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Antagonistic and plant growth promoting *Bacillus* sp. MBRL 576 enhances the growth and yield of rice (*Oryza sativa* L.)

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ABSTRACT

A Bacillus sp. strain MBRL 576 isolated from rice rhizosphere was tested for antagonism against six rice fungal pathogens, and further evaluated for plant growth promotion in rice under in vitro condition. The results indicated that the Bacillus sp. MBRL 576 showed antagonistic activity against all the tested fungal pathogens by producing diffusible and volatile compound(s) and fungal cell wall degrading enzymes such as β-1,3-glucanase, β-1,4-glucanase, lipase and protease. Besides antagonism, this strain also produced 10 µg/ml of IAA, solubilize inorganic phosphate up to 88 µg/ml, catecholate type of siderophore and ACC deaminase. Rice seeds treated with Bacillus sp. strain MBRL 576 significantly increased the growth and grain yield under net house condition and even under pathogen challenged conditions. Overall, this strain could be used as potential bioinoculant to enhance growth promotion of agricultural crops including rice.

Key words: *Bacillus sp. MBRL 576, rice, antagonistic activity, plant growth promoting traits, vigor index, grain yield*

INTRODUCTION

Rice (*Oryza sativa* L.) is one of the most important cereal crops in the world, feeding more than 50% of the world's population (Gyaneshwar et al., 2001). There is a need for an increase in global productivity in order to provide sufficient food for the increasing human population. However, plant pathogens including fungi have severely affected crop production and quality, and are the most visible threats to sustainable food production (Kumar and Dangar, 2013; Kumar and Mishra, 2014). Current methods in agriculture such as massive use of chemical pesticides and fertilizers pose serious environmental and health problems (Gunnell et al., 2007; Leach and Mumford, 2008; Kumar et al., 2017a; 2017b). The use of indigenous bacteria having biocontrol and/or plant growth promotion activity for agricultural crops especially rice holds great promise for sustainable, ecofriendly and productive as a promising supplement or alternative to the use of synthetic agrochemicals (Kumar et al., 2013).

Microorganisms that can grow in the rhizosphere are ideal for use as biocontrol agents, since they provide the front line defense for protection against plant pathogens (Kumar et al., 2016a). Strains of the genus Bacillus are among the most commonly reported plant growth promoting rhizobacteria (PGPR) (Compant et al., 2005; Govindasamy et al., 2010; Kumar et al., 2016b). Some species of the genus Bacillus have shown promising results for the biological control of various plant pathogens as well as growth promoters of some crops (Podile and Laxmi, 1998), as they are abundant in the soil and produce heat resistant spores that survive and remain metabolically active under harsh environmental conditions (Rodgers, 1989). Bacillus spp. are nonpathogenic, easy to cultivate, and protein and metabolite secretors. These characteristics make them appropriate for the formulation of stable and viable biological products that can be used for soil-borne disease management (Govindasamy et al., 2010; Kloepper, 1997). Products for plant disease biocontrol containing Bacillus subtilis and other *Bacillus* spp. have

been used over the past years as seed dressings in several crops (Schisler et al., 2004).

The principal mechanisms of growth promotion by Bacillus species include production of indole acetic acid (IAA) (Mishra and Kumar, 2012), siderophores (Bharucha et al., 2013) and aminocylopropane carboxylate (ACC) deaminase (Ghosh et al. 2003; Govindasamy et al., 2008), nitrogen fixation (Kumar et al., 2017c), solubilization and mobilization of phosphate (P) (Minaxi et al., 2012; Kumar et al., 2016b; Sahoo et al., 2017). *Bacillus* spp. also protect the plants against disease by production of antibiotics (Vanittanakom et al., 1986; Tan et al., 2013), volatile compounds (Ryu et al., 2003, 2004) and fungal cell wall degrading enzymes (Basha and Ulaganathan, 2002; Compant et al., 2005). There are several reports on *Bacillus* spp. enhancing seed germination and growth of rice plants (Mishra and Kumar 2012; Ng et al. 2012; Ji et al. 2014). Considering the above facts, the aim of the present study was to assess the biocontrol and plant growth ability of *Bacillus* sp. in rice.

MATERIALS AND METHODS

Isolation and screening of rhizospheric bacteria

Healthy rice plants (variety, *Jatra*) from 3 sites were uprooted from rice fields adjoining the Manipur University campus, India (24.75° N, 93.92° E). Soils closely adhering to the roots were collected using sterile spatula and Bacillus sp. were isolated by heat treatment method (80 °C, 15 min) on nutrient agar (NA) medium by serial dilution technique. Colonies with distinct morphologies were selected and subcultured to get pure cultures. Pure cultures were subjected to preliminary antagonistic assays by dual culture method (described in the following section) against rice fungal pathogens. The best strain MBRL 576 was selected, characterized and further screened for other antagonistic and plant growth promoting traits. The strain was preserved in agar slants (4 °C) and as glycerol suspensions (20% v/v, -20 °C) for further use.

Molecular characterization of the strain

Extraction of genomic DNA and PCR amplification of 16S rRNA gene were carried out as described by Li et al. (2007). The sequences were analyzed against the GenBank and the Ez-Taxon server databases (Kim et al., 2012). Phylogenetic tree based on neighbour-joining method (Saitou and Nei, 1987) was constructed using MEGA 5.2 software package (Tamura et al., 2011).

Test organisms

The rice fungal pathogens *viz., Curvularia oryzae* MTCC 2605, *Fusarium oxysporum* MTCC 287, *Helminthosporum oryzae* MTCC 3717, Pyricularia oryzae MTCC 1477, *Rhizoctonia oryzae-sativae* MTCC 2162, and *Rhizoctonia solani* MTCC 4633 were procured from Microbial Type Culture Collection (MTCC), Institute of Microbial Technology (IMTECH), Chandigarh, India. The strains were grown and maintained on potato dextrose agar (PDA).

In vitro biocontrol assays

Antagonism by diffusible and volatile compound(s)

The antifungal bioassay due to production of diffusible compound(s) was done using dual culture method according to Khamna et al. (2009) with few modifications. Bacterial culture was streaked in circular pattern at the corner of the PDA plates. Fungal plugs (6 mm) were then placed at the center of the plates. The antifungal bioassay due to production of volatile compound(s) (VOCs) was done according to Trivedi et al. (2008) with few modifications. Strain was allowed to grow on NA until full growth. The lids of the plates were then replaced with PDA plates containing fungal plugs at the centre. In the control plates, lids of the NA plates without the isolate were replaced with PDA plates containing fungal plugs at the centre. Plates were wrapped with parafilm. Plates were kept incubated at 30°C. Mycelial growth inhibition was calculated using the formula: (C-T)/C x 100, where C is the growth of the test pathogen in the control plate (measured in mm radius), and T is the growth of test pathogen in the test plate (measured in mm). The inhibition zone was measured after the fungal mycelia in control plates reached the edges of the plates.

Antifungal bioassay by culture filtrates

For bioassays of antifungal compounds in the culture filtrates, the isolate was inoculated in five different liquid media to test the influence of growth media on the production of antifungal metabolites. The media used

were nutrient broth (NB), trypticase soya broth (TSB), King's B (KB) medium, YPG1 (Hu et al., 2008) and YPG1-Ca (Hu et al., 2008). The culture was kept incubated (150 rpm, 30 °C, 48 h), followed by centrifugation (10,000 rpm, 10 min). The supernatants collected were filtered through sterile membrane filters (0.2 mm pore size). The culture filtrate was subjected to various treatments such as incubation at room temperature (denoted as R), heating in water bath at 80 °C for 20 min (H) and autoclaving at 121°C for 15 min (S). 0.1 ml each of the treated filtrates was incorporated into the agar wells on PDA plates and fungal agar plugs were then placed at the centers of the plates. Sterile distilled water (SDW) was incorporated in the control plates instead of culture filtrates. Clearing zones of inhibition were measured in mm.

Fungal cell wall degrading enzyme production

Colloidal chitin from chitin (shrimp shells) was prepared according to Reid and Ogrydiazk (1981). A loop full of 2 d old culture was transferred into sterile vials containing 2 ml of semi-solid NA (0.1% agar), and gently shaken for uniformity. Then it was spot inoculated on colloidal chitin agar plate and chitinase production was screened according to Hsu and Lockwood (1975). β-1,3-glucanase and β-1,4-glucanase production were assayed according to Srividya et al. (2012) and Ariffin et al. (2006), respectively. Lipase and protease production were screened according to Cappuccino and Sherman (1992) on tributyrin agar and skim milk agar, respectively.

In vitro plant growth promoting (PGP) assays

IAA production

Production of IAA was detected according to the method of Khamna et al. (2010). A loopful of culture was inoculated in NB containing 2 mg/ml of L-Tryptophan (Trp) and kept incubated under shaking conditions (150 rpm, 30°C, 2 d). The culture broths were centrifuged (10,000 rpm, 10 min) and 1 ml of the supernatant was mixed with 2 ml of Salkowski reagent. Appearance of pink colour indicated IAA production.

For quantitative assay of IAA production, the strain was cultured on NB medium containing 2 mg/ml of Trp under shaking conditions (150 rpm, 30 °C). 5 ml aliquot was withdrawn periodically from the culture flask at 24 h intervals and centrifuged (10,000 rpm, 10 min). 1 ml supernatant was mixed with 2 ml Salkowski reagent and kept incubated for 20 min at room temperature. Absorbance was measured at 530 nm and the amount of IAA produced was calculated from the standard IAA curve. The amount of IAA produced was correlated with the culture growth (measured as dry cell mass).

Phosphate (P) solubilization

P solubilization was determined by spot inoculation of a loopful of MBRL 576 in the National Botanical Research Institute's phosphate growth medium containing bromophenol blue (NBRIP-BPB) (Mehta and Nautiyal, 2001). Plates were kept incubated at 30 °C for 4 d. Halo zone formation surrounding the colony indicated P solubilization.

Quantitative estimation of phosphate solubilization was done according to Kapri and Tewari (2010). Strain was inoculated in 100 ml of NBRIP medium (pH 7) and kept incubated in a shaker (150 rpm, 30 °C). 5 ml aliquots were withdrawn periodically at 24 h intervals, centrifuged (10,000 rpm, 10 min) and the supernatants were analyzed for pH and P concentration. The amount of P in the culture supernatants were estimated using the Fiske and Subbarow method (1925) and expressed as equivalent P (μg/ml). KH_2PO_4 was used as the standard.

Siderophore production

Siderophore production was determined as described by You et al. (2005) in NA containing Chrome azurol S (CAS). Siderophore type (catechol or hydroxamate) was determined according to the method of Arnow (1937) and Meyer et al. (1995).

Quantitative estimation of siderophore production was done by CAS-shuttle assay (Payne 1994) using five iron-deficient liquid media *viz.,* modified Gause No.1 (GM), Cas-amino acid (CAA), NB, Succinic acid (SM) and Barbhaiya and Rao (BR) media. 5 ml aliquot were withdrawn periodically from the culture flasks at 24 h intervals, centrifuged (10,000 rpm, 10 min) and 1 ml of supernatant was mixed with 1 ml of CAS reagent. Absorbance was measured at 630 nm against a reference consisting of 1 ml of

uninoculated broth and 1 ml of CAS reagent. The amount of siderophore produced (percentage siderophore units) was calculated by using the formula: (Ar - As)/Ar x 100, where, Ar = Absorbance of reference at 630 nm (CAS reagent), and As = Absorbance of sample at 630 nm.

Aminocylopropane carboxylate (ACC) deaminase production

ACC deaminase activity was screened according to El-Tarabily (2008) using the nitrogen-free Dworkin and Foster's minimal salts agar medium (DF) (Dworkin and Foster, 1958). The medium was supplemented with 3 mM ACC per liter as a sole nitrogen source. The heat-labile ACC was filter sterilized through sterile membrane filter (0.22 μm Millipore size) and the filtrate was added to the DF medium after autoclaving. Strain was streaked on DF agar medium plates amended with either $(NH_4)_2SO_4$ or ACC. The plates were kept incubated at 30°C for 4 d. Strain was re-inoculated and kept incubated under the same experimental conditions. Growth on DF medium indicated ACC deaminase production.

In vitro seed germination test (vigor index)

Strain was grown on NB for 3 d and harvested by centrifugation (10,000 rpm, 10 min). The pellets collected were washed thrice with sterile distilled water (SDW) and 6 different concentrations of the culture suspensions were prepared (3x107, 6x107, 1.2 x108, 1.8 x108, 2.4 x108, 3 x108 cfu/ml). Rice (variety: *Jatra*) seeds were surface sterilized with 70% ethanol for 5 min followed by 0.2% sodium hypochlorite for 5 min and rinsed four times with SDW. Sterilized seeds were soaked in the cell suspensions prepared earlier and kept under shaking conditions (150 rpm, 30°C, 2 h). The seeds were then transferred to sterile plates containing wetted filter papers (10 seeds per plate). Untreated seeds soaked in SDW were used as the control. Plates were incubated at 28°C. The number of germinated seeds, root lengths and shoot lengths were noted after 4 d of incubation and vigor index calculated using the following formula (Abdul-Baki and Anderson 1973): Vigor index = Percent germination × Seedling length, where seedling length = (mean shoot length + mean root length).

Vigor index was also calculated under pathogen challenged conditions. Rice seeds were sterilized with the same method as stated above. Sterilized seeds were soaked in cell suspensions corresponding to highest vigor index in the above experiment and kept incubated under shaking conditions (150 rpm, 30°C, 2 h). Sterilized filter paper used in the earlier experiment was replaced by minimal media containing potato dextrose (HiMedia, 0.48 % w/v) and agar (0.8% w/v). 10 seeds were placed in the petri plates leaving 1 cm away from the margins. Fungal plugs were placed in the centre of the plates. Sterilized seeds soaked in SDW were also placed in plates containing only the fungal plugs and in control plates. Plates were kept incubated at 28°C. The growth parameters such as the number of germinated seeds, root lengths and shoot lengths were noted after 8 d and vigor indices were calculated using the above formula.

Growth promotion under nethouse conditions

Fungal plugs were inoculated in 100 ml of potato dextrose broth (PDB) and kept incubated under shaking conditions (150 rpm, 30°C) for 5 d. The inside of earthen pots were lined with polythene in order to prevent water leakage and 3 kg (approx.) of sandy-loam soil (after drying for 1 month) was placed in each pot. The upper part of the soil (approx. 3 cm) was removed and 20 ml of each fungal broth was spread uniformly and, it was then replaced/cover with the same soil. The soil was inoculated with the fungus and kept for 2 d. Surface sterilized rice seeds (variety: *Jatra*) were added into the culture broth (2.4 x 108 cfu/ml, 2 d old). The flasks were kept under shaking conditions for 2 h (150 rpm, 30°C). For control and fungal treated pots, rice seeds were inoculated in SDW. 6 rice seeds were placed in each pot by removing the upper layer of the soil (approximately 3 cm). For the bioinoculant treated pots, 15 ml of the culture broth was spread by removing the upper layer of the soils (approximately 3 cm) surrounding the seeds. The pots were then covered with the same soils. 10 pots were kept for each treatment in a randomized block design. Pots were watered on alternate days with tap (non-sterilized) water. After one week, the plants were thinned to 4 plants per pot. Plants of 5 pots for each treatment were harvested after 45 d and selected parameters such as root and shoot lengths, fresh root and shoot weights, and dry root and shoot weights were measured. The remaining pots were kept for grain yield measurement in which plants were further

thinned to 2 plants per pot.

Statistical analysis

All data were subjected to one-way analysis of variance (ANOVA) followed by independent t-test at $P \leq 0.05$ using the SPSS 16 software (SPSS Inc).

RESULTS AND DISCUSSION

Of 42 bacteria obtained from rice rhizospheric soil, strain MBRL 576 was selected for further study as it showed antagonistic activity against all the test pathogens studied.

Characterization of isolate MBRL 576

The genomic DNA of isolate MBRL 576 was extracted and amplified for 16S-rRNA and sent for sequencing. The 98.2% sequence of 16S rRNA of this isolate matched with *Bacillus tequilensis* 10bT. Phylogenetic analysis of MBRL 576 sequences also showed the similarity with other Bacillus sp. (Fig. 1). The 16S rRNA gene sequence of the *Bacillus* sp. strain MBRL 576 was deposited in NCBI GenBank under the accession number KC577143.

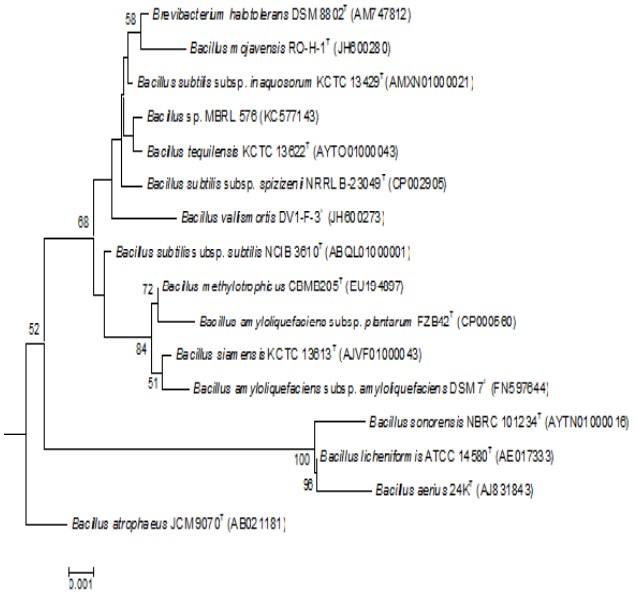

Fig. 1. Neighbour joining phylogenetic tree based on 16S rRNA gene sequences showing the position of *Bacillus* sp. strain MBRL 576 with the genus *Bacillus*. Nodes indicate the boostrap values as shown in percentages of 1000 replicates; only values above 50% are shown. Bar, 0.001 substitutions per nucleotide position.

In vitro biocontrol assay

Strain MBRL 576 showed inhibition against all the indicator fungal pathogens [by diffusible compound(s)] but it failed to inhibit mycelial growth of *F. oxysporum* by VOCs. The strain exhibited highest mycelial growth inhibition by diffusible compound(s) against *P. oryzae* (70.5%) and lowest against *R. oryzae-sativae* (40%) (Fig. 2). MBRL 576 also exhibited highest mycelial growth inhibition by VOCs against *R. solani* (60%) and lowest against *R. oryzae-sativae* (10%) (Fig. 2).

Culture filtrates showed significant clearing zones of inhibition (7 to 23 mm) and among the five different production media, TSB was found to be the best medium for production of antifungal metabolites(s). Other growth media showed very less or no inhibition. The antifungal metabolite(s) produced by MBRL 576 may be thermostable as it exhibit antifungal activities even after autoclaving at 121°C. The culture filtrate failed to exhibit inhibition against *R. oryzae-sativae*

Table 1. Inhibition of mycelial growth by culture filtrates of *Bacillus* sp. MBRL 576.

Media	Inhibition zone size (mm)																	
	MTCC 4633			MTCC 2162			MTCC 3717			MTCC 1477			MTCC 287			MTCC 2605		
	R	H	S	R	H	S	R	H	S	R	H	S	R	H	S	R	H	S
NB	-	-	-	-	-	-	23	23	17	12	12	12	14	14	14	22	22	20
TSB	10	10	10	-	-	-	18	18	15	17	15	15	14	14	14	20	20	18
KB	8	8	8	-	-	-	14	14	13	10	8	8	7	7	7	16	13	13
YPG1	-	-	-	-	-	-	7	7	7	ND	ND	ND	-	-	-	8	8	7
YPG-Ca	-	-	-	-	-	-	7	7	7	ND	ND	ND	-	-	-	7	7	7

Note: NB: Nutrient broth, TSB: Trypticase soya broth, KB: King's B, YPG1: yeast extract 1g/L; peptone 10g/L; glucose 10 g/L, YPG1-Ca: yeast extract 1 g/L; peptone 10 g/L; glucose 10 g/L; $CaCl_2.2H_2O$ 5g/L, R: Room temperature, H: Heat treatment (80^0C, 15 min), S: Autoclaved (121^0C, 20 min), ND: Not determined.

Table 2. *In vitro* seed germination tests for *Bacillus* sp. MBRL 576 at different inoculum sizes.

Treatment	Inoculum size (x 10^8cfu/ml)	Germination percent	Root Length* (cm)	Shoot Length* (cm)	Vigor Index
Control	00	85	2.47±1.3a	0.28±0.4a	233.75
	0.3	90	2.47±1.4a	0.80±0.5b	320.4
	0.6	92.5	3.01±1.5b	0.81±0.5b	353.35
MBRL 576	1.2	95	3.20±1.2c	0.45±0.5b	346.75
	1.8	100	5.27±1.3d	0.78±1.6b	605
	2.4	100	6.36±1.2e	0.84±0.4b	720
	3	100	3.73±1.9d	0.36±0.3a	409

*Values with the same letter within a column are not significant at P ≤ 0.05.

(Table 1). The strain also showed positive results for β-1,3-glucanase, β-1,4-glucanase, lipase and protease activities but negative for chitinase production.

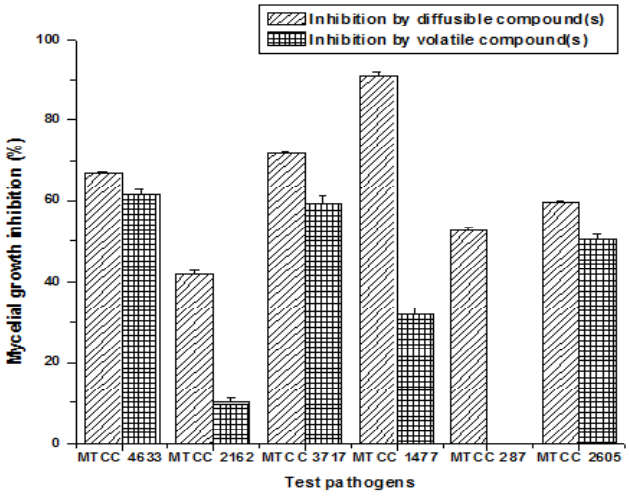

Fig. 2. Mycelial growth inhibition of fungal test pathogens by Bacillus sp. strain MBRL 576 due to diffusible and volatile compound(s). Note: MTCC 4633, *Rhizoctonia solani* ; MTCC 2162, *Rhizoctonia oryzaesativae*; MTCC 3717, ***Helminthosporum oryzae***; MTCC 1477, *Pyricularia oryzae*; MTCC 287, *Fusarium oxysporum*; MTCC 2605, *Curvularia oryzae*

In vitro PGP assays

MBRL 576 showed positive results for all the PGP traits

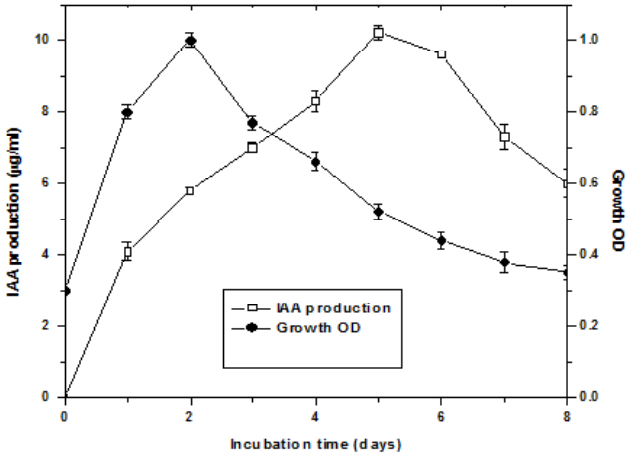

Fig. 3. IAA production by *Bacillus* sp. MBRL 576 in presence of 2 mg/ml of Trp.

Table 3. *In vitro* seed germination for Bacillus sp. MBRL 576 challenged with fungal pathogens (inoculums size, 2.4 x 10^8 cfu/ml).

Treatment	Germination Percent	Root length* (cm)	Shoot length* (cm)	Vigor index
Control	100	2.71±1.30c	4.68±0.55b	739
MBRL 576	100	2.64±0.56c	5.30±0.63d	794
MTCC 4633	76	0.99±1.14a	1.94±2.11a	222.6
MTCC 4633+MBRL 576	100	3.52±1.27d	4.36±1.06b	788
MTCC 2162	100	1.97±1.05a	4.75±0.91b	672
MTCC 2162+MBRL 576	100	2.97±1.09d	5.25±1.76c	822
MTCC 3717	90	0.81±0.74a	3.35±2.01a	374.4
MTCC 3717+MBRL 576	95	2.40±1.44b	4.21±1.67b	627.9
MTCC 1477	80	1.04±0.93a	2.38±1.57a	273.6
MTCC 1477 + MBRL 576	100	2.79±0.53d	3.99±0.64b	678
MTCC 287	100	2.14±0.72a	4.76±0.90b	690
MTCC 287+MBRL 576	100	3.59±1.53d	5.28±0.78d	887

*Values with the same letter within a column are not significant at P ≤0.05.

Table 4. Different growth parameters of rice plants (*Jatra*) inoculated with *Bacillus* sp. MBRL 576, plants treated with MBRL 576 and challenged by pathogens, and plants challenged with pathogen alone.

Treatment	Length (cm)		Fresh weight (g)		Dry weight (g)	
	Root*	Shoot*	Root*	Shoot*	Root*	Shoot*
Control	19.45±0.83c	41.85±3.42c	0.14±0.03c	0.23±0.03a	0.05±0.001b	0.08±0.002a
MBRL 576	19.85±0.16d	43.93±4.20c	0.17±0.01c	0.35±0.03a	0.06±0.001c	0.10±0.002b
MTCC 4633	16.95±0.66a	39.05±1.70b	0.07±0.004a	0.32±0.05a	0.04±0.004b	0.09±0.003a
MTCC 4633 + MBRL 576	18.05±1.20b	43.70±1.03d	0.10±0.002b	0.25±0.02a	0.04±0.003b	0.09±0.002a
MTCC 3717	15.68±0.76a	37.85±1.19a	0.04±0.005a	0.21±0.02a	0.03±0.001a	0.07±0.001a
MTCC 3717 + MBRL 576	16.90±1.02a	44.90±1.50d	0.09±0.004a	0.34±0.02a	0.04±0.008b	0.10±0.002b
MTCC 1477	15.68±0.59a	38.32±1.22a	0.05±0.003a	0.14±0.05a	0.02±0.001a	0.05±0.001a
MTCC 1477 + MBRL 576	19.35±0.60c	45.18±1.70d	0.12±0.02c	0.29±0.03a	0.05±0.002b	0.09±0.002a

* Values with the same letter within a column are not significant at P ≤0.05.

Table 5. Different grain yield parameters of rice plants (*Jatra)* inoculated with *Bacillus* sp. MBRL 576, plants treated with MBRL 576 and challenged with pathogens, and plants challenged with pathogen alone.

Treatment	No. of panicles per plant	No. of grains per panicle*	Total grain yield per plant (g)*	10 grains weight (g)*	Total No. of grains per plant*	No. of filled grains*	No. of unfilled grains*
Control	1±00	71.5±19d	1.02± 0.86b	0.13±0.05b	71.5±19c	31±31a	40.5±12d
MBRL 576	1.5±0.7	57.5±3.53b	2.07±0.88f	0.25±0.007g	85±35.3e	74.5±16.2d	10.5±0.70b
MTCC 4633	1±00	66.5±12c	1.28±0.77c	0.14±0.01c	66.5±12b	44.5±31.8a	22±19.7c
MTCC 4633 + MBRL 576	1±00	72.5±7.77d	1.74±0.16f	0.22±0.02f	72.5±7.77d	67.5±7.7c	5±1a
MTCC 3717	1±00	62.5±16.2c	1.62±0.36e	0.23±0.02f	62.5±16.2b	58.5±23.4b	4±2.8a
MTCC 3717 + MBRL 576	1±00	79.5±28.9e	1.88±0.48f	0.21±0.04e	79.5±28.9e	73.5±20.5d	6±1.41a
MTCC 1477	1±00	26±8.44a	0.60±0.25a	0.10±0.03a	26±26.2a	21±19.5a	5±7.07a
MTCC 1477 + MBRL 576	1±00	69±15.5c	1.48±0.77d	0.21±0.06d	69±15.5b	53±18.2b	16±15.5b

* Values with the same letter within a column are not significant at P ≤ 0.05.

assayed, including IAA production, P solubilization, siderophore production and ACC deaminase production. It could produce 10 µg/ml IAA after 5 d of incubation (Fig. 3) and solubilize P up to 88 µg/ml after 9 d of incubation, with a corresponding decrease in pH of the medium (from pH 7 to 4.56) (Fig. 4). MBRL 576 produced catecholate type of siderophore and among different production media, CAA (56% siderophore units, after 3 d of incubation) was found to be the best medium. NB and GM were found to be other media suitable for siderophore production, whereas, SM and BR were found to be poor media (Fig. 5). Strain MBRL 576 also showed positive results for ACC deaminase production.

In vitro seed germination test (Vigor index)

Among the different inoculum densities, inoculum size corresponding to 2.4x108 cfu/ml showed highest vigor index value of 720 (Table 2). MBRL 576 treated rice seedlings showed higher vigor indices and significant increases (P≤0.05) in shoot lengths over the control. MBRL 576 treated rice seedlings challenged with pathogens exhibited higher germination percentages and significant increased (P≤0.05) in root and shoot lengths over seedlings challenged with pathogens only (Table 3).

Growth promotion under nethouse conditions

MBRL 576 treated rice plants showed significant

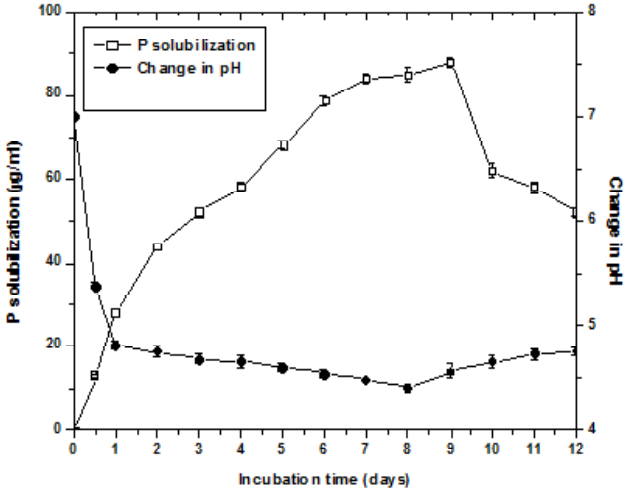

Fig. 4. P solubilization by *Bacillus* sp. MBRL 576.

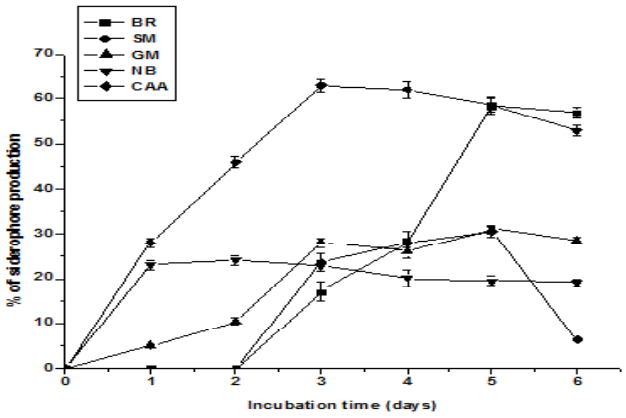

Fig. 5. Comparative siderophore production by *Bacillus* sp. MBRL 576 in different media.

increased (P≤0.05) in root lengths and dry root weights over the control, MBRL 576 treated rice plants challenged with pathogen and plants challenged with pathogen only in absence of the bioinoculant. Bioinoculant treated rice plants challenged with two different pathogens (except *H. oryzae*) also showed significant increased (P≤0.05) in root and shoot lengths, and fresh and dry root weights over pathogen alone treated plants (Table 4).

MBRL 576 treated rice plants showed higher number of panicles and significant increase (P≤0.05) in total grain yields, total number of grains and number of filled grains per plant over the control plant and bioinoculant treated plants challenged with pathogens and plants challenged with pathogens only in absence of the bioinoculant. MBRL 576 treated rice plants challenged with pathogens also showed similar results (except strain challenged with *H. oryzae*) over rice plants challenged with pathogens only (Table 5).

Bacillus is one of the most effective bacterial genera known for biocontrol agents that have shown suppressive effects against a wider range of plant pathogens, and Bacillus antibiotics are generally assumed responsible for the biocontrol activity (Helbig et al., 1998; Govindasamy et al., 2010; Mojica-Marin et al., 2008). In the present study, *Bacillus* sp. strain MBRL 576 showed significant mycelial growth inhibition against all the tested rice fungal pathogens. Antifungal activity by this strain may be mediated by antibiotics, VOCs, siderophores and fungal cell wall degrading enzymes. The antifungal compounds in the culture filtrates may be heat stable as they showed activity even after heat sterilization, though there was slight decrease in the size of the inhibition zone. Similarly, culture filtrates of *B. subtilis* have been shown to inhibit mycelial growth of *P. grisea* and *R. solani* even after sterilization (heat stable) but were more effective with the non-heat treated filtrates (Leelasuphakul et al., 2006). Heat stabilities of the antagonistic activity may be due to production of heat stable antibiotics and decrease in the inhibition zone sizes after heat sterilization may be due to production of heat labile β-1,3-glucanase and β-1,4-glucanase in the culture filtrates as reported by Leelasuphakul et al. (2006). VOCs produced by MBRL 576 failed to inhibit radial growth of *F. oxysporum*. Similarly, Kai et al. (2008) have reported that VOCs produced by *B. subtilis* failed to inhibit growth of *F. solani* and *F. culmorum*.

MBRL 576 also showed positive results for PGP traits such as phosphate solubilization, IAA, and siderophore production. IAA producing B. subtilis and *B. amyloliquefaciens* strains significantly promoted growth of rice seedlings (Mishra and Kumar, 2012). MBRL 576 could solubilize phosphate with decrease in the pH of the medium. This may be due to production of low molecular weight organic acids (Bnayahu, 1991; Rodriguez et al., 2004). Production of siderophores is one of the important mechanisms used by PGPR to promote plant growth and also for antagonism against

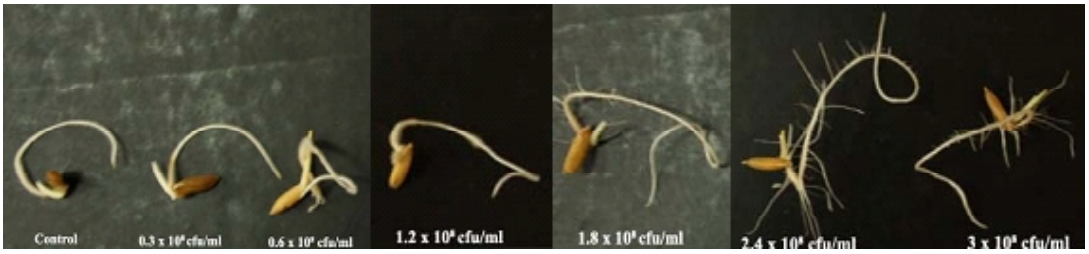

Fig. 6. *In vitro* rice seed germination by MBRL 576 at different inoculums sizes.

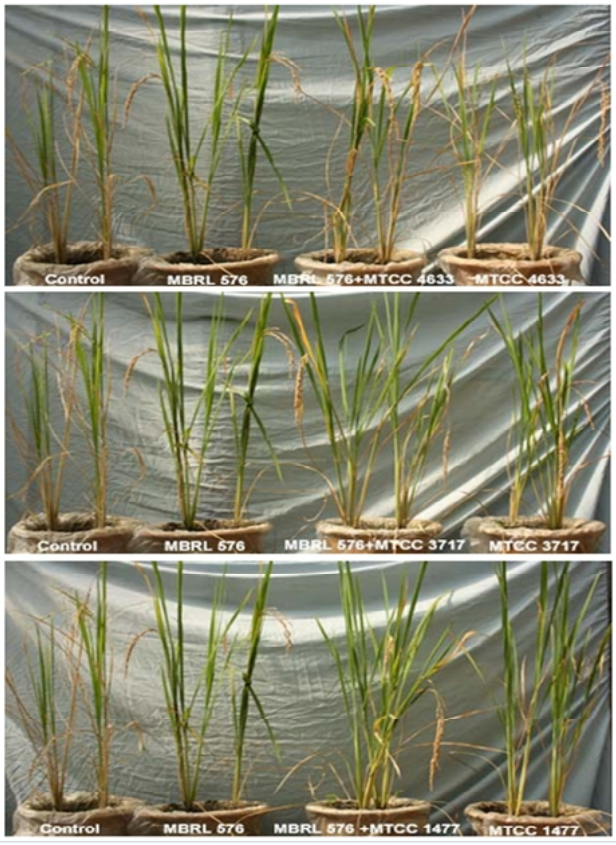

Fig. 7. Grain yields of rice plants (Jatra) inoculated with *Bacillus* sp. MBRL 576, plants inoculated with MBRL 576 and challenged by pathogens, and plants challenged with pathogen alone.

phytopathogens. MBRL 576 could produce significant amounts of siderophores. Bharucha et al. (2013) reported that *Bacillus* spp. produced 84% of siderophore units (catecholate type) and the purified siderophore suppressed the growth of *F. oxysporum* and *Aspergillus niger.* Rice seeds treated with a siderophore producing *B. subtilis* strain significantly increased seedling emergence rate and promoted the growth of seedlings (Kumar et al., 2011). Rice growth promotion by MBRL 576 may be due to presence of various PGP traits.

MBRL 576 increased germination percentages and vigor indices in rice seedlings. Rice seeds treated with *B. amyloliquefaciens* have been reported earlier to have significantly increased germination percentages and vigor indices (Ng et al., 2012). Similarly, treatment of rice seedlings with a *B. amyloliquefaciens* strain promoted the growth of rice plants under gnotobiotic conditions (Mishra and Kumar, 2012). Under nethouse conditions, MBRL 576 significantly promoted growth of rice plants. Rice seeds treated with a *B. subtilis* strain showed significantly increased germination percentages and enhanced growth of rice seedlings under greenhouse conditions (Kumar et al., 2011). Strain MBRL 576 enhances grain yield production in rice plants under nethouse conditions. Similarly, *Bacillus* sp. RM-2 promote growth and grain yield production of cowpea under greenhouse conditions (Menaxi et al., 2012).

Strain MBRL 576 was found to be closely related to *B. tequilensis*. Dastager et al. (2011) have reported a *B. tequilensis* strain that solubilize 28.3 µg/ml phosphates with decrease in pH of the medium (from pH 7 to 5). They also reported that the strain could produce 127.5µg/ml IAA when the medium was supplemented with Trp (10 mg/ml). The strain also showed positive results for siderophore production. Treatment of cut stems with the strain showed significant increases the in number of roots and root and shoots lengths of black pepper plants (Dastager et al., 2011).

CONCLUSION

Overall, the present study concludes that Bacillus sp. MBRL 576 showed a wider antagonistic activity against various fungal pathogens causing rice diseases. Production of fungal cell wall degrading enzymes such as β-1,3-glucanase, β-1,4-glucanase, lipase and protease could be one of the reasons to inhibit the tested pathogens. Strain MBRL 576 increased the vigor index and growth of rice seedlings even under pathogen challenged conditions. Rice seeds treated with MBRL 576 significantly increased the growth and grain yield production under nethouse conditions. Production of good amount of plant growth promoting traits such as IAA, siderophore, phosphate solubilization and ACC deaminase production by Bacillus sp may be responsible for enhancing rice growth. The strain has the potential for used as biocontrol and plant growth promoting agents for rice cultivation under field conditions.

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