1.16
T. cucumeris + E. cloacae (BRHS/R-71)	2.56±0.09	70.23±1.24
T. cucumeris + P. ploymyxa (BRHS/R-72)	2.33±0.11	72.90±1.22
T. cucumeris + B. altitudinis (BRHS/S-73)	2.10±0.08	75.50±1.40
T. cucumeris + B. methylothophicus (BRHS/P-91)	2.77±0.15	67.79±1.63
T. cucumeris + Bukholderia sp. (BRHS/P-92)	2.25±0.18	73.83±1.44
T. cucumeris + B. aerophilus (BRHS/B-104)	2.50±0.15	70.93±1.26

Values are mean of three replicate experiments.±= Standard Error.

species of the genus *Bacillus.* The optimal tree with the sum of branch length = 7.99112791 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap tests (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. Codon positions included were 1^{st} + 2^{nd} +3^{rd} + non-coding. There were a total of 356 positions in the final dataset. The isolates *B. pumilus* and *B. altitudinis* were found to be clustered with the other *Bacillii* strains thus confirming their identity (Fig. 1).

Biocontrol of Seedling blight of B. juncea caused by T. cucumeris

Effect of the seven PGPR isolates (single application) *viz, B. pumilus, B. altitudinis, B. aerophilus, B. methylotrophicus, Paenibacillus*

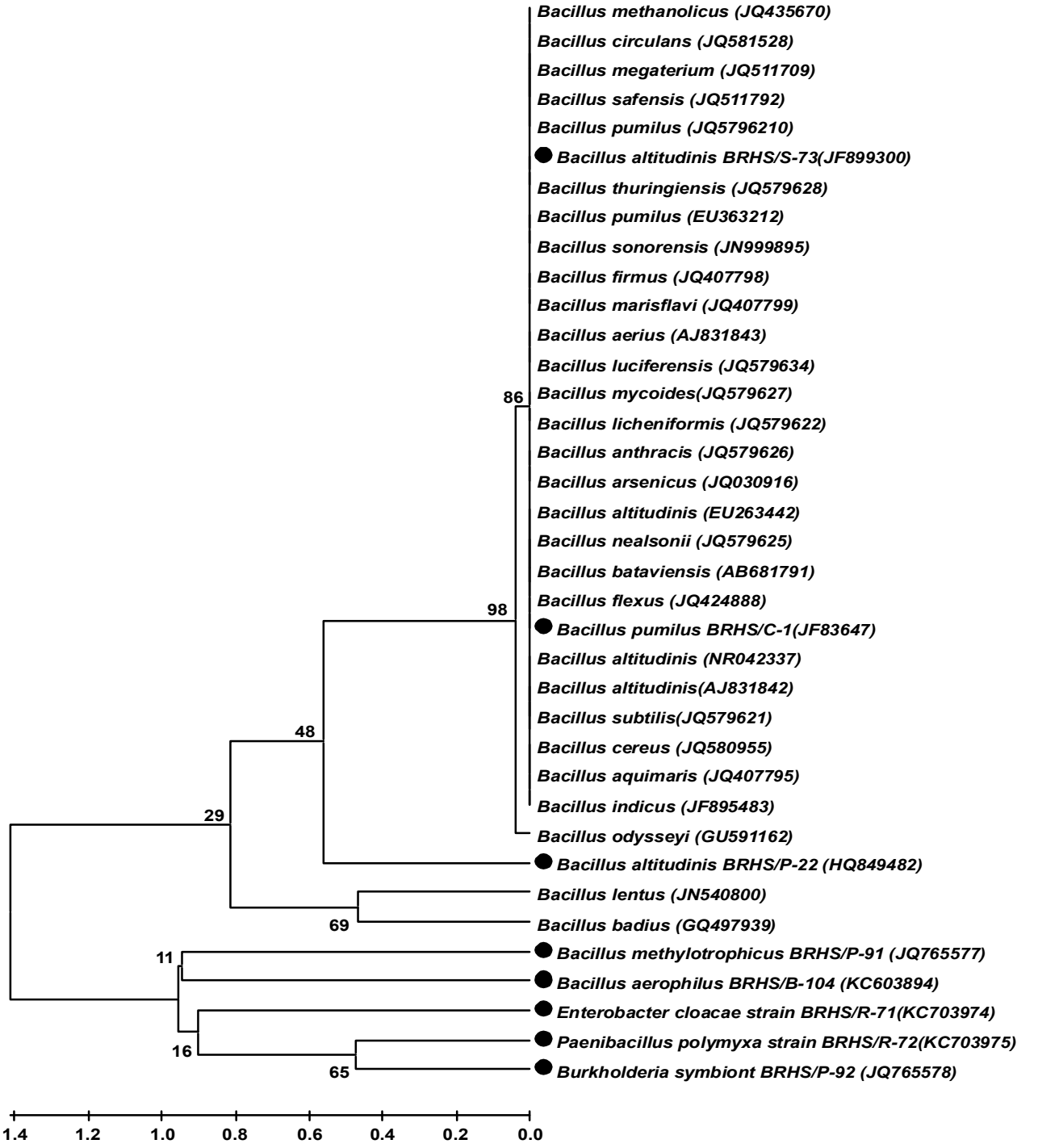

Fig. 1: Phylogenetic tree based on 16S rRNA gene sequences showing the phylogenetic relationship between different PGPR isolates The optimal tree with the sum of branch length = 7.99112791 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches

polymyxa, Enterobacter cloacae and *Burkholderia symbiont* on development of seedling blight disease of *B. juncea* caused by *T. cucumeris* in pot condition was evaluated. Seeds were primed prior to sowing in the pots infested with pathogen and after the emergence of seedlings; PGPR was applied to the rhizosphere soil. Disease assessment was carried out after 2, 4, 6, 10, 12 and 14 days of drench application of PGPR. It was observed that seed bacterization by PGPR isolates followed by drench application in pathogen infested soils were effective in reducing seedling blight incidence. The disease severity

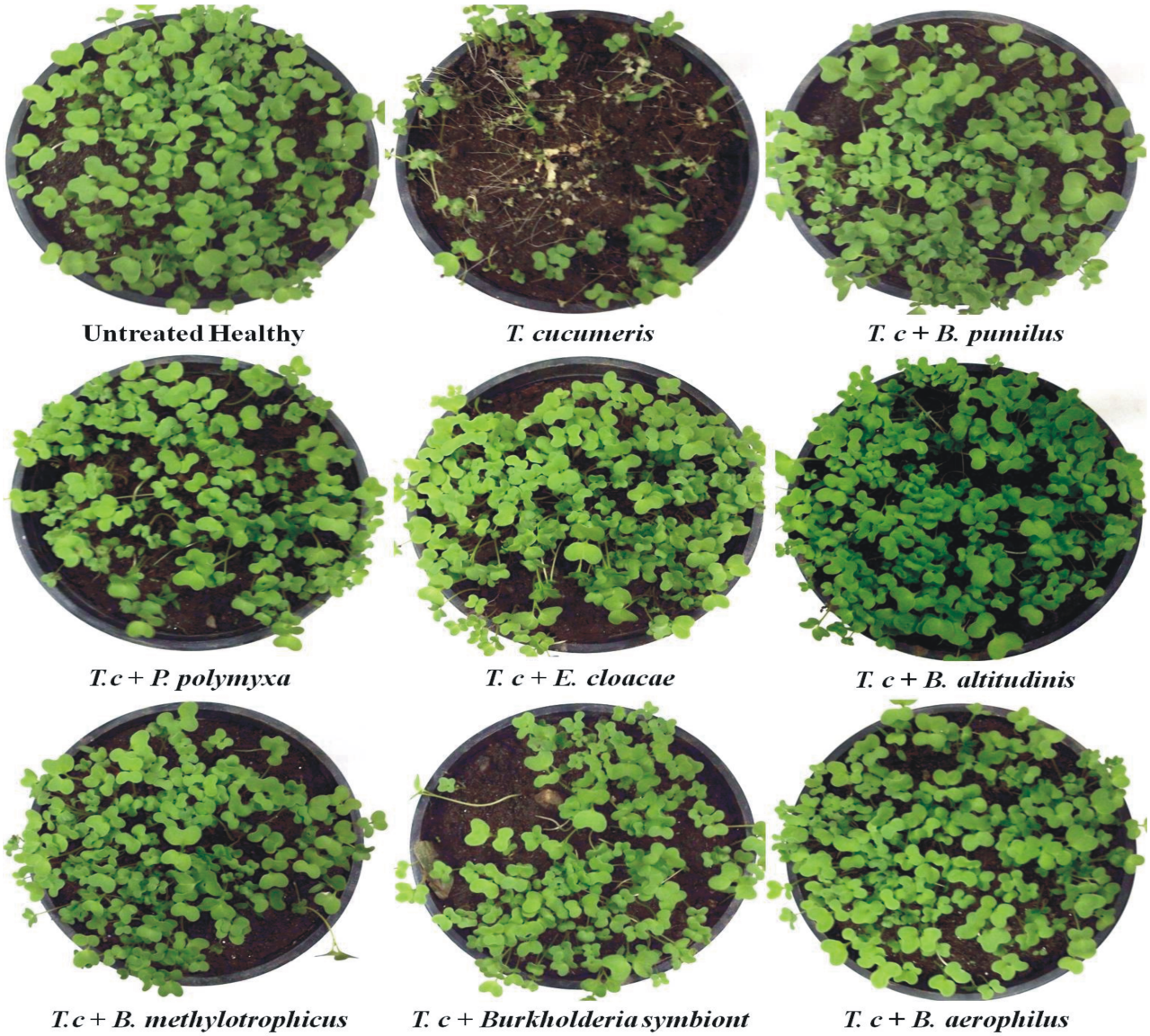

Fig. 2: Experimental setup showing various treatments to evaluate the effect of selected PGPR on seedling wilt of *B. juncea* caused by *T. cucumeris (T.c)*
Untreated Healthy (UH); Untreated Inoculated (UI)- *T. cucumeris;* Treated Inoculated (TI)*: T.c + Bp; T.c + P.p; T.c + E.c; T.c + B.a; T.c + B. m; T.c +B.s ; T.c + B. aero*

Table 3 : NCBI Genbank and NBAIM Accession numbers of PGPR isolates

Sl.No	Isolate	Identified as	NCBI GenBank Acc. No.*	NBAIM Accession Number
1	BRHS/C-1	*Bacillus pumilus*	JF836847	NAIMCC-B01483
2	BRHS/R-71	*Enterobacter cloacae*	KC703974	NAIMCC-B01486
3	BRHS/R-72	*Paenibacillus polymyxa*	KC703775	NAIMCC-B01491
4	BRHS/S-73	*Bacillus altitudinis*	JF899300	NAIMCC-B01485
5	BRHS/P-91	*Bacillus methylotrophicus*	JQ765577	NAIMCC-B01492
6	BRHS/P-92	*Burkholderia symbiont*	JQ765578	NAIMCC-B01489
7	BRHS/B-104	*Bacillus aerophylus*	KC603894	NAIMCC-B01490

*Sequences deposited in NCBI GenBank database. Sequence homology of query and reference >98%.

Table 4: Seedling blight development in the roots of *Brassica juncea* in presence and absence of PGPR isolates in pot conditions

Treatments	Days after PGPR Treatment						
	2d	4d	6d	8d	10d	12d	14d
Thanatephorus cucumeris	2.00	2.25	3.00	3.15	3.50	5.00	6.00
T. cucumeris + B. pumilus	0.25	0.50	0.50	0.75	0.75	1.00	1.15
T. cucumeris + B. altitudinis	0.15	0.50	0.50	0.75	0.75	1.00	1.00
T. cucumeris + B. aerophilus	0.15	0.25	0.35	0.45	0.50	0.50	0.75
T. cucumeris + B. methylotrophicus	0.25	0.75	0.85	1.00	1.15	1.25	1.50
T. cucumeris + P. polymyxa	0.25	0.50	0.50	0.75	1.15	1.25	1.30
T. cucumeris + E. cloacae	0.25	0.75	1.00	1.00	1.15	1.45	1.50
T. cucumeris + B. symbiont	0.25	0.50	0.50	0.75	1.15	1.50	1.75

Disease 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 – Extensive rotting of the collar region of the root 60-70% root and leaves withered; 5- 80%of the root effected 80-85% of the shoot and leaves withered; 6-entire plants dies with upper withered leaves still remaining attached; roots fully rotted. Values are average of three replicate sets (10 plants each). ± = Standard Error.

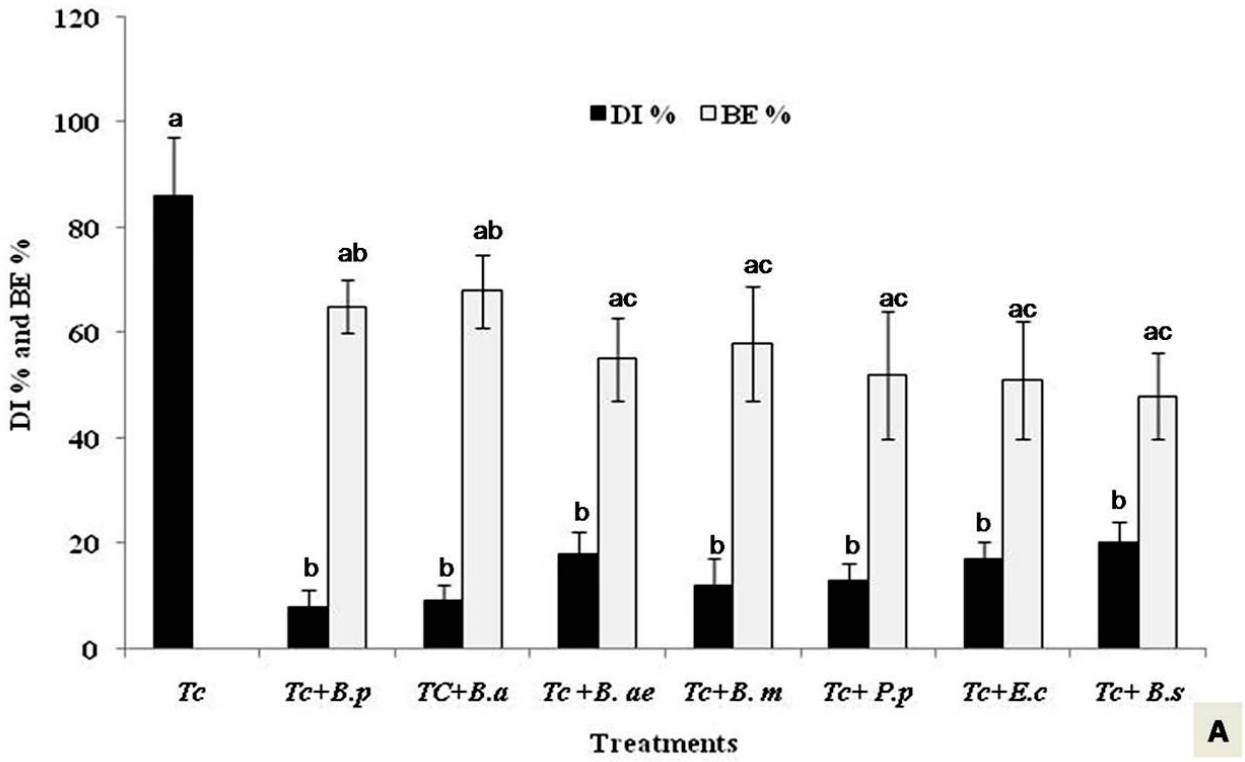

Fig. 3: Effect of selected PGPR on Disease Incidence (DI) of seedling wilt in *B. juncea* caused by *T. cucumeris* and Biocontrol Efficacy (BE) of applied PGPR against *T. cucumeris, Tc= T. cucumeris, Bp= B. pumilus, Ba=B. altitudinis, B ar=B. aerophilus, Bm=B. methylotrophicus, Pp=P. ploymyx, Ec=E. cloacae,Bs=Burkholderia symbiont.* Bars with different letters indicate significant differences between untreated inoculated (*T. cucumeris*) and Treated (ab, abab, *P* =0.01; abac, *P*=0.05). Similar letters indicate insignificant.

Table 5: PTA-ELISA values and colour intensity of dot immunobinding assay (DIBA) of PGPR applied soil antigens using PAb of *Thanatephorus cumeris*

Antigens from rhizosphere of	Treatment	ELISA A_{405} values*	DIBA Colour intensity of dots **
	T. c	2.016±0.143[a]	+++
	T. c + B. p	0.820±0.044[c]	+
B. juncea	*T. c + B. a*	0.711±0.074[b]	+
	T. c + B. ar	1.020±0.044[b]	++
	T. c + P. p	0.972±0.047[c]	+
	T. c + E. c	1.220±0.421[b]	++
	T. c + B. s	1.166±0.446[b]	++

*Average of three replicates, ±=SE ; PAb dilution 1: 100: Alkaline phosphatase dilution 1:10,000; Substrate NBT/BCIP

**+= Light purple; +++ Purple; +++ = deep purple. Different letters indicate significant differences between ELISA values of Untreated inoculated and treated inoculated (ab, P =0.01; ac, P=0.05). Similar letters indicate insignificant.

Tc= T. cucumeris, Bp= B. pumilus, Ba=B. altitudinis, B ar=B. aerophilus,Bm=B. methylotrophicus, Pp=P. ploymyx, Ec=E. cloacae,Bs=Burkholderia symbiont.

increased with time and reached a maximum of 6 at the end of 14 days. Among all the bacterial isolates tested, *B. pumilus* and *B. altitudinis* were found to be the most effective in single application. The disease development between the treated and the control untreated pots were significant however it was not significant among different treatments (Table 4, Fig. 2).

Disease incidence (DI %) and biocontrol efficacy (BE %)

The disease severity in *B. juncea* inoculated with only *T. cucumeris* increased with time, reaching a maximum of 86% at the end of 14 d. In contrast, treatments containing primed seeds and PGPR showed maximum disease severity of only 20%. Lowest disease incidence (DI%) was observed in treatments containing *B. altitudinis* (6.66%) followed by *B. pumilus* (7.66%). Among all the treatments, highest Biocontrol Efficacy (BE%) of 74% was shown by *B. altitudinis* followed by *B. pumilus* 65% (Fig. 3). Regardless of the values expressed, *B. pumilus* and *B. altitudinis* could significantly (P = 0.01) reduce the occurrence of seedling blight disease of *B. juncea* caused by *T. cucumeris* under pot conditions.

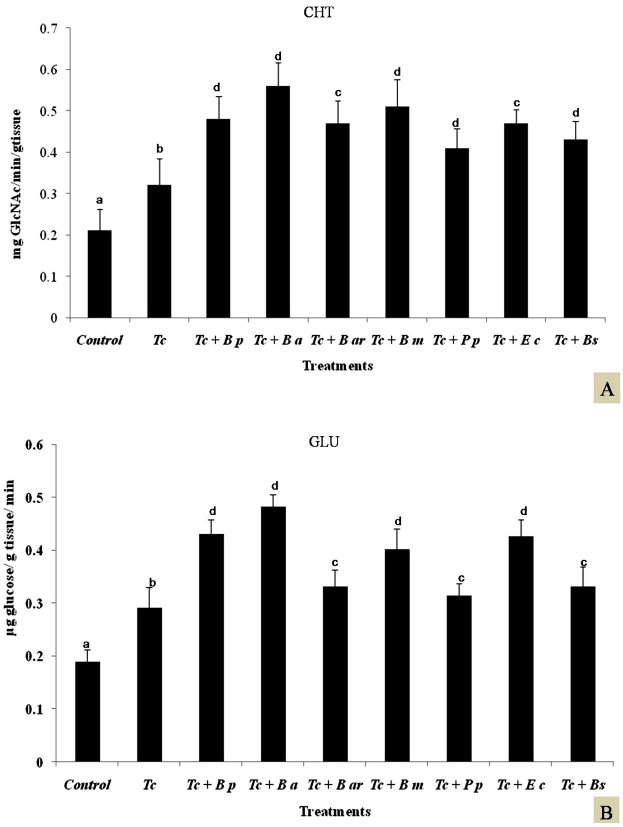

Fig. 4: Activities of (A) Chitinase (CHT) and (B) β-1, 3 glucanase (GLU) in the seedlings of *B. juncea* after 36h of PGPR treatment. *Tc= T. cucumeris, Bp= B. pumilus, Ba=B. altitudinis, B ar=B. aerophilus,Bm=B. methylotrophicus, Pp=P. ploymyxa, Ec=E. cloacae,Bs=Burkholderia symbiont.* Data of three replicate experiments and SE. Bars with different letters indicate significant differences between control and treated (ab, ac P =0.01; ad, P=0.05). Similar letters indicate insignificant.

Plant defense and activities of defense enzymes

Seed bacterization followed by drench application of PGPR was found to affect the biochemical responses of *B. juncea* seedlings in the presence of pathogen- *T. cucumeris*. The reduction in seedling blight incidence by PGPR isolates were noted and the conferment of resistance towards the fungal pathogen was evaluated in terms of enhancement of key defense related enzymes- CHT, GLU, PAL and POX in the seedlings after 36 h of bacterial application to the rhizosphere. The results showed that the activities of all the tested enzymes was higher in plants treated with PGPR isolates (P=0.01). Over all *B. altitudinis* and *B. pumilus* were found to induce resistance against *T. cucumeris* more effectively than the other tested PGPR isolates when applied singly (Figs. 4 & 5).

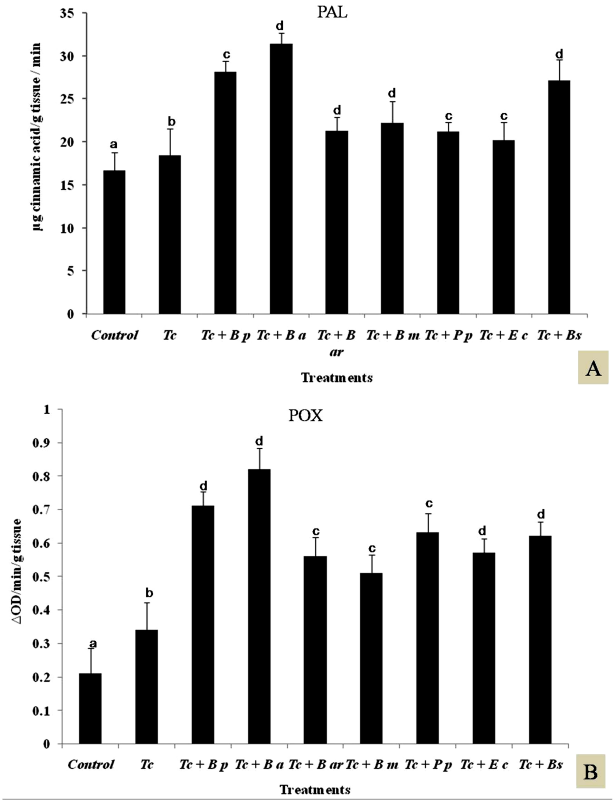

Fig. 5: Activities of (A) Phenylalanine Ammonia Lyase (PAL) and (B) Peroxidase (POX). in the seedlings of *B. juncea* after 36h of PGPR treatment. *Tc= T. cucumeris, Bp= B. pumilus, Ba=B. altitudinis, B ar=B. aerophilus,Bm=B. methylotrophicus, Pp=P. ploymyxa, Ec=E. cloacae,Bs=Burkholderia symbiont.* Data of three replicate experiments and SE. Bars with different letters indicate significant differences between control and treated (ab, ac *P* =0.01; ad, *P*=0.05). Similar letters indicate insignificant

Effect of PGPR on population of T. cucumeris in soil

Pathogen population in the rhizosphere soil of *B. juncea* in untreated and treated soils was determined immunologically using PAbs raised against the pathogen. DIBA and ELISA were conducted after 14 days of PGPR application to the rhizosphere. Results revealed that application of PGPR in the rhizosphere significantly reduced the pathogen population in the rhizosphere soil of the tested crop (Table 5).

DISCUSSION

In the present investigation rhizospheric bacteria were isolated from soils collected from different geographic location of Darjeeling Hills and were screened for *in vitro* PGP characteristics like phosphate solubilization, chitinase, siderophore, HCN, IAA and ACC deaminase production as well as their potential to inhibit *Talaromyces flavus* a causal agent of seedling blight of *B. juncea*. All these characters are considered to be the most important PGP traits and this type of microorganisms play an important role in sustainable agriculture (Bhusan *et al.* 2013; Baker, 2018). Microorganisms with simultaneous phosphate solubilizing and biocontrol potential are the best bioinoculants which can be used as potential plant growth promoters and biocontrol agents (Sunar *et al.* 2017). Selected bacteria showing multiple PGP traits were identified as *Bacillus pumilus* (NAIMCC-B01483), *B. altitudinis* (NAIMCC-B01485), *B. methylotrophicus* (NAIMCC-B01492), *B. aerophilus* (NAIMCC-B01490), *Burkholderia symbiont* (NAIMCC-B01489), *Enterobacter cloacae* (NAIMCC-B01486), *Paenibacillus polymyxa* (NAIMCC-B01491) on the basis of their rDNA sequences. In our previous studies we had demonstrated that the analysis of aligned rRNA gene sequences is a reliable clustering strategy for identification purposes in a variety of taxonomic groups and systemic level. The study showed that it is 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 (Chakraborty *et al.* 2011). In our present investigation, we tested the ability of these seven potential isolates to manage seedling blight of *B. juncea* (common Indian mustard) caused by *T. cucumeris* under pot conditions. Seed bacterization followed by direct application of PGPR as soil drench was found to be effective in preventing the incidence of seedling blight of *B. juncea*. Among the treatments, *B. pumilus* and *B. altitudinis* were found to be the most effective isolates. The current strain of *B. altitudinis* has also been tested for its positive role in plant growth promotion and disease management in crop plants (Sunar *et al.* 2015). The reduction of disease may be due to the ability of these isolates to inhibit growth of the pathogen in the soil by direct antagonism, siderophore production or HCN production. Siderophores sequester ferric iron in the form that cannot be used by the pathogen thereby reducing its number and/or activity. The secondary metabolites produced by certain species of *Bacillus* and *Pseudomonas* have also been reported to have antibacterial or antifungal activity against phytopathogenic microorganisms (Compant *et al.* 2005; Chakraborty *et al.*; 2014, Ossowicki *et al.* 2017; Sahni and Prasad, 2020).

In our present investigation, PGPR were first coated on the seeds (seed biopriming) and the same bacteria were applied as soil drench to the pathogen infested soil. Seed priming with living bacterial inoculum is termed as biopriming that involves the application of plant growth promoting rhizobacteria. It increases speed and uniformity of germination. Seed biopriming allows the bacteria to enter/adhere the seeds and also acclimatization of bacteria in the prevalent conditions (Mahmood *et al.* 2016). Biopriming has been applied in various crops for the biocontrol of several diseases. Abuamsha *et al.* (2011) successfully demonstrated that biopriming the oilseed rape cultivars with PGPR could control the pathogen *Leptosphaeria maculans* causing blackleg disease upto 71.6%. Along with the crop productivity, biopriming can also be favored as the potential technique for biocontrol of several plant pathogens (Mahmood *et al.* 2016; Takishita *et al.* 2018). Apart from seed bio-priming we also adopted another approach of directly applying the PGPR in the soil keeping in mind that application of the inoculum directly to the soil is favored when there is threat of presence of antagonistic microbes / pathogens on the plant tissues. As a result to this approach the pathogen population was significantly reduced. Thus this two way approach was successful in our present investigation to control the seedling blight incidence up to 74%. In order to determine whether the tested PGPR isolates elicits ISR in *B. juncea*, several biochemical analyses were conducted. The major components analyzed in the present study included PR proteins-chitinase (CHT), â-1, 3-glucanase (GLU) and defense related enzymes peroxidase (POX) and phenylalanine ammonia lyase (PAL) which increased significantly after the application of PGPR in pathogen infested soil. Increase in the activities of these enzymes is related to enhanced defense against different fungal pathogens and has been successfully demonstrated in studies conducted by Chakraborty *et al.* (2004, 2009, 2014), Sunar *et al.* (2014, 2017); Lu *et al.* 2017 and Jayapala *et al.* 2019 where PGPR like *B. altitudinis*, *Bacillus megaterium, B. pumilus* and *Ochrobactrum anthropi* were successfully utilized to overcome several root diseases. Pathogen was applied to sterile soil prior to PGPR inoculation which was intended to mimic the field conditions. The control treatments had the same soil from the same natural environment, which indicated that the observed differences were due to the introduced bacterial isolate. Our results suggested that seed bacterization and direct application of these PGPR resulted in induction of resistance in *B. juncea* against *T. cucumeris* and at the same time reduced their population in the soil. *Bacillus*-based biocontrol agents play a fundamental role in the field of biopesticides. Many *Bacillus* species have proved to be effective against a broad range of plant pathogens. They have been reported as plant growth promoter, systemic resistance inducer, and used for production of a broad range of antimicrobial compounds (lipopeptides, antibiotics and enzymes) and competitors for growth factors (space and nutrients) with other pathogenic microorganisms through colonization (Shafi *et al.* 2017). Thus it is clear from the results of our present study that rhizosphere soil microorganisms have the ability to induce resistance in crop plants against fungal pathogens efficiently by combination of mechanisms. Further, our current investigation also suggests that *Bacillus pumilus* (NAIMCC-B01483) and *B. altitudinis* (NAIMCC-B01485) are the most potential PGPR isolated from Darjeeling Hills which can be suitable candidates for developing microbial formulations for betterment of plant growth and improving their health status.

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