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Bioprospecting and Antifungal Potential of Novel Endophytic *Bacillus* in Rice against Sheath Blight Disease Incited By *Rhizoctonia solani*

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Sheath blight of rice caused by *Rhizoctonia solani* (teleomorph: *Thanatephorus cucumeris*) has become a major constraint to rice production. The pathogen is cosmopolitan with a very wide host range and attacks large number of crop plants and weeds. Various strategies have been undertaken to manage the disease but viable control measure is lacking till date. In this context, biological control of the disease using Plant Growth Promoting Endophytic bacteria (PGPE) is a potential alternative to the presently available chemical control methods. In this study, a total of 80 endophytic bacteria have been isolated from different rice tissues *viz.*, root, stem and leaves and characterized. They were further narrowed down to 20 strains on the basis of *in vitro* production of antifungal antibiotics, indole acetic acid, siderophore, phosphate solubilization, hydrolytic enzymes and were also evaluated for antagonism against rice sheath blight pathogen (*Rhizoctonia solani*). These isolates were evaluated in greenhouse in reducing sheath blight incidence and for significant enhancement in growth (root and shoot length) and yield of rice plants. These isolates were further characterized for for the presence of chitinase gene (chiA) and β-1,3 glucanase gene using polymerase chain reaction. Most of the isolates could amplify the bacillomycin, chitinase gene and β-1,3-glucanase genes encoding a product size of 395, 270 bp and 415 bp respectively. Among the 20 isolates assayed *B. subtilis* (EBPBS4) was highly effective in reducing the sheath blight incidence. This could also inhibit the mycelial growth of *R. solani* under *in vitro* conditions when compared to other isolates. It can be inferred from the study that, mycolytic enzymes producing *Bacillus* spp. play a major role in the lysis of the mycelium of *R. solani* and could be used for the management of sheath blight of rice. PCR amplification of bacillomycin (bmyB), chitinase and β 1-3glucanase genes suggests that amplified genes from the *Bacillus* could have a role to further define the biocontrol activities in the suppression of *R. solani*.

Key words: *Bacillus subtilis,* Bacillomycin, β-1, 3-glucanase, *Rhizoctonia solani*.

Increasing global population creates significant concern over the agricultural production to cope with requirement of sufficient food for all. In this context, management of diseases in cereal crops is vital to alleviate food shortages and to improve efficiency in food production. Among cereals, Rice (*Oryza sativa* L.) is the world's most important crop and India is the second largest producer and contributing approximately 90 % to world's rice production (IRRI, 2014). Rice productivity, however, remained virtually stagnant from past few decades because of its susceptibility to sheath blight disease.

Rice sheath blight caused by *R. solani* has been regarded as an important disease in all rice-growing countries (Eizenga *et al.* 2002; Boukaew *et al.,* 2013). The synergistic interaction of these pathogens can cause more than 5- 55% yield losses, if proper and timely measures are not taken to manage these pathogens (Pandey *et al.* 2007). Moreover, farmers heavily rely on fungicides

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to manage these soil-borne pathogens. Because of concerns regarding both human health and environment, viable alternatives to these chemicals are being sought (Franks *et al.,* 2006). It has been recognized that a large number of naturally occurring rhizospheric bacteria are antagonistic towards plant pathogens and as a result may offer a viable substitute for abandoning the use of these chemicals to enhance crop growth and yield.

Plant rhizosphere is a versatile and dynamic ecological environment of intense plant–microbe interactions for harnessing essential micro- and macro-nutrients from a limited nutrient pool (Solanki *et al.,* 2012a). We focused on bacterial genera that are often found in large populations in soils with general disease suppression, such as gram positive spore-forming species belonging to Bacillus and Bacillus derived genera. Bacillus has many characteristics as an excellent biocontrol agent, including the production of diverse antibiotics (Solanki *et al.* 2012b; Liu *et al.* 2006), formation of viable spores (Cenci *et al.,* 2006), promotion of plant growth (Ryu *et al.* 2004), and ubiquitous presence in soil (Gajbhiye *et al.,* 2010). Some of the well documented characteristics of *Bacillus* spp. related to soil fertility and plant nutrition mobilization are the production of bacterial phytohormones and solubilization of mineral phosphates (Calvo *et al.* 2010), which allow them to inhabit diverse niches in agro-ecosystems. Additionally, strains of Bacillus have also paramount advantages over other biocontrol bacteria in several manners such as they are mostly soil inhabitants, have long shelf life and also impart phosphorus- solubilization, resulting in improved growth and yield of crops (Quan *et al.,* 2006).

Considering the multiple applications of *Bacillus* spp., it is essential to study their PGPR activity (Huang et al. 2007), which is useful in designing strategies to exploit them as bioinoculants for sustainable and integrated disease management without causing harm to the environment and farmers. Moreover, there is a very limited knowledge regarding the biological suppression of rice pathogens by the application of PGPE. Thus, the major objectives of present investigation were to (1) investigate the antagonistic ability of *Bacillus* spp. isolated from different regions of Tamil Nadu against rice pathogens; (2) evaluate their plant growth promotion activities in order to use them further as inoculant strains.

MATERIALS AND METHODS

Survey

An extensive survey was made in the different districts of Tamil Nadu to collect different tissues of rice and its rhizosphere soil for isolation of endophytic bacteria and rhizobacteria, respectively. The sheath blight disease severity was also surveyed and the pathogen, *Rhizoctonia solani* isolated and maintained in pure culture.

Isolation of endophytes

Rice root and stems sections (2-3 cm long) was made using a sterile scalpel. Samples was weighed and surface sterilized with 1% sodium hypochlorite (NaOCl) in 0.05% triton X-100 for 10 minutes and rinsed four times in 0.02 M sterile potassium phosphate buffer pH 7.0 (PB). A 0.1 ml aliquot is taken from the final buffer wash and transferred to 9.9 ml tryptic soy broth (TSB) to serve as sterility check. Samples were discarded if growth is detected in the sterility check samples (agitating samples in TSB, Hi Media Code No. M 011, at 28±2ºC) within 48 h. Each sample (0.5g) was triturated with a sterile mortar and pestle in 9.5 ml of the final buffer wash. Serial dilutions up to (10^{10}) of the triturate were made in PB. Each dilution of every sample is plated (0.1 ml) on three plates each of Nutrient agar (NA g/l; peptone 5, beef extract 2, and agar 20, pH 5.0) (Harish *et al*., 2008b). The plates were incubated at 28±2ºC for 48-72 h. At each sampling date and for each treatment one representative of each bacterium, as evident from their colony type and morphology is transferred to fresh NA Medium plates to establish pure cultures.

Phenotypic characterization of bacterial isolates

Native endophytic bacterial isolates were characterized based on morphology and biochemical methods. Colony morphology of isolates was studied under a stereoscope microscope (Leica). This included shape, edge, elevation, surface and pigmentation. The Bacterial identification was carried out on the basis on Bergeys Manual of Systematic Bacteriology (Clauss and Berkeley, 1986). The *Bacillus* species were estimated on the basis of morphological and physiological characteristics.

Biochemical characterization of *Bacillus subtilis*

Characterization included growth at different temperatures and the ability to utilize different substrates as a sole carbon source. Other than this the bacterial biochemical response was tested by using ready biochemical kit for the specific identification of gram positive rods (Rapid biochemical identification test kits- KB002 Hi Assorted TM Biochemical test, HiMedia laboratories Pvt. Ltd). Results of these tests were scored either as positive or negative and grouped with the aid of manual in the kit.

Evaluation of effective PGPR strains on seed germination and seedling vigour Seed germination assay

The experiment was conducted to assess the influence of 20 selected efficient isolates on seed germination and tested for their plant growth promotion traits

Growth promotion test

Bacillus subtillis strains were grown in 250 ml conical flasks containing 100 ml of NA broth for 48 h on a rotary shaker (150 rev min-1) at 28±2ºC. Cells were removed by centrifugation at 8000 rpm for 10 min at 4ºC and washed in sterile water. The pellet was resuspended in small quantity of sterile distilled water and then diluted with adequate amount of sterile distilled water.

Seeds of rice were surface sterilized with two per cent sodium hypochlorite for 30 sec, rinsed in sterile distilled water and dried overnight under sterile steam air. *B. subtilis* strains inoculated into NA broth was taken in a Petri dish. One gram of seeds were soaked in 10 ml of bacterial suspension (containing $3x10^8$ cfu/ml) for 2 h and dried overnight in a sterile Petri dish.

Plant growth-promoting activity of *B. subtilis* strains were assessed based on the seedling vigour index by the standard roll towel method (ISTA, 1993). Twenty bacterized seeds were kept over the presoaked germination paper. The seeds were held in position by placing another presoaked germination paper strip and gently pressed. The polythene sheet along with seeds were then rolled and incubated in growth chamber for 15 days. Three replications were maintained for each treatment. The root length and shoot length of individual seedlings were measured and the germination percentage of seeds was also calculated.

The vigour index was calculated by using the formula as described by Abdul Baki and Anderson (1973).

Vigour Index = (Mean root length + Mean shoot length) x Germination (%).

Characterization for plant growth promoting traits

The growth promotion traits of endophytic bacterial isolates were evaluated by performing standard protocol for the estimation of indole acetic acid (IAA), P-solubilization, siderophore, hydrogen cyanide (HCN) and ammonia production according to Glickmann and Dessaux (1995), Brick *et al.* (1991), Schwyn and Neilands (1987), Lorck (1948) and Dey *et al.* (2004), respectively. For P-solubilization, plates containing Pikovskaya's media amended with tri-calcium phosphate were observed for clearing or solubilization zones around the colonies. Similarly, siderophore production was confirmed by observing clear halo zone formation on Chrome Azurol S medium. Productions of hydrolytic enzymes (chitinase, b-glucanase, and protease) were determined according to Solanki *et al.* (2012a). All assays were repeated thrice with three replications

Tests for antagonism

The pathogenic isolates of *Rhizoctonia solani* were isolated from sheath blight infected rice tissues. *Bacillus* strains were evaluated for their in vitro antifungal activity in dual culture assays on potato dextrose agar (PDA) with the plant pathogen *R. solani* of 7 mm diameter taken from an actively growing culture and was placed at centre on the surface of the PDA plate. *Bacillus* strains grown in nutrient broth to a concentration of $6x10^8$ cell per ml were streaked in a straight line on the edge of the NA + PDA (1:1) plate (3 cm from the centre) containing fungal disk, followed by incubation at 28 ± 2 ºC for 5 days or till the fungal mycelia completely covered the entire plate in control. Plate inoculated with fungal disk alone was used as a control. The antifungal activity was evaluated by measuring the growth inhibition against test fungi. The Percentage of inhibition was calculated as follows:

Percent inhibition =[(C - T)] /C* 100

where, C is the radial growth of fungus in control, and T is the radial growth of the fungus in the presence of test organism. The strains

exhibiting more than 50 % of mycelial growth inhibition were considered as promising antagonist. All assays were repeated thrice with three replications.

To study antifungal activity of cell free crude extract of selected screened bacteria against fungal pathogens, bacterial strains were grown in yeast extract-glucose (YEG) medium for three days in an orbital incubator shaker with 80 rpm at 30 ± 2 ºC. Culture filtrate was separated from

bacterial cells through centrifugation (10,000 rpm for 10 min at 20 ºC) and filter sterilized (0.2 mm pore size), and was stored at -20 ºC until further use.

Hyphal growth inhibition by culture filtrates

The culture filtrates of the *B. subtilis* were mixed (1:1) with double strength (2×) of melted (and cooled at 45 æ%C) sterile PDA and poured onto 9 cm diameter plates. Experiments were replicated three times. A 9mm agar plug of an actively growing fungal mycelium of *R. solani* cultures was placed on the center of the test agars. The fungal cultures were further incubated under moist conditions at 25 æ%C for 2 days (*R. solani*). Two perpendicular directions of radial growth of the fungal colony were measured using a stereo zoom microscope. The percentage of hyphal growth inhibition was calculated as described earlier (Gamliel, 1989).

The enzyme assay

Glucanase activity was determined by a colorimetric method (Burner, 1964). The amount of reducing sugar released from laminarin (Sigma Chemical Co. St. Louis, MO) was measured. The standard assay contained 10 µl of the crude enzyme solution and 90 µl of 5 mg/ml laminarin in 0.1M sodium acetate buffer pH 5.0. After incubation at 40 æ%C with gentle shaking for 10 min, the reaction was stopped by boiling for 5 min and 0.2 ml of 1% dinitrosalicylate (DNS) and 0.2 ml of sodium acetate buffer were added and boiled for another 5 min, then placed in an ice bath and 0.9 ml distilled H2O was added. The optical absorption was measured at 540 nm. The amount of reducing sugar released was calculated. expressed in units (mol glucose equivalent/min). Proteins concentrations were determined by the method of Bradford (1976) and bovine serum albumin was used as standard.

DNA extraction from isolates

Bacillus spp. was grown in nutrient broth or on nutrient agar plates at 28 °C. Total DNA (including chromosomal and plasmid DNA) was extracted as described by Robertson *et al*. (1990) with slight modifications. Cultures grown for 16 h in nutrient broth were centrifuged into a pellet, washed in TE (10 mM Tris pH 7.5/1 mM EDTA pH 8.0) and suspended in 10% sucrose. Cells were incubated at 37 °C in lysozyme solution (5 mg/ml lysozyme, 50 Mm Tris pH 7.5, 10 mM EDTA pH 8.0), followed by addition of 20% SDS containing 0.3% beta-mercaptoethanol. DNA was purified by organic extraction and ethanol precipitation. Purified DNA was quantified by UV spectrophotometry.

Polymerase Chain Reaction

To prove strains as *Bacillus* sp., 16S rRNA interveining sequence specific BCF1 (CGGGAGGCAGCAGTAGGGAAT); BCR2 (CTCCCCAGGCGGAGTGCTTAAT) primers were used to get an amplicon size of 546 bp. Amplification of 16S rRNA gene was performed from the genomic DNA of strains using universal primers BCF1 (CGGGAGGCAGCAGTAGGGAAT); BCR2 (CTCCCCAGGCGGAGTGCTTAAT) primers. PCR cocktail (20 µl) contained 10 pM of each primer, 50 ng of genomic DNA, 10X Taq DNA polymerase buffer, 1 U of Taq DNA polymerase (Banglore Genei, India) and 2.5 mM of each dNTP. Amplification was performed in a thermo cycler (Quanta,) at 95 ºC for 5 min, followed by 30 cycles of 1 min at 95 ºC, 1 min at 55ºC and 1 min at 72 ºC with a final extension at 72ºC for 5 min. A 5 µl aliquot of each amplified product was electrophoresed on 1.2 % agarose gel along with 100 bp DNA ladder as marker in 1X TAE buffer at 80 V for 45 min, stained with ethidium bromide and visualized with a UV transilluminator.

Detection of bacillomycin (bmyB), chitinase and ²-glucanase genes

Primers and all the reagents were obtained from Bangalore Genei Pvt. Ltd., Bangalore, India. The primers used for molecular analyses are given in Table 4. Genomic DNA of *Bacillus* strains was extracted by cell lysis method and PCR amplification of bacillomycin (bmyB) and b-glucanase genes was performed using the PCR primers: For bmyB gene, F-5'-TGAAACAAA GGCATATGCTC-3' and R-5'-AAAAATGCATCTGCC GTTCC-3' (Joshi and McSpadden Gardener, 2006) and ²-glucanase gene, F-5'-AATGGCGGTGTATTCCTTGACC-3' and R-

5'-GCGCGTAGTCACAGTCAAAGTT-3' (Solanki *et al.* 2012b). PCR cocktail (25 µl) contained 10 pM of each primer, 50 ng of genomic DNA, 10X Taq DNA polymerase buffer, 1.0 U of Taq DNA polymerase (Banglore GeNei, India) and 2.5 mM of each dNTP. Amplification was performed in a thermo cycler (Bio-Rad Laboratories, CA, USA) at 94 ºC for 4 min; 35 cycles of denaturation at 92 ºC for 1 min, annealing at 54 ºC for bmyB and 55.5 ºC for ²-glucanase for 45 s, and extension at 72 ºC for 1 min each, followed by a 7 min extension at 72 ºC. The PCR products were resolved on 1.2 % agarose gel with 100 bp ladder.

Statistical Analysis

The data were statistically analyzed using the IRRISTAT version 92 developed by the International Rice Research Institute Biometrics unit, the Philippines Gomez and Gomez, 1984. The percentage values of the disease index were arcsine transformed. Data were subjected to analysis of variance (ANOVA) at significant level ($P < 0.05$) and means were compared by Duncan's Multiple Range Test (DMRT).

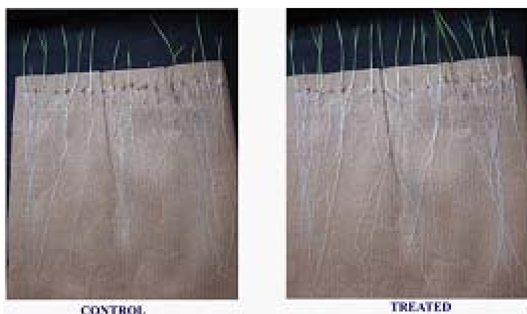

Fig. 1. Evaluation of effective PGPR strains on seeds germination and seedling vigour

Fig. 2. Effect of bioformulation of Enophytic *Bacillius* strain (EB PBS$) on rice seed under pot culture condition

RESULTS AND DISCUSSION

Invitro screening of strains and test for antagonism

A total of 80 bacilli were selectively isolated from the rice tissues of different districts of Tamil Nadu. A total of twenty efficient endophytic *Bacillus* strains were selected on the basis of colour, morphological characteristics viz., colony morphology (shape, margin, elevation and surface) and cell morphology (Gram s reaction, cell shape and arrangement) and were studied in detail (Table 1). Bacteria exhibited wide morphological variation. Maximum variation in colony shape *viz.*, irregular, circular. Maximum of the obtained bacterial from different rice variety samples were Gram positive Bacilli with mucoid texture, entirely edged and creamy white in appearance. All the isolates were tested for *in vitro* screening against rice sheath blight pathogens (Fig. 2). Out of them, only twenty bacterial (EBPBS4, EBAM1, EBWL8, EB MD6, EBWL10, EBAL1,EBAM2, EBAS3, EBMD2, EBMD5, EBMD1, EBPBS 5, EBMD7, EBCO2, EBAL4, EBAS2, EBMD4, EBWL7, EBCO3 and EBTN1) isolates displayed strong antagonistic activity and showed significant inhibitory effect on mycelial growth against *R. solani* (Table 2) These results were in accordance with Solanki *et al*. (2012a), where they reported the antagonistic potential of *Bacillus megaterium, B. subtilis, B. amyloliquefaciens* and *Bacillus* spp. This finding supports earlier observations that, virtually all natural and agricultural soils studied so far possessed some ability to suppress the activity of soil-borne plant pathogens due to the presence and activity of soil microorganisms, generally referred as soil suppressions (Postma *et al.,* 2008).

Among twenty strains, eight strains *viz.,* EBPBS4, EBAM1, EBWL8, EB MD6, EBWL10, EBAL1 and EBAM2 showed positive reaction to malonate, sucrose, mannitol, glucose, arabinose and trehalose utilization tests. All the twenty five strains showed negative reaction to Voges

Fig. 3. Antogonstic activity of EB PBS$ against *Rhizoctonia solani* under invitro

Table 1. Bio chemical Characterization of *Bacillus* spp.

S. No	Isolates	Simple staining	Gram staining	KOH test	Starch hydrolysis	Gelatin hydrolysis	Anaerobic groewth	Citrate utilization	Growth in 7% NaCl	Growth at 45ºC	Endospore staining	Catalase activity	P Solubilization activity	Sideophore production
1.	EBPBS4	Long rod	+	-	+	+	+	+	+	+	+	+	+	+++
2.	EBAM1	rod	+	-	+	+	+	+	+	+	+	+	+	++
3.	EB MD6	rod	+	-	+	+	+	+	+	+	+	+	+	+++
4.	EBWL8	Long rod	+	-	+	+	+	+	+	+	+	+	+	+
5.	EBWL10	rod	+	-	+	+	+	+	+	+	+	-	+	++
6.	EBAL1	rod	+	-	+	+	+	+	+	+	+	+	+	+
7.	EBAM2	rod	+	-	+	+	+	+	+	+	+	+	+	+++
8.	EBAS3	Short rod	+	-	+	+	+	-	+	+	+	-	+	-
9.	EBMD2	rod	+	-	+	+	+	-	+	+	+	-	+	+++
10.	EBMD5	rod	+	-	+	+	+	+	+	+	+	+	+	++
11.	EBMD1	rod	+	-	+	+	+	+	+	+	+	-	+	+
12.	EBPBS 5	rod	+	-	+	+	+	-	+	+	+	+	+	++
13.	EBMD7	rod	+	-	+	+	+	-	+	+	+	-	+	-
14.	EBCO2	rod	+	+	+	+	+	+	+	+	+	+	+	+
15.	EBAL4	rod	+	+	+	+	+	+	+	+	+	+	+	++
16.	EBAS2	rod	+	-	+	+	+	+	+	+	+	+	+	-
17.	EBMD4	rod	+	-	+	+	+	+	+	+	+	+	+	+
18.	EBWL7	rod	+	-	+	+	+	+	+	+	+	+	+	+
19.	EBCO3	rod	+	-	+	+	+	+	+	+	+	+	+	+
20.	EBTN1	rod	+	-	+	+	+	+	+	+	+	+	-	+

Proskauer's and ONPG tests.

Plant growth promoting activity of bacterial isolates

Twenty effective isolates obtained in the present study were mostly gram positive rods and were subjected to PGPR properties determining tests (Kloepper *et al.*, 1980) i.e. siderophore production and phosphate solublization test. Appearance of clear zone indicated the positive result i.e., phosphate solubilization and Siderophore production was indicated by yellow halos around the colonies. Out of 20 only 15 isolates showed positive test namely EBPBS4, EBAM1, EBWL8, EB MD6, EBWL10, EBAL1,EBAM2, EBAS3, EBMD2, EBMD5, EBMD1, EBPBS 5, EBMD7, EBCO2, EBAL4, EBAS2, EBMD4, EBWL7, EBCO3 and EBTN1. All the isolates were then subjected to HCN production test (Rezzonico *et al.,* 2007). Appearance of reddish brown zone around the inoculated colony shows positive result. Among the 20 isolates some showed strong results while other gave moderate and low results. Isolates which gave negative result had PGPR property because HCN is a lethal byproduct which is harmful for the plant growth promotion. HCN, produced by many soil microorganisms and it is postulated to play a role in biological control of pathogens (Defago *et al.,* 1990). Production of HCN by certain strains of fluorescent pseudomonads has been involved in the suppression of soil borne pathogens (Siddiqui *et al.,* 2006). The isolates were also subjected to siderophore production test. Siderophores are low molecular weight, extracellular compounds with a high affinity for ferric iron, that are secreted by microorganisms to take up iron from the environment (Sharma and Johri, 2003) and their mode of action in suppression of disease were thought to be solely based on competition for iron with the pathogen (Duijff *et al.*, 1997). On the basis of above three test screening of isolates were done. A total twenty isolates were further selected and subjected for IAA production test. (Fig. 1). Indole acetic acid (IAA) production was detected as described by Brick *et al.,* (1991).The ability of bacteria to produce IAA in the rhizosphere depends on the availability of precursors and uptake of microbial IAA by plant.

Table 2. Screening of endophytic *Bacillus* spp. against *R. solani*

S. no.	Isolates	Mycelial growth (cm)	Per cent inhibition (C-T/C*100)	S. no.	Isolates	Mycelial growth (cm)	Per cent inhibition (C-T/C*100)
1.	EB MD 1	4.30	52.22	23.	EB CO4	5.00	44.44
2.	EB MD 2	4.20	53.33	24.	EB AL 1	4.00	55.56
3.	EB MD 3	5.00	44.44	25..	EB AL 2	4.80	46.67
4.	EB MD 4	4.60	48.89	26.	EB AL 3	5.00	44.44
5.	EB MD 5	4.20	53.33	27.	EB AL 4	4.50	50.00
6.	EB MD 6	4.00	55.56	28.	EB AL 5	5.00	44.44
7.	EB MD 7	4.50	50.00	29.	EB AM 1	3.70	58.89
8.	EB MD 8	5.00	44.44	30.	EB AM 2	4.10	54.44
9.	EB MD 9	5.60	37.78	31.	EB AS 1	5.80	35.56
10.	EB MD 11	5.60	37.78	32.	EB AS 2	4.50	50.00
11.	EB PBS 1	6.00	33.33	33.	EB AS3	4.10	54.44
12.	EB PBS 2	5.00	44.44	34.	EB TN1	4.80	46.67
13.	EB PBS 3	5.10	43.33	35.	EB TN2	5.20	42.22
14.	EB PBS 4	3.70	58.89	36.	EB TN3	4.90	45.56
15.	EB PBS 5	4.40	51.11	37.	EB TN4	5.10	43.33
16.	EB WL 6	6.00	33.33	38.	EB TN 5	5.30	41.11
17.	EB WL 7	4.60	48.89	39.	EB TN 6	5.20	42.22
18.	EB WL 8	4.00	55.56	40.	EBCD 1	5.80	35.55
19.	EB WL 9	5.00	44.44	41.	EB CD2	5.70	36.67
20.	EB WL 10	4.00	55.56	42.	EB NG1	4.80	46.67
21.	EB CO 2	4.50	50.00	43.	EB CP1	4.90	45.57
22.	EB CO 3	4.70	47.78	44.	Control	9.00	0.00

Growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere and other PGP activities (Glick, 1995). Production of IAA by *Bacillus is* a general characteristic of our test isolates. Higher level of IAA production by *Pseudomonas* was recorded by other workers (Xie *et al* 1996). Production of IAA was indicated by appearance of pink colour. After qualitative analysis these isolates were subjected for quantitative production of IAA which were tabulated. Isolates EB PBS 4 showed maximum IAA production and least by isolates EB AM1 which was obtained manually from standard graph as shown in Fig. 2. These isolates (EBPBS4, EBAM1, EBWL8, EB MD6, EBWL10, EBAL1, EBAM2, EBAS3, EBMD2, EBMD5, EBMD1, EBPBS 5, EBMD7, EBCO2, EBAL4, EBAS2, EBMD4, EBWL7, EBCO3 and EBTN1) were then subjected to biochemical characterization for the determination of probable microorganism. *Bacillus* species were estimated by morphologies and physiology characteristics based techniques.

These isolates were then assessed to know their influence on the seed germination by measuring the shoot and root length. The growth promoting activity of 20 isolates of PGPR was tested for seed germination and seedling vigour by using tomato seeds in roll towel methods. The data on seed germination and seedling vigour of rice as influenced by seed bacterization with different endophytic bacterial isolates by roll towel method are given in Table 3. and Fig 3,4 In general, the seed germination ranged from 90 per cent (uninoculated control) to 100 percent (EB PBS 4). All the strains, EBAM1, EBWL8, EB MD6, EBWL10, EBAL1,EBAM2, EBAS3, EBMD2, EBMD5, EBMD1, EBPBS 5, EBMD7, EBCO2, EBAL4, EBAS2, EBMD4, EBWL7, EBCO3 and EBTN1 significantly improved seed germination when compared to the uninoculated control (UIC). The isolate EB PBS 4 showed significantly increased seed germination (100%) and also shoot and root length as well as enhanced vigour index of 25.87, 8.95 and 3486 respectively.

Molecular characterization and accession numbers

Based on 16S rRNA gene partial sequencing, similarity values >99 % suggested that all strains belongs to genus *Bacillus* and *Bacillus*

Table 3. Effect of bio-formulation of *B. subtilis* on plant growth promoting traits in rice under *invitro* conditions

S. No	Treatments	Germination %	Root length (cm)	Shoot length (cm)	Vigour Index
1	EBPBS4	100	8.95	25.87	3482.00
2	EBAM1	99	8.87	23.89	3243.24
3	EB MD6	98	8.51	23.01	3088.96
4	EBWL8	96	6.90	21.80	2755.20
5	EBWL10	98	7.12	21.68	2822.40
6	EBAL1	97	8.18	23.72	3094.30
7	EBAM2	95	7.77	22.66	2890.85
8	EBAS3	98	7.48	20.97	2788.10
9	EBMD2	94	6.17	21.62	2612.26
10	EBMD5	90	5.80	18.00	2142.00
11	EBMD1	92	5.12	18.20	2145.44
12	EBPBS 5	95	6.51	20.80	2594.45
13	EBMD7	94	6.02	19.30	2380.08
14	EBCO2	95	5.40	20.50	2460.50
15	EBAL4	92	6.00	18.80	2281.60
16	EBAS2	96	7.08	20.90	2686.08
17	EBMD4	94	6.21	17.30	2209.94
18	EBWL7	90	5.40	18.50	2151.00
19	EBCO3	90	6.00	19.80	2322.00
20	EBTN1	93	7.10	20.10	2529.60
21	CONTROL	75	4.59	10.10	1101.75

Table 4. Primers and PCR conditions

S. no	Name		Sequence 5' to 3'	Base pair	PCR condition		
1.	16S r RNA	BCF1	BCF1 (CGGGAGGCAGCAGTAGGGAAT)	546	Initial denaturation	94 °C; 3 min.	30 cycles
					Denaturation	94 °C; 1 min.	
					Annealing	55 °C; 1 min.	
		BCR2	(CTCCCCAGGCGGAGTGCTTAAT)		Extension	72 °C; 90 sec	
					Final extension	72 °C; 10 min.	
2.	Bacillomycin D	BymbF	TGAAACAAA GGCATATGCTC	875	Initial denaturation	94 °C; 3 min.	35 cycles
					Denaturation	94 °C; 1 min.	
					Annealing	60 °C; 30 sec	
		BymbR	AAAAATGCATCTGCC GTTCC		Extension	72 °C; 105 sec	
					Final extension	72 °C; 6 min.	
3.	Chi A	Chi A F	GATATCGACTGGGAGTTCCC	270	Initial denaturation	94 °C; 3 min.	35 cycles
					Denaturation	94 °C; 1 min.	
					Annealing	52 °C; 1 min.	
		Chi A R	CATAGAAGTCGTAGGTCATC		Extension	72 °C; 2 min.	
					Final extension	72 °C; 5 min.	
4.	^{2} 1-3 glucanase	gluF	AATGGCGGTGTATTCCTTGACC	415	Initial denaturation	94 °C; 5 min.	35 cycles
					Denaturation	94 °C; 1 min.	
					Annealing	55.5°C; 30 sec	
		gluR	GCGCGTAGTCACAGTCAAAGT		Extension	72 °C; 1 min.	
					Final extension	72 °C; 10 min.	

derived genera, *viz. B. subtilus* (EB PBS 4) B. cereus (EB WL), *B. amyloliqueifaciens*. Partial 16S rRNA gene sequences of the strains were submitted to NCBI GenBank under accessions numbers

Detection of antibiotic genes

An important role of hydrolytic enzymes has been well documented as a variety of microorganisms that exhibit hyper-parasitic activity, attacking pathogens by excreting these enzymes (Chernin and Chet, 2002). Detection of amplified genetic markers (bacillomycin, chitinase and b-glucanase) in antagonistic *Bacillus* strains was shown in Table 4. Bacillomycin (bmyB) gene amplification showed one specific band at around 395 bp length in all *Bacillus* spp. and amplification of chitinase and β-glucanase gene was also specific on the 270 and 415 bp respectively in all the twenty strains (Fig. 6).

In addition, PCR amplification was confirmed the presence of the bacillomycin (bmyB) and β-glucanase genes. Similarly, bmyB and β-glucanase genes have been reported from different *Bacillus* strains using PCR techniques (Joshi and McSpadden Gardener, 2006; Solanki *et al.,* 2012b). This suggests that amplified genes from the *Bacillus* could also have a role to further define

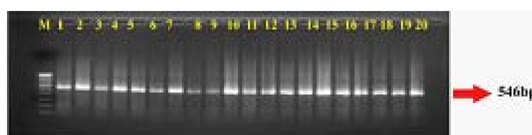

Fig. 4. PCR based ampilification of ITS region of Endopytic *Bacillius* spp

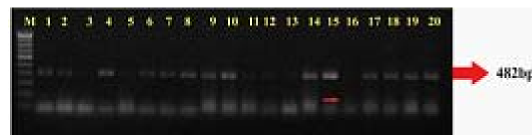

Fig. 5. PCR based ampilification of bacillomycin (bymC) gene

Fig. 6. Detection of chitinase gene in Endophytic *Bacillus* spp

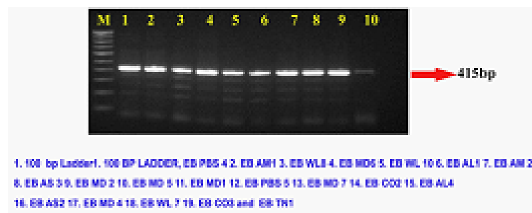

Fig. 7. PCR based detection of glucanase gene in Endophytic *Bacillius* spp

Table 5. Effect of bio-formulation of *B.subtilis* treatments on the plant growth parameters and sheath blight incidence of rice (BPT 5204)under glass house condition.

Sl. No.	Treatments	Plant height (cm)	No of tillers	Percent disease Index –PDI	Percent reduction over control	Grain yield /plant (g)
1	ST with EBPBS410ml/kg	57.0^{def}	7.8^{ab}	42 .00de(40.39)	46.15	12.13^{g}
2	SD with EBPBS4500ml/ha	56.0^{ef}	7.4^{bc}	46^{c}(42.70)	41.03	11.05^{h}
3	SA with EBPBS41000ml/ha @ monthly interval	55.1^{fg}	6.9^{cd}	51^{b}(45.57)	34.62	12.57^{e}
4	FS with EBPBS41000ml/ha	56.4^{ef}	6.4^{d}	38^{f}(38.05)	51.28	12.25^{fg}
5	ST+SD	57.8^{ef}	7.2b^{c}	43^{cd}(40.97)	44.87	13.06^{c}
6	ST+SD+SA	62.5^{b}	7.8^{ab}	34^{g}(35.66)	56.41	14.97^{b}
7	ST+SD+FS	60.1^{bc}	7.4^{bc}	39^{ef}(38.64)	50.00	12.35^{f}
8	SA+FS	59.6^{cd}	7.6^{ab}	33^{g}(35.05)	57.69	12.90^{cd}
9	ST+SD+SA+FS @monthly interval	65.0^{a}	8.2^{a}	31^{g}(33.82)	60.26	16.96^{a}
10	Talc formulation of *B.subtilis*	58.6^{cde}	7.5^{bc}	40^{def}(39.22)	48.72	12.83^{d}
11	Carbendazim @0.05%	56.2^{ef}	7.3^{bc}	14^{h}(21.96)	82.05	09.66^{i}
12	Control	53.1^{g}	6.0^{e}	78^{a}(62.08)	0.00	08.65^{j}

Values are the mean of three replications . Means in a column followed by same superscript letters are not significantly different according to Duncan's multiple range test at $P = 0.05$.

the diversity, ecology, and biocontrol activities of *B. subtilis* in the suppression of soil-borne pathogens.

Overall, the present study is successful in selecting effective strains of *Bacillus* from rice endophyte to manage soil-borne pathogens and we are reporting *B. subtilis* EB-PBS 4 as an effective biocontrol agent of rice sheath blight with the production of diffusible and volatile antibiotics, siderophore, IAA and phosphate solubilization. Hence, they can be used under field conditions as an effective biocontrol agent promotingplant growth with reduced disease incidence. They could be incorporated within an integrated disease management package including moderately resistant cultivars, limited fungicide application and effective cultural practices.

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