Isolation and Identification of Potential Phosphate Solubilizing Bacteria from the Rhizoplane of *Oryza sativa* L. cv. BR29 of Bangladesh

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A total of 30 bacteria were isolated from the rhizoplane of rice cv. BR29 cultivated in Mymensingh, Bangladesh and from the seedlings obtained from surface-sterilized seeds of BR29. Upon screening, 6 isolates showed varying levels of phosphate solubilizing activity in both agar plate and broth assays using National Botanical Research Institute's phosphate medium. The bacterial isolates were identified based on their phenotypic and 16S rRNA genes sequencing data as *Acinetobacter* sp. BR-12, *Klebsiella* sp. BR-15, *Acinetobacter* sp. BR-25, *Enterobacter* sp. BR-26, *Microbacterium* sp. BRS-1 and *Pseudomonas* sp. BRS-2. The BR-25 exhibited highest phosphate solubilizing activity followed by BR-15. They grew rapidly in the liquid medium at pH 5 and 7 but almost no growth occurred at pH 3. The pH value of the culture medium was decreased with bacterial growth suggesting that they might secrete organic acids to solubilize insoluble phosphorus. Scanning electron microscope analysis of two-week-old rice seedlings germinated from seeds previously inoculated with BR-25 and BR-15 revealed dense colonization at the root surfaces presumably using fimbriae on the bacterial cells.

Key words: Phosphate Solubilizing Bacteria, *Oryza sativa* L., Root Colonization

Introduction

Phosphorus is one of the essential mineral macronutrients, which are required for maximum yield of agriculturally important crops. Most agricultural soils contain large reserves of phosphorus, a considerable part of which has accumulated as a consequence of regular applications of phosphate fertilizers (Richardson, 1994). However, a large portion of soluble inorganic phosphate applied to soil as chemical fertilizer is rapidly immobilized soon after application and becomes unavailable to plants (Dey, 1988; Yadav and Dadarwal, 1997). Farmers are thus asked to apply phosphorus fertilizers in several-fold excess in order to overcome this problem. Therefore, the release of insoluble and fixed forms of phosphorus is an important aspect of increasing soil phosphorus availability.

Plant root-associated phosphate solubilizing bacteria (PSB) have been considered as one of the possible alternatives for inorganic phosphate fertilizers for promoting plant growth and yield (de-Freitas *et al.,* 1997; Rodríguez and Fraga, 1999; Richardson, 2001; Vessey, 2003; Thakuria *et al.,* 2004). Seed or soil inoculation with PSB is known to improve the solubilization of fixed soil phosphorus and applied phosphates, resulting in higher crop yield (Yahya and Al-Azawi, 1989; Abd-Alla, 1994; Mehta and Nautiyal, 2001). In fact, PSB render more phosphates into the soluble form than required for their growth and metabolism by secreting organic acids and/or enzymes (*e.g.* phosphatases), the surplus get the plants (Vessey, 2003). The interest in PSB has increased due to the prospective use of efficient strains as bio-inoculant (biofertilizer) components in organic agriculture, which is emerging as an alternative to chemical inputs in intensive agriculture (Ryder *et al.,* 1994; Bashan and Holguin, 1998). However, their root colonization, persistence and performance in the rhizosphere are severely affected by environmental factors, especially under stressful soil conditions.

Availability of phosphorus is low at both low and high pH values under upland conditions and high under wetland rice culture. Again P availabil-

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ity in Bangladesh soils is low in rabi season due to low temperature and increases in kharif season with the rise of temperature. Phosphorus recovery is usually very low (8–20%) in rice in Bangladesh (Anonymous, 1997). Due to high cost of mineral superphosphate fertilizers, the resource-poor farmers of Bangladesh often fail to apply recommended doses of P fertilizers to the soil. Although a small country, the diversity of soils and vegetation in Bangladesh is high. Under these conditions, there is a prospect of using PSB inocula in rice and other crop production systems. The rice soils of Bangladesh are highly fertilized with enormous quantities of inorganic phosphate fertilizers (Anonymous, 1997). A significant reduction in the use of phosphate fertilizer could be achieved if solubilization of soil-insoluble phosphorus is made available to crop plants (Rodríguez and Fraga, 1999; Vessey, 2003; Thakuria *et al.,* 2004).

However, very few attempts have been made to isolate and characterize the potential PSB from the rhizosphere of rice in Bangladesh. Hence, little information is available concerning phosphate solubilizing bacteria and their ability to colonize rice roots in Bangladesh.

The objective of this study was to isolate and characterize PSB from the rhizoplane of rice cultivated in an Old Brahmaputra Alluvium soil in Bangladesh. The ability of persistence and root colonization by two promising PSB, *Acinetobacter* sp. BR-25 and *Klebsiella* sp. BR-15, upon seed inoculation were examined by scanning electron microscopy.

Materials and Methods

Isolation of the rhizoplane bacteria

Root samples of rice (*Oryza sativa* L.) cv. BR29 (45 days old) cultivated in silt loam soils of Old Brahmaputra Alluvium tract (Sonatola series) in Mymensingh, Bangladesh, were collected on March 15, 2004 and then washed gently with sterilized water to remove soils. Rhizoplane bacteria were isolated and purified on NBA (nutrient broth agar) medium using an aliquot of root washings as done earlier (Williams and Asher, 1996) and preserved in 20% glycerol at −80 °C until used in the bioassays. The cultivated soils had the average pH 6.7, organic carbon 0.7%, organic matter 1.21%, total N 0.12%, available P 12.09 μg/ml, exchangeable K 0.16%, and available sulfur 9.12 μg/ml. For seed-associated bacteria, rice cv. BR29 seeds were kept under running water for 48 h. Imbibed seeds were surface-sterilized with 70% EtOH for 1 min followed by 2.5% NaOCl solution for 15 min and finally washed well with sterilized water. Sterilized seeds were sown in a culture tube containing 10 ml of 1/5 Hoagland's S medium supplemented with 0.3% gellan gum [Hoagland's S medium (g l^{-1}): $Ca(NO_3)_2 \cdot 4H_2O$ (1.18), KNO_3 (0.505), $MgSO_4 \cdot 7H_2O$ (0.493), KH_2PO_4, (0.272)]. Culture tubes were then kept in the phytotron (23 °C, 16 h light and 8 h dark). After 15 d of sowing, 10 μl of gellan gum medium were taken out using a sterile micro tip from the rhizosphere of individual rice seedlings and inoculated on NBA plates, serially diluted by streak culture and purified by repeated culture of individual colony on the same medium. These procedures resulted in two seed endophytic bacteria, BRS-1 and BRS-2, from rice.

Identification of phosphate solubilizing bacteria

Four potent phosphate solubilizing bacterial isolates from the rhizoplane of field-grown rice and two seed endophytes were identified based on their 16S rRNA genes sequencing. For determination of the 16S rRNA sequences of the active isolates, chromosomal DNA was extracted and quantified using the Isoplant II (Wako Pure Chemical Industries) kit and Genequant pro (Biochrom Ltd., Cambridge, UK), respectively. The 16S rDNA region was amplified by PCR using a HotStarTaq (Qiagen, Hilden, Germany) kit and the universal primers 27F and 1525R for the 16S rRNA gene (Weisburg *et al.,* 1991). The thermal profile and conditioning step followed by PCR direct-sequencing for the 1.5 kb PCR product as the template with the universal primers 1080R, 1112F, 926F and 803R was performed as described earlier (Deora *et al.,* 2005). Purification and analysis of the labeled PCR-direct mixture was performed by a sequencer (ABI Prism® 310 Genetic analyzer, Applied Biosystems) according to the instructions of the manufacturer. The 16S rRNA sequences of the strains were aligned with reference sequences obtained from the BLASTN database on DDBJ (DNA Data Base of Japan) website.

Screening for phosphate solubilizing bacteria

All bacterial strains were tested by an agar assay using National Botanical Research Institute's phosphate (NBRIP) medium supplemented with

1.5% Bacto-agar (Difco Laboratories, Detroit, MI, USA) (Nautiyal, 1999). The NBRIP growth medium contained (g/l): glucose (10), $Ca_3(PO_4)_2$ (5), $MgCl_2 \cdot 6H_2O$ (5), $MgSO_4 \cdot 7H_2O$ (0.25), KCl (0.2) and $(NH_4)_2SO_4$ (0.1). Four strains per plate were stabbed in triplicate using sterile toothpicks. The halo and colony diameters were measured after 14 d of incubation of the plates at 25 °C. The ability of the bacteria to solubilize insoluble phosphate was described by the solubilization index [= the ratio of the total diameter (colony + halo zone) to the colony diameter (Edi Premono *et al.,* 1996)].

Quantitative estimation of phosphate solubilization in broth assay by rice rhizoplane bacteria

The quantitative bioassay was carried out using Erlenmeyer flasks (100 ml) containing 10 ml of NBRIP broth medium inoculated with the bacteria at around 10^8–10^9 CFU/ml. Autoclaved uninoculated NBRIP medium served as control. The flasks were incubated for 2 d at 30 °C on a shaker at 180 rpm. The cultures were harvested by centrifugation for 15 min at 8,000 rpm and 4 °C. Supernatant was decanted and autoclaved at 121 °C for 20 min. Autoclaved samples were then filtered through a 4.5 μm membrane. Available phosphorus content in the culture supernatant as well as control (supernatant obtained from no bacteria inoculation) was estimated using the vanado-molybdate colorimetric method by measuring the absorbance at a wavelength of 420 nm. Each treatment was replicated three times and data were expressed as the mean value ± standard error.

Bacterial growth and pH value of the culture medium

To analyze whether bacteria can grow in a range of pH 3 to 7 and to test their ability to change the pH value of the medium, all the six active strains of PSB were inoculated separately in test tubes (18×1.6 cm) containing 10 ml of potato dextrose broth at varying pH levels. Bacteria were grown in a shaking incubator (100 rpm) for 8 d at 25 °C. The optical density of the bacteria and pH value of the medium were estimated after 2 d intervals using a spectrophotometer (at 595 nm) (TECAN GENios, Toronto, Ontario, Canada) and a pH meter (Horiba, B-212, Kyoto, Japan), respectively. Each treatment was replicated three times and data were expressed as the mean value.

Transmission and scanning electron microscopy

Morphology of bacteria cultured in liquid NBA medium under static culture conditions for 48 h was observed using an H-800 transmission electron microscope (HITACHI) following the procedures described previously (Islam *et al.,* 2005). Surface-sterilized seeds were inoculated with individual bacteria (*ca.* 10^8 CFU/seed) and then sown and grown in a test tube containing 10 ml of 1/5 Hoagland's S medium supplemented with 0.3% gellan gum (Islam *et al.,* 2005). *In vivo* root colonization of 2-week-old seedlings was studied following the protocol described earlier using a JEOL JSM-6301F scanning electron microscope (Islam *et al.,* 2005).

Results and Discussion

Isolation and screening of phosphate solubilizing bacteria on agar assay

We isolated and purified a total of 30 bacterial strains from the rhizoplane of rice by repeated streak culture on NBA medium. Initially, all isolates were tested for their phosphate solubilizing activity by an agar assay using NBRIP medium supplemented with 1.5% Bacto-agar (Nautiyal, 1999). The phosphate solubilization index of the rice isolates varied from 1.2 to 6.7 (Table I). The isolate BR-25 exhibited the highest phosphate solubilization index (6.7) followed by BR-15 (4.8) when calcium phosphate was used as P source. However, none of them showed any phosphate solubilizing activity when $AlPO_4$ or $FePO_4$ were used as P sources (data not shown). Many species of rhizobacteria including bacilli, rhizobia and pseudomonads can solubilize insoluble phosphates in the agar assay *in vitro* (Nautiyal, 1999; Thakuria *et al.,* 2004).

Quantification of phosphate solubilizing activity and identification of bacteria

Phosphate solubilization ability of all rice isolates was further evaluated in NBRIP liquid broth medium. Reasonably, all active rice isolates showed consistent results in solubilizing phosphate from calcium phosphate in both liquid broth and agar assays (Table I). Similar consistent results of phosphate solubilization by PSB in both agar and broth assay were observed earlier (Nautiyal, 1999).

Bacterial isolate source	Phosphate solubilization	
	index[a] in agar assay	μg/ml in broth assay
Field-grown rice		
Acinetobacter sp. BR-12	4.6	387 ± 19
Klebsiella sp. BR-15	4.8	395 ± 65
Acinetobacter sp. BR-25	6.7	524 ± 10
Enterobacter sp. BR-26	3.0	206 ± 9
BR-11	1.6	40 ± 4
BR-13	2.0	94 ± 7
BR-14	1.4	68 ± 4
BR-17	1.2	2 ± 3
BR-21	1.6	73 ± 9
BR-22	1.6	11 ± 6
BR-23	1.6	8 ± 6
BR-24	2.0	86 ± 8
In vitro-grown rice after surface-sterilization of seeds		
Microbacterium sp. BRS-1	2.0	97 ± 6
Pseudomonas sp. BRS-2	2.5	132 ± 8

Table I. Calcium phosphate solubilization by bacterial isolates in agar and broth assay using National Botanical Research Institute's phophate (NBRIP) growth medium.

None of the isolates showed any activity against $AlPO_4$ or $FePO_4$.
Bacterial population inoculated in liquid culture was 10^8–10^9 CFU/ml.
[a] Solublization index = total diameter (colony + halo zone)/colony diameter.

Four potent P solubilizing isolates from the rhizoplane of field-grown rice and two seed endophytes were identified based on their phenotypic and 16S rRNA genes sequencing as *Acinetobacter* sp. BR-12, *Klebsiella* sp. BR-15, *Acinetobacter* sp. BR-25, *Enterobacter* sp. BR-26, *Microbacterium* sp. BRS-1 and *Pseudomonas* sp. BRS-2. The 16S rDNA sequences of these active isolates viz. BR-12, BR-15, BR-25, BR-26, BRS-1 and BRS-2 have been deposited in DDBJ (DNA Database of Japan) under the accession numbers AB267073, AB267070, AB267071, AB267068, AB267072 and AB267069, respectively.

Maximum phosphate solubilizing activity was recorded in *Acinetobacter* sp. BR-25 and *Klebsiella* sp. BR-15. Time-course observation in the broth assay showed that both of them can equally solubilize increasing amounts of phosphate with time until 16 h (data not shown). The ability of phosphate solubilization by plant-associated *Pseudomonas, Klebsiella, Enterobacter* and *Microbacterium* species have been reported in several papers, however, reports on root-associated *Acinetobacter* sp. and their phosphate solubilizing activity are very rare (Rodríguez and Fraga, 1999; Vessey, 2003; Peix *et al.,* 2003). Several early studies revealed that *Acinetobacter* spp. are normally present in activated sludge (Buchan, 1983; Sidat *et al.,* 1999). They are efficient in storing polyphosphate intracellularly and can remove and release phosphorus from the sludge. High phosphate solubilizing activity exhibited by *Acinetobacter* spp. isolated from the rice rhizoplane of Bangladesh and their ability to colonize rice roots upon seed inoculation warrant further investigation to test its performance in field conditions. Several earlier studies showed that phosphate solubilizing rhizobacteria enhance the growth and yield of many economically important crops (deFreitas *et al.,* 1997; Rodríguez and Fraga, 1999).

Effect of pH value of the culture medium on growth of bacteria

Optical density of culture medium revealed that the rice rhizoplane PSB exhibited almost similar and rapid growth at pH 5 and 7, and very slow or no growth at pH 3 (Fig. 1A). This result is reasonable because these bacteria were isolated from the rhizoplane of rice cultivated in a high land having the soil pH 6.7. The growth of *Acinetobacter* sp. BR-12, *Klebsiella* sp. BR-15 and *Enterobacter* sp. BR-26 reached the peak within 2 to 4 days. The other bacterial isolates grew steadily with time until the 8th day of culture. *Acinetobacter* spp. is the predominant microorganism involved in enhanced phosphorus uptake from the activated sludge (Sidat *et al.,* 1999) but the mechanism of its luxury phosphorus uptake is not fully elucidated. Our results also suggest that *Acinetobacter* spp. and other rice isolates grow well in neutral medium and are less tolerant to high acidic conditions.

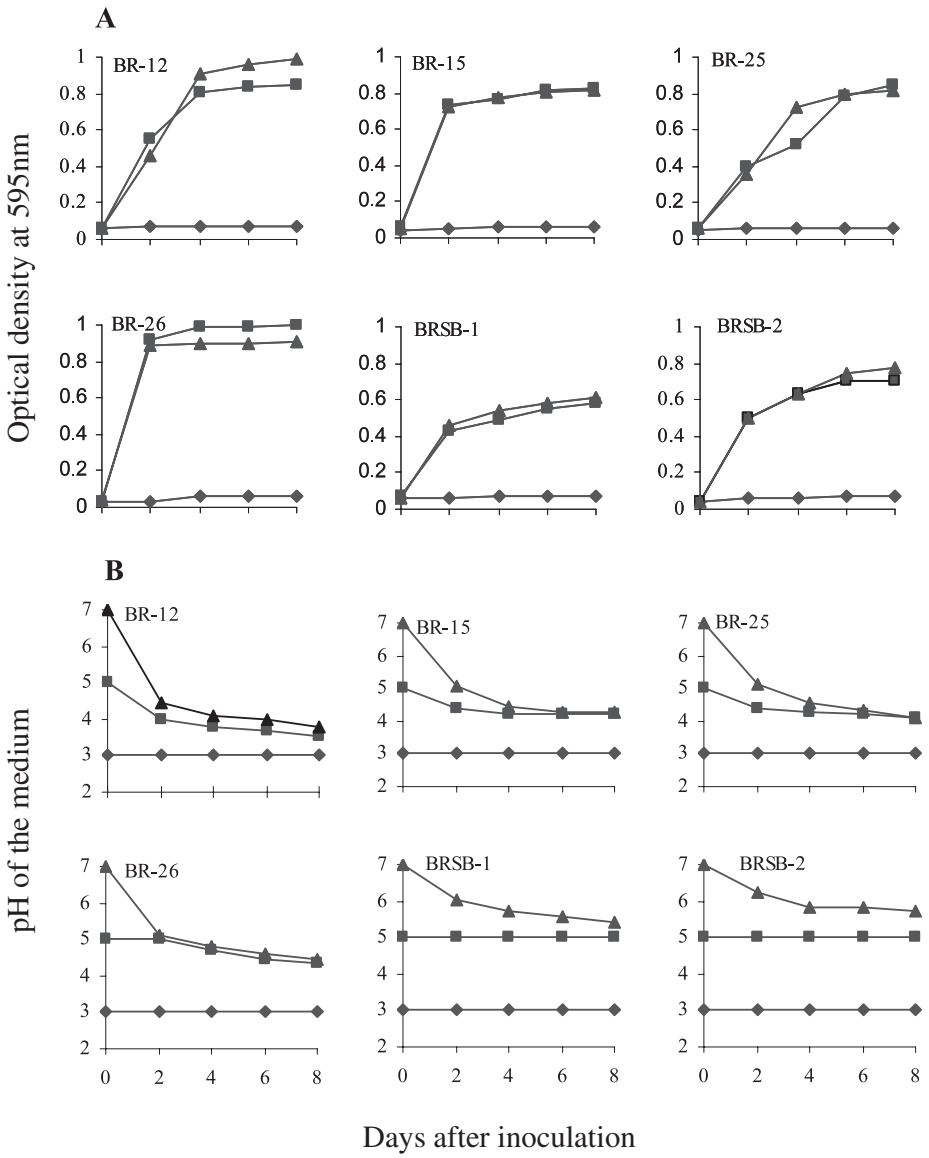

Fig. 1. Changes of the growth of bacteria reperesented by the optical density (A) and the pH value of the culture medium (B) with time of inoculation in National Botanical Research Institute's phophate (NBRIP) medium.
—◆— pH 3.0; —■— pH 5.0; —▲— pH 7.0. Each experiment was replicated three times.

Changes of pH value of culture medium with growth of bacteria

The pH value of the culture medium of all rhizoplane bacteria was decreased with time to pH 5 and 7 except for two rice seed endophytes (Fig. 1B). This is reasonable because these bacteria were isolated from the rhizoplane of rice grown in upland soils having pH 6.7. This result indicates that they secreted organic acids into the medium to solubilize calcium phosphate. The production of organic acids such as gluconic, 2-ketogluconic, lactic, isovaleric, isobutyric, acetic, oxalic, citric acid by phosphate solubilizing bacteria has been well

documented (Rodríguez and Fraga, 1999). Almost no change of pH value of culture medium was observed in case of the rice endophytes at pH 3 and 5, however, a slight decrease of pH value was observed when grown at pH 7. These results suggest that *Acinetobacter* sp. and *Klebsiella* exist in the rhizoplane of rice grown in Bangladesh and might help plants to take up phosphorus by roots.

Although the mechanism of the bacterial phosphate solubilizing activity observed in the current study is not clear at present, all of the isolates slightly decreased the pH value of the culture medium suggesting that organic acid secretion by the bacteria might play a role in phosphate solubilizing activity (Fig. 1B). Several lines of evidence suggest that the principal mechanism for mineral phosphate solubilization is the production of organic acids, and acid phosphatases by PSB play a major role in the mineralization of organic phosphorous in soil (Goldstein, 1986; Rodríguez and Fraga, 1999; Vessey, 2003; Thakuria *et al*., 2004).

Morphology of bacteria and their colonization on rice roots

Transmission electron microscopic observation revealed that bacteria isolated from the rhizoplane of field-grown rice possessed dense fimbriae but those isolated from the roots of rice seedlings grown from the surface-sterilized rice seeds in gnotobiotic Hoagland's gel medium had no fimbriae (data not shown). Fimbriae are filamentous protein appendages on bacterial cell surface; their known function is adhesion to surfaces of the plants or other objects (Korhonen *et al*., 1986). Our observation suggests that rhizoplane bacteria generally possessed fimbriae on their cells while the endophytes may lack these hairy protein appendages as they remain inside the plant. A further study with a high number of bacterial endophytes is needed to confirm this hypothesis.

To evaluate the ability of *Acinetobacter* sp. BR-25 and *Klebsiella* sp. BR-15 to colonize rice roots, we inoculated surface-sterilized seeds of rice cv. BR29 with an aqueous suspension of bacterial cells (*ca*. 10^8 CFU/seed), followed by incubation in a test tube (18 cm long and 1.5 cm i. d.) containing 1/5 strength of Hoagland's solution with gellan gum. Using scanning electron microscopy (SEM) we observed that roots of seedlings grown from seeds previously inoculated with bacteria vigorously colonized and attached in a unilaminar fashion (Fig. 2). Although bacterial biofilm was observed throughout the rice roots (Figs. 2A, B, D), higher density of micro-colonies was always found at the root-hair zones (Fig. 2C), however, root-hairs were almost free from the bacteria in both cases. These results suggest that inoculation of rice seeds with phosphate solubilizing rhizoplane bacteria might be a useful way for improving the phosphorus uptake by rice plants in Bangladesh as both tested bacteria showed high multiplication in the rhizoplane upon seed inoculation.

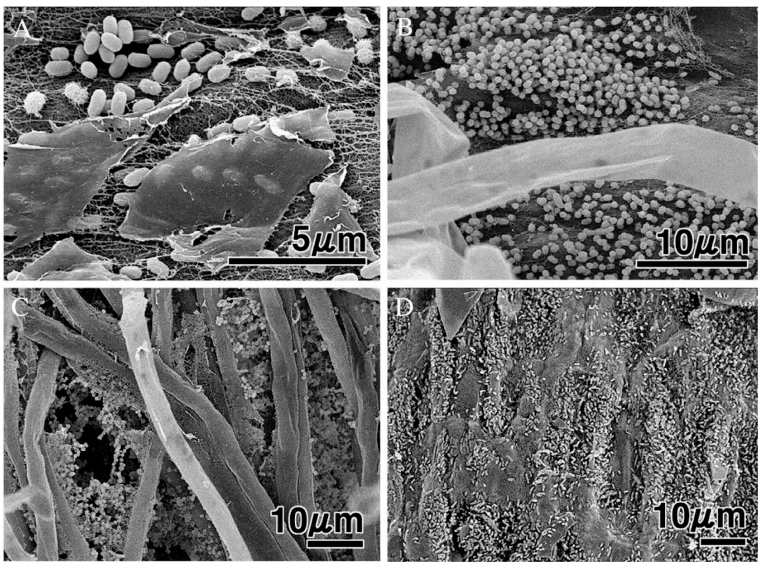

Fig. 2. Scanning electron micrographs showing dense colonization of *Acinetobacter* sp. BR-25 (A–C) and *Klebsiella* sp. BR-15 (D) on the surface of roots (cv. BR29) of rice seedlings from seeds previously inoculated with bacteria.

The solubilization of phosphorus in the rhizosphere is the most common mode of action implicated in plant growth promoting rhizobacteria that increase the nutrient availability to host plants (Richardson, 2001). Examples of recently studied associations include *Azotobacter chroococcum* and wheat (Kumar and Narula, 1999), *Bacillus circulans* and *Cladosporium herbarum* and wheat (Singh and Kapoor, 1999), *Bacillus* sp. and five crop species (Pal, 1998), *Enterobacter agglomerans* and tomato (Kim *et al.,* 1998), *Pseudomonas chlororaphis* and *P. putida* and soybean (Cattelan *et al.,* 1999), *Rhizobium* sp. and *Bradyrhizobium japonicum* and radish (Antoun *et al.,* 1998), and *Rhizobium leguminosarum* bv. *phaseoli* and maize (Chabot *et al.,* 1998). Phosphate solubilizing bacteria are common in rhizospheres (*e.g.*, Nautiyal *et al.,* 2000; Vazquez *et al*., 2000).

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