



PLANT GROWTH PROMOTING AND ANTAGONISTIC ACTIVITY OF *Bacillus* STRAINS ISOLATED FROM RICE RHIZOSPHERE

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) are advantageous bacteria that inhabit around plant roots and enhance plant growth by a wide variety of direct and indirect mechanisms. Utilization of PGPR is now gradually increasing in agriculture and offers an attractive way to replace chemical fertilizers, pesticides, and supplements. In our present study six endospore forming bacterial isolates that were screened from rhizosphere of rice showed potential plant growth promoting (PGP) and antagonistic activities. Based on morphological and biochemical analyses the isolates were identified as *Bacillus* sp. Among the isolates (FB1-FB-6), all the strains produced IAA, FB3 produced siderophore and phytase. All the isolates produced lytic enzymes, and FB1-FB6 solubilized various sources of organic and inorganic phosphates as well as zinc. Strains FB1-FB6 also strongly inhibited the growth of several phytopathogens such as *Macrophomina phaseolina*, *Fusarium oxysporum*, *Alternaria alternata* and *Colletotricum* sp. *in vitro*. Since, *Bacillus* sp. FB1-FB6 shows PGP and antifungal activities so it can be surmised that the isolated strains have strong potential to be successful biofertilizers and bioenhancers.

KEY WORDS: PGPR, *Bacillus* sp., Phosphate solubilization, and Antagonistic activity.



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INTRODUCTION

Plant growth promoting rhizobacteria are a group of bacteria that actively colonize plant roots and exert positive effect on growth and productivity. The mechanisms by which PGPRs promote plant growth are not yet properly elucidated. The mechanisms thought to involve: - the ability to produce phytohormones, symbiotic N₂ fixation, production of siderophores, the synthesis of antibiotics, enzymes and/or fungicidal compounds and also - solubilisation of mineral phosphates and other nutrients. In the context of increasing international concern for food and environmental quality, the use of PGPR for reducing chemical inputs in agriculture is a potentially important issue. PGPR have been applied to various crops to enhance growth, seed emergence and crop yield, and some have been commercialized.¹⁻⁴ Generally 2–5% of rhizosphere dwelling bacteria are PGPR. Among them, spore-forming bacilli have an advantage over the non-spore formers such as *Pseudomonas*, because spores are more robust and resistant to the elevated temperature and high concentrations of chemicals.⁵ *Bacillus* spp. are considered to be the safe microorganisms that hold remarkable abilities for synthesizing a vast array of beneficial substances.⁶ *Bacillus* spp. having potent plant growth promoting traits such as IAA production, phosphate solubilization, nitrogen fixation, and biocontrol attributes like production of HCN, siderophore, hydrolytic enzymes and antibiotics have been isolated from soybean.⁷ Several other workers have also found the biocontrol activities of *Bacillus* against many common phytopathogens.⁸⁻⁹ Rice is the most important staple food in several developing countries, and chemical fertilizer is the most important input required for rice cultivation. The high-yielding rice variety has resulted in an increase in rice production but requires large amounts of chemical fertilizers. But the use of chemical fertilizers is not eco-friendly due to its harmful effect on microorganisms, plants as well as soil. In order to make rice cultivation sustainable and less dependent on chemical fertilizers, it is important to know how to use PGPR. There are many evidences which also attest that PGPR increase growth and productivity of many commercial crops including rice⁴, wheat¹⁰, cucumber¹¹, maize¹², cotton¹³, and banana.¹⁴ Hence, the present study was designed to isolate and characterize bacilli from the rhizosphere of rice having PGPR and antagonistic traits so that they can be exploited as a potential bioinoculant for rice.

MATERIALS AND METHODS

Isolation and identification of *Bacillus* spp

Soil samples (1 g) from rhizosphere of rice were heat-treated (80°C), transferred to 9 ml sterile distilled water and mixed thoroughly by shaking the flask on a rotatory shaker for 5 min. After serial dilution 0.1 ml suspension was spread over nutrient agar plates in triplicates. Inoculated plates were incubated at 30 ± 1 °C for 24–48 h. Rough and abundant colonies with waxy growth (1–4 mm diam) and irregular spreading edge were obtained. The selected isolates were maintained on the NAM (nutrient agar medium) slants at 4°C for further use. The bacterial isolates were identified by using cultural,

morphological and biochemical characteristics features described in Bergey's manual of determinative bacteriology¹⁵ and stored at 4°C on slants and maintained through sub-culturing. The isolates were characterized by Gram staining, motility test, Methyl Red, Voges-Proskauer, Citrate, oxidase test, catalase test, H₂S production and starch hydrolysis as per the standard methods.^{16,17}

Sources of fungal pathogen

Four plant pathogenic fungi *Macrophomina phaseolina*, *Fusarium oxysporum*, *Colletotrichum* sp. *Alternaria alternata* were used in present study. The fungal cultures were obtained from the culture collection of Microbiology and Microbial biotechnology laboratory, Department of Botany, University of Gour Banga.

Phosphate solubilization

Phosphate solubilization test was performed by spot inoculation of test organisms on both National Botanical Research Institute's Phosphate Solubilization-Bromophenol Blue medium (NBRIP)¹⁸ and Pikovskaya's medium.¹⁹ The plates were incubated at 30±1 °C for 4-5 days. Formation of a clear zone around the colony was considered positive for phosphate solubilization.

IAA Production

IAA production by bacterial isolates was tested following the methods of Gordon and Weber.²⁰ The bacterial isolates were grown on Nutrient broth (NB) and incubated at 30°C for 24 h and were centrifuged to collect supernatant. Two drops of O-phosphoric acid was added to supernatant, appearance of pink colour confirmed the production of IAA.

HCN Production²¹

To determine production of hydrogen cyanide (HCN), The cultures of bacterial isolates growing in the log phase were streaked on agar plates supplemented with 4.4 g glycine/L with simultaneous addition of filter paper soaked in 0.5% picric acid in 1% Na₂CO₃ in the upper lid of the plate and plates were sealed with parafilm, and the plates were incubated at 30±1°C. The uninoculated plates were served as control. The plates were examined putatively for the color change for the confirmation of HCN production.

Siderophore production

For this determination, a method followed by Schwyn and Neilands²² was adopted utilizing Chromeazurol S (CAS) medium. The log phase-grown cultures of bacterial isolates were spot-inoculated on CAS medium followed by incubation at 30±1°C for 48-72 h. Plates were examined for the formation of orange to yellow halo zones around the developed colonies as confirmation of siderophore production.

Zinc solubilization

Zinc solubilization ability of the isolates was detected by spotting the log phase culture of bacterial strains on Tris-minimal medium plates having zinc phosphate, and zinc carbonate as source of insoluble inorganic zinc along with bromophenol blue as pH indicator. The inoculated plates were then incubated at 30 ± 1 °C for 3 days, and observed for the clearing zone around the

colonies (due to the solubilization of inorganic zinc by bacteria).

Phytase production

Phytase activity of individual strain was detected by spot inoculation of log phase culture on phytase screening media having sodium phytate as sole source of organic phosphate. Plates were then incubated at 28 ± 1 °C for 3 days and observed for halos around the colonies.

Organic acid production

Bacterial cultures were grown in MM9 agar medium using methyl red as an indicator and observed for decline in pH from yellow to pink below pH 5.0. Isolates having the ability to produce organic acid resulted in a pink zone around the colony.

Ammonia production

Pepton water is used for tested ammonia production. Freshly growing culture were inoculated in pepton water and incubated for 48-72 hours at 30°C. Nessler's reagent was added in each tube, changes in colour from brown to yellow was positive test for ammonia production.²³

EXTRA CELLULAR ENZYME ACTIVITY

Chitinase activity

At first colloidal chitin was prepared by using the method of Berger and Reynolds.²⁴ Then chitinase detection agar (CDA) plates were prepared. Then bacteria were spot inoculated. The plates were incubated at 30 ± 1 °C for 5 days. After incubation formation of clear zone indicates the production of chitinase.²⁵

Amylase activity

The bacterial isolates were spot inoculated on starch agar medium plates and incubate at 30 ± 1 °C for 48 h. After the incubation period, the plates were flooded with iodine solution and zone appearances surrounding colonies indicate the production of amylase.²⁶

Cellulase activity

The bacterial isolates were spot inoculated on Carboxy methyl cellulose (CMC) agar plates and incubate at 30 ± 1 °C for 5 days. After the incubation period, the zone formation surrounding colonies indicate the production of cellulase.²⁷

Protease activity

The bacterial isolates were spot inoculated on Skim milk agar plates and incubate at 30 ± 1 °C for 3 days. After the incubation period, the zone formation surrounding colonies indicate the production of protease.²⁸

Production of oxalate oxidase enzyme

Production of oxalate oxidase was studied following the method of Dickman and Mitra.²⁹ and Savchuck.³⁰ Bacterial strains was spotted on agar plate containing oxalic acid degradation selective medium (for utilization of ammonium oxalate as sole carbon source) and incubated in dark at 30 ± 1 °C for 7 days. These plates were observed for clearing zone around bacterial growth indicating the presence of enzyme oxalate oxidase, which can inhibit the growth of some selective plant pathogens such as *S.sclerotiorum*.³¹

ANTAGONISM AGAINST PHYTOPATHOGENS

All *Bacillus* isolates were tested for their antagonistic activities against various plant pathogenic fungi, including *F. oxysporum*, *M. phaseolina*, *Alternaria alternata* and *Colletotrichum* sp., in the PDA medium. *Bacillus* isolates were spot-inoculated on the agar medium 2 cm away from the center of fungal disk showing mycelial growth. Antagonistic activity was recorded on the 5th day after incubation at 28 ± 1 °C. The zones of inhibition were measured and the plates without bacterial inoculums were used as a control. The value of inhibition was measured by using the formula $C-T \times 100/C$ (T, treatment; C, control).⁵

RESULTS

Morphological, physiological and biochemical characteristics of all six isolates showed them as Gram-positive, rod shaped, endospore former with white, dry and fold, opaque and irregular edged colonies on NAM plates. All of them found positive for catalase, oxidase, glucose and almost all were negative for H₂S production, methyl red and glucosamine. Most of them tolerated temperature up to 60°C to 80° C. comparison of biochemical character with Bergey' s manual of bacteriology revealed that isolates belongs to *Bacillus* .

In vitro PLANT GROWTH PROMOTING ACTIVITY

All the isolates were tested for plant growth promoting activity like phosphate and zinc solubilization, IAA production, HCN production, siderophore production, ammonia production, and phytase production.

Phosphate solubilization

The isolates were streaked on both PKV and NBRIP medium to evaluate their ability to solubilize phosphate. All isolates produced yellow zone of various degrees around the colonies (Table 1). The isolates FB3, FB5 and FB6 showed maximum zone where as isolates FB4 and FB2 showed comparatively less phosphate solubilization efficiency (Fig. 1A and 1B).

IAA production

All the isolates used in present study (FB1-FB6) produced IAA. The results confirmed that all the bacterial isolates were able to develop a pink color in their respective culture broth with or without the addition of tryptophan. Development of pink color was common in all the strains (Table 1).

HCN production

HCN production was tested solid agar plate supplemented with glycine. All of these (FB1-FB6) bacterial strains showed negative result in HCN production (Table 1).

Siderophore production

Siderophore production was assessed using blue coloured CAS medium. Among the isolates, only FB3, produced a yellow zone around bacterial colony (Table 1), which indicates its capacity to produce siderophore.

Zinc solubilization

For screening of zinc solubilization capacity isolates were grown on modified Pikovskaya medium which contain zinc oxide as source of zinc. Out of six isolates,

the isolates (FB1, FB4, FB5 and FB6) solubilise zinc indicating in changes in pH as indicated by change in colour around bacterial spot on the medium. (Table 1, Fig. 1C).

Phytase production

All the isolates of *Bacillus* were tested for phytase production in phytase screening medium (PSM) supplemented with sodium phytate. The isolate FB3 was able to solubilise sodium phytate by formation of a halo zone around its spot indicating the release of free P on medium. (Table1). Other isolates could not show any visible change on PSM medium.

Organic acid production

Organic acid production was assessed using M9 minimal medium. All the strains used in present study could not produce organic acid. (Table1).

Ammonia production

Out of six isolates of *Bacillus*, FB2 produce low amount of ammonia while FB3, FB5, FB6 produce high amount of ammonia and FB1, FB4 produce moderate amount of ammonia by developing brown to yellow colour. (Table1).

Table 1
Plant growth promoting attributes of *Bacillus* spp.
(FB1-FB6) isolated from rice rhizosphere.

Isolates	Phosphate sol.		IAA Production	HCN determination	Siderophore production	Zinc sol.	Phytase production	Organic acid production	Ammonia production
	NBRIP	PKV							
FB1	+++	++	+	-	-	+	-	-	++
FB2	++	+	+	-	-	-	-	-	+
FB3	+++	+++	++	-	+	-	+	-	+++
FB4	+	+	++	-	-	+	-	-	++
FB5	+++	+++	+++	-	-	+++	-	-	++++
FB6	+++	+++	+++	-	-	+++	-	-	+++

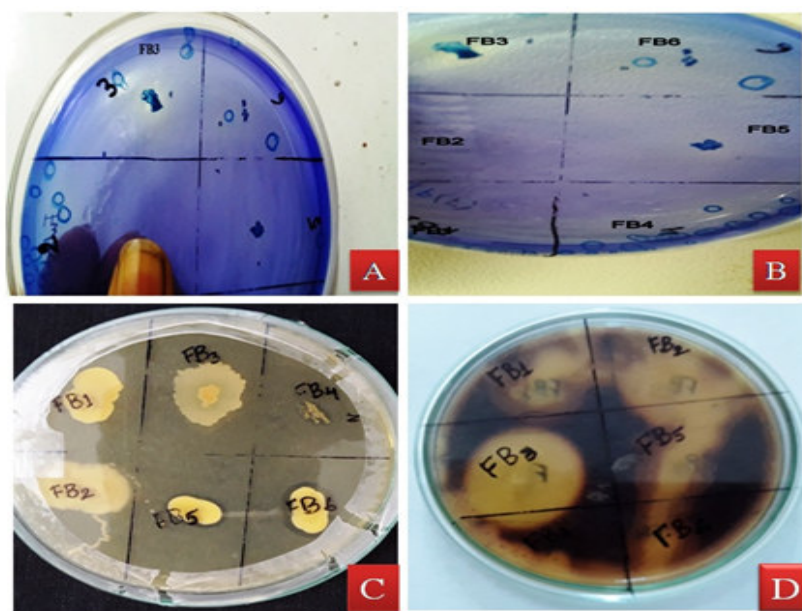


Figure 1
In vitro PGPR activity of the *Bacillus* sp. Phosphate solubilization (A-FB3), (B-FB1-FB6); Zinc solubilization (C-FB1-FB6); Chitinase activity (D-FB1-FB6).

CELL WALL DEGRADING ENZYMES

All the isolates were screened for production of cell wall degrading enzymes. The isolates showed clear zone on CDA plates indicating their ability to produce chitinase enzyme (Table 2, Fig. 1D). All *Bacillus* isolates screened on Starch Agar medium plates displayed amylase activity, by creating a halo zone around the bacterial spot on the medium (Table 2, Fig. 2D). On carboxy methyl cellulose (CMC) agar plates, all *Bacillus* isolates showed cellulase activity by producing zone around the

bacterial spot on the medium (Fig. 2A and 2B). Every *Bacillus* isolates spotted on Skim milk agar agar plates displayed protease activity, by creating a halo zone around the bacterial spot on the medium (Table 2, Fig. 2C).

Oxalate oxidase

None of these isolates produced clear halo around bacterial spot on plate containing oxalic acid degradation selective medium, which is indicative of not Oxalate oxidase production. (Table 2).

Table 2
Production of mycolytic enzymes by Bacillus spp. (FB1-FB6)

Isolates	Chitinases	Amylase	Cellulase	Protease	Oxalate oxidase
FB1	++	+++	+++	+++	—
FB2	++	+++	+++	+++	—
FB3	+++	++++	++++	+++	—
FB4	—	++	+++	+++	—
FB5	++	++	+++	+++	—
FB6	++	++	+++	+++	—

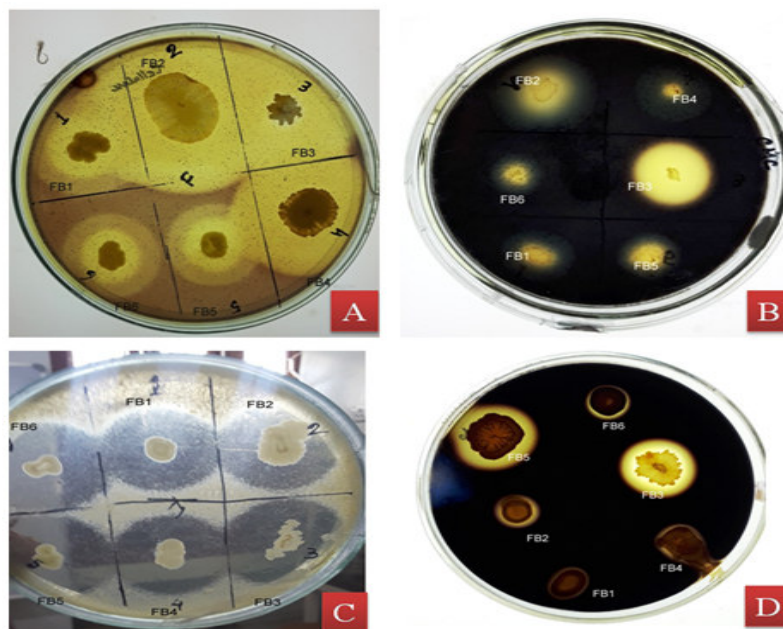


Figure 2
Production of cell wall degrading enzymes by the Bacillus sp. (FB1-FB6); Cellulase (A), (B); Protease (C); Amylase (D).

ANTAGONISM AGAINST PHYTOPATHOGENS

All the strains were screened for antagonistic activity and results were exhibited in Table 3 and Fig. 3. Among the isolates FB1 showed antagonistic activity against *Fusarium oxysporum* (40.3%), *Macrophomina phaseolina* (55.6%) and *Alternaria alternata* (56.7%), but it could not show antagonistic activity against *Colitroticum sp.* The isolate FB2 showed antagonistic activity against all the pathogens used in study. Highest inhibition was observed against *Macrophomina phaseolina* (55.8%) and *Alternaria alternata* (53.1%). The isolate also showed inhibition zone against *Colitroticum sp.*(35.6%),*Fusarium oxysporum* (32.7%). The isolate FB3 showed a commendable antagonistic activity against all the pathogens used in study. Inhibition was observed against *Macrophomina*

phaseolina (63.8%), *Alternaria alternata* (64.3%),*Colitroticum sp.* (56.1%),*Fusarium oxysporum* (52.9%). The isolate FB4 showed less antagonistic activity against *Macrophomina phaseolina* (32.4%). But showed good against *Colitroticum sp.* (54.2%),*Fusarium oxysporum* (50.3%),and *Alternaria alternata*(46.1%).The isolate FB5 also showed a commendable antagonistic activity against all the pathogens used in study. Inhibition was observed against *Macrophomina phaseolina* (55.9%), *Alternaria alternata* (60.7%),*Colitroticum sp.*(63.8%),*Fusarium oxysporum* (59.6%). The isolate FB6 showed a little antagonistic activity against *Macrophomina phaseolina* (41.1%) and *Alternaria alternata* (38.7%) but antagonistic activity against *Colitroticum sp.* (58.2%),*Fusarium oxysporum*(60.3%) was promising.

Table 3
Antagonistic attributes of Bacillus spp. (FB1-FB6) isolated from rice rhizosphere against phytopathogens.

Isolates	<i>Colitroticum sp.</i>	<i>Fusarium oxysporum</i>	<i>Macrophomina phaseolina</i>	<i>Alternaria alternata</i>
FB1	—	+	++	++
FB2	++	+	+++	+++
FB3	+++	+++	+++	+++
FB4	+++	+++	+	+++
FB5	+++	+++	++	+++
FB6	+++	+++	+	+

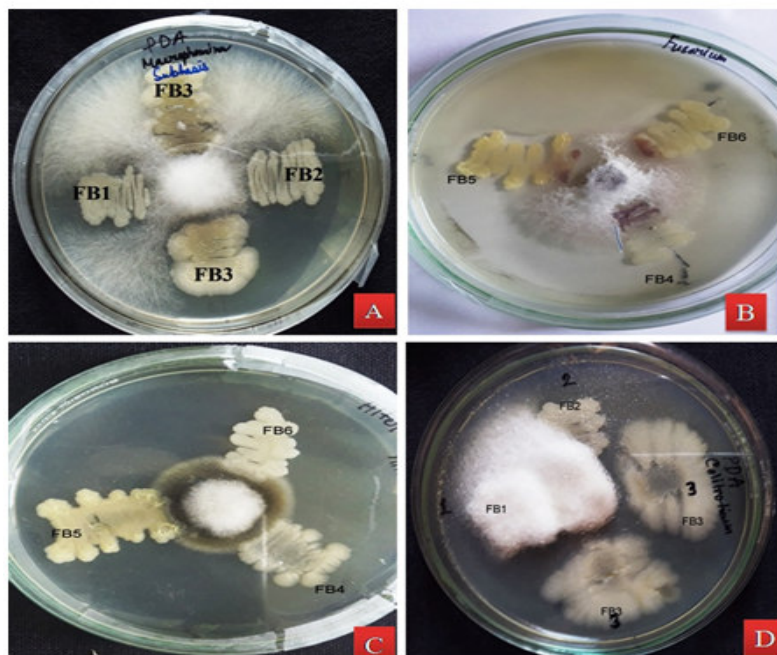


Figure3

Antagonism by *Bacillus* sp against fungal pathogen; *Macrophomina phaseolina* (A); *Fusarium oxysporum* (B); *Alternaria alternata* (C); *Colitroticum* sp (D).

DISCUSSION

The rhizosphere is a dynamic root region creating the ecological environment for plant-microbe interaction and provision of essential macro- and micronutrients from a limited nutrient pool.^{32,33} PGPR colonize plant roots and provoke plant growth and development by a wide variety of mechanisms. The mechanism by which PGPR stimulate plant growth is not yet elucidated, although several hypotheses such as production of phytohormones, suppression of plant pathogens, activation of phosphate solubilization, and promote mineral nutrient uptake are usually believed to be involved^{34, 37, 4}. In our experiments, spore forming *Bacillus* strains were isolated and were identified based on phenotypic characters and some biochemical features. The isolates were screened for their ability to utilize inorganic phosphates (zinc phosphate, tri-calcium phosphate), and organic phosphates (sodium phytate) and production of cell wall hydrolytic extracellular enzymes such as chitinase, cellulase, amylase, protease (Table 2). Furthermore, this isolate was found to be producer of IAA, siderophore etc. Isolation of *Bacillus* species from the rhizosphere of different crops has widely been studied previously. Presence of all the PGP traits was reported in *Bacillus circulans* MTCC 8983 by Mehta et al.³⁸ Several mechanisms of action have been proposed for PGPR increasing nutrient level, nitrogen fixation, increasing beneficial symbioses, enhancing root surface area, and combination of multiple modes; IAA and soluble phosphate production are among those several mechanisms of action. A single PGPR may demonstrate several modes of action.^{39, 40} The bacteria, which can solubilized phosphate, zinc have the beneficial effect on plant growth promotion have been reported by Iqbal et al.⁴¹ PGPR solubilize precipitated phosphates and make the phosphate available to rice plants that represent a possible mechanism of plant growth promotion.⁴² In our

study the isolates of *Bacillus* spp. (FB1-FB6) solubilised phosphate as well as zinc. FB3 also solubilize organic phosphate (calcium phytate). They were also able to produce IAA. Production of IAA and soluble phosphate are the most common mechanisms of action implicated in PGPR and indeed microbes demonstrating these attributes are wide spread in rhizosphere.^{39, 40} *In vitro* inhibition of various phytopathogens by *B. subtilis* ME488 has also been reported by Chung et al.⁸ *In vitro* IAA production by *Bacillus* spp. in significant amount has also been reported by Singh et al.⁴³ and^{38, 5}. In a study Shrestha et al.⁴⁴ showed that *Bacillus*-type bacteria are master in rice plants as antagonists against pathogens. *Bacillus* spp. have been pre-eminently used to control various crop diseases.^{45, 46} A *Bacillus* strain BPR7 produced mycolytic enzymes viz., chitinase, β -1,3-glucanase and β -1,4-glucanase which thought to be degrade the components of fungal cell wall such as chitin, β -1,3-glucan and glucosidic bonds.⁵ All strains (FB1-FB6) showed high level of antagonism against pathogens used in this study. Antagonistic activity against target phytopathogens by (FB1-FB6) may be due to the production of antimicrobial substances such as chitinolytic enzymes, cellulase, antibiotics, siderophore and nutrient competition.^{8, 33, 34} Other possible mechanism by which *Bacillus* can protect plants from pathogens is the competition for nutrients and suitable niches on rhizosphere.⁴⁷ A number of studies suggest that PGPR enhances the growth, seed emergence, crop yield contribute to the protection of plant against certain pathogens and pests.^{1,2,48,49,50,51}

CONCLUSION

From the above study it can be concluded that all the isolates used in present study showed PGPR activities at various degree. The isolate FB3 and FB5 showed possibility to be exploited as biocontrol agent and may be used as bioinoculant to induce systemic resistance in

rice against phytopathogens, thus proving an alternative to the hazardous chemical fertilizers.

CONFLICT OF INTEREST

Conflict of interest declared none.

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