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Stress induced phosphate solubilization by *Arthrobacter* sp. and *Bacillus* sp. isolated from tomato rhizosphere

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Abstract

The importance of rhizospheric microbial phosphate solubilization has now been well documented. However, the performance of these microbes is greatly affected by various environmental stresses such as salt stress, pH stress, temperature stress etc. In this study, two stress tolerant phosphate solubilizing rhizobacteria *Arthrobacter* sp. and *Bacillus* sp. have been isolated from tomato rhizosphere and characterized with various morphological and biochemical tests. Phosphate solubilizing bacteria were screened on the basis of their phosphate solubilization and strains with high phosphate solubilizing ability were then tested against wide range of temperature, pH, and salt stresses. Their ability to solubilize other insoluble phosphates, such as ferric phosphate ($FePO_4$) and aluminum phosphate ($AlPO_4$) was also studied. In addition to phosphate solubilizing ability these strains also demonstrated various plant growth promoting and biocontrol activities including indole acetic acid (IAA) production. These two strains have the potential to be used as plant growth promoting rhizobacteria (PGPR).

Keywords: Rhizobacteria, phosphate solubilization, environmental stress, plant growth promoting rhizobacteria.

Introduction

Phosphorus (P) is an essential nutrient for plant growth and development constituting up to 0.2% plant dry weight (Harrison et al., 2002). Phosphorus is typically insoluble or poorly soluble in soils. Although the average P content of soils is about 0.05% (W/W), only 0.1 % of the total phosphorus exists in plant accessible form (Illmer and Schimmer, 1995). As a result large amounts of soluble forms of P fertilizers are applied to attain maximum crop production. However, the applied soluble forms of P fertilizers are easily precipitated into insoluble forms such as tricalcium phosphate [$Ca_3(PO_4)_2$], $FePO_4$, and $AlPO_4$ (Achal et al., 2007). It has been found that approximately 75–90% of applied P fertilizer is precipitated by Ca, Fe and Al metal cations and these insoluble forms are not efficiently taken up by the plants. This again leads to an excess application of P fertilizer to crop fields (Khan et al., 2007). The unavailable phosphates built up in soils are enough to sustain maximum crop yields globally for about 100 years (Goldstein et al., 1993; Khan et al., 2007). Additionally, excess P application also enhances the potential for P loss to surface waters through overland or subsurface flow, which accelerates freshwater eutrophication. Plants take up inorganic phosphate in two soluble forms: the monobasic ($H_2PO_4^-$) and the dibasic (HPO_4^{2-}) ions (Vessey, 2003). Some soil microorganisms are able to solubilize these insoluble P forms through the process of organic acid production, chelation, and ion exchange reactions and make them available to plants (Vessey, 2003). Seed or soil inoculations with phosphate solubilizing microbes (PSM) have largely been used to improve crop growth and production by solubilizing of fixed and applied phosphates (Nauyital et al., 2000). The existence of microorganisms able to solubilize various forms of calcium phosphate has been reported frequently but relatively few studies investigated the solubilization of other phosphates such as $AlPO_4$ and $FePO_4$. Microbes in alkaline soils in India are confronted with high salt, high pH, and high temperature and microbial phosphate solubilization is highly sensitive to these environmental stresses (Johri et al., 1999). The production of food and forage in semiarid and arid regions of the world can be increased by the application of PSMs capable of withstanding such abiotic stresses. Moreover, PSMs may also show plant growth promoting activities such as indole acetic (IAA), gibberellic acid, cytokinins, ethylene production, hydrogen cyanide (HCN) production, asymbiotic nitrogen fixation and resistance to soil borne pathogens etc (Cattelan et al., 1999). The aforementioned characteristics are necessary for an efficient biofertilizer (Ahmad et al., 2008). The objective of our study was to isolate and characterize phosphate solubilizing rhizobacteria that are able to solubilize various insoluble phosphates efficiently under environmental stresses.

Materials and methods

Isolation of phosphate solubilizing rhizobacteria

Phosphate solubilizing rhizobacteria were isolated from the rhizosphere of tomato grown in a tropical agricultural field at Kalyani, West Bengal India (22°59′N, 88°28′E). The soil in Kalyani is typically mild alkaline alluvial soil. The soils adhered to tomato roots were collected in sterile distilled water prior to serial dilution. Serially diluted (up to 10^{-5}) sample aliquots were spread onto Petri plates containing Pikovskaya (PKV) agar (Pikovskaya, 1948). Appearance of halo zones around some of the colonies suggested their phosphate solubilizing ability (Vyas et al., 2007). Eleven bacterial colonies were isolated from 10^{-3} dilution and were inoculated separately into conical flasks containing Pikovskayas broth and incubated at room temperature (25±2 ºC) on an orbital shaker for 2 days. Three replicated cultures were centrifuged at 8000g for 20 minutes at room temperature (25±2 ºC) and 2 ml aliquots of the supernatant were taken and soluble phosphorous estimated colorimetrically following the chloromolybdic acid - stannous chloride method (Jackson, 1967) at 600 nm. The corresponding amount of soluble phosphate was calculated from a standard curve of KH_2PO_4 (9 points; $r^2 = 0.99$). Two strains (labeled TRSB10 and TRSB 16) with highest phosphate solubilizing efficiency were used for further characterization (efficiency at solubilizing $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$ for 6 days). The $Ca_3(PO_4)_2$ solubilization assay was performed in similar way as described above. For $AlPO_4$ and $FePO_4$ solubilization a modified Pikovskaya's broth was used. The broth contained 4.0 g/l $AlPO_4$ or 6.0g/l $FePO_4.2H_2O$, yielding an equivalent amount of phosphorus as in the standard PVK medium (5.0g/l $Ca_3(PO_4)_2$) , together with 0.5 g $CaCO_3$ per liter to avoid lowering of pH in the broth. Estimation of the number of colony forming units (CFU) in Pikovskaya's broth was done for 6 days. Phosphate solubilization is primarily contributed by the production of organic acids which reduces the pH of the medium (Vessey, 2003). Therefore, the pH of Pikovskaya's broth (control and inoculated) was measured for 6 days.

Estimation of stress induced phosphate solubilizing capacity

For determination of phosphate solubilization under salt, pH, and temperature stressed conditions, Pikovskaya's broth with $Ca_3(PO_4)_2$ was used. Pikovskaya's broth (100 ml) with different concentrations of NaCl (0%, 2.5%, 5%, 10% and 20% w/v) was prepared for salt induced phosphate solubilization. To induce pH stress the pH of Pikovskayas broth was adjusted to 5 different levels (pH 8, 9, 10, 11) by 1N HCl or 1M NaOH. Flasks with salt and pH stress induced Pikovskaya's broth were inoculated with the two bacterial strains and incubated at room temperature (25±2 ºC) for 3 days. For estimation of high temperature induced phosphate solubilization, Pikovskaya's broth (100 ml) was inoculated with the strains were incubated for 3 days at three different temperatures (37^0C, 45^0C or 50^0C). In all cases, the quantity of solubilized $Ca_3(PO_4)_2$ was measured colorimetrically as described above.

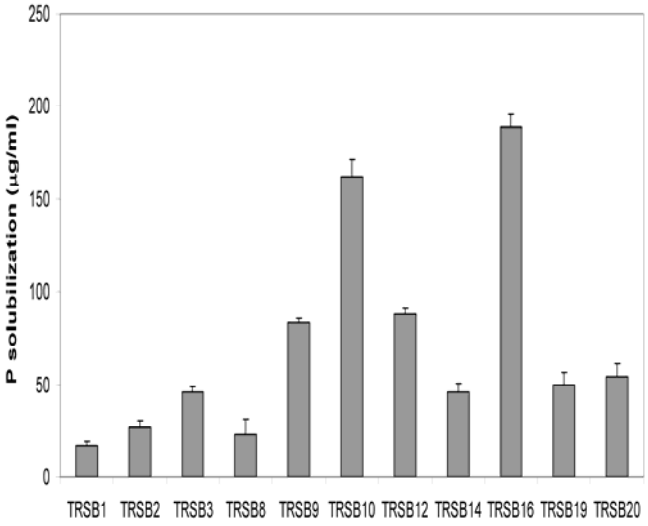

Fig 1. Tricalcium phosphate solubilization by various strains isolated from tomato rhizosphere. Each value is the mean of three replicates. Error bars show one standard error of the mean.

Table 1. F values showing the effect of time, cfu, and pH on $Ca_3(PO_4)_2$ solubilization.

	TRSB 10	TRSB 16
Time	131.9***	79.79***
Cfu	93.14***	129.1***
pH	1.517	131.1***
Time:cfu	14.72**	59.23***
Time:pH	2.920	71.64***
Cfu:pH	4.658	21.20**
Time:cfu:pH	7.2119*	9.396*

*** P<0.001 ** P<0.01 * P<0.05

Morphological and biochemical characterization

Morphological and biochemical characteristics were studied according to a microbiology manual (Cappucino and Sherman, 1982).

Study of PGPR characteristics

Production of IAA was estimated according to a modified Brick et al. (1991) method (Ahmad et al., 2008). Two bacterial strains were inoculated in 100 ml Pikovskaya broth and incubated for 24 hrs at room temperature (25±2 ºC). One ml of the inoculated broth for each strain was recultured in freshly prepared 100 ml Pikovskaya broth with 1 ml of 0.2 % L-tryptophan. These cultures were incubated at room temperature (25±2 ºC) for a further 24 hrs and 5ml aliquots were centrifuged at 8000 *g* for 10 min. Two ml of supernatant were mixed with 4 ml of freshly prepared Salkawaski reagent **(**100 ml of 35 % perchloric acid plus 2 ml 0.5 M $FeCl_3$ solution). The intensity of the resultant pink color was measured at 530 nm after 30 min of dark incubation.

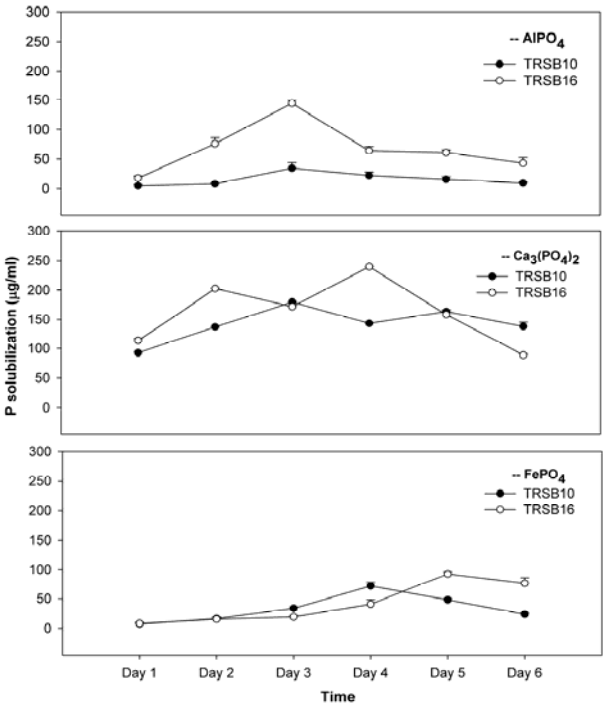

Fig 2. Solubilization various phosphates by *Arthrobacter* sp. (TRSB10) and *Bacillus* sp. (TRSB16) Each value is the mean of three replicates. Error bars show one standard error of the mean.

The corresponding amount of IAA was calculated from a standard curve (7 points; $r^2 = 0.98$). Production of HCN was detected according to Bakker and Schipper (1987) method. Single isolates were streaked onto petri plates of solidified King's B medium and a single disc of filter paper was placed in the lid of each petri plate. The petri plates were then sealed with parafilm® and incubated at 25^0C for 4 days, whereas one uninoculated set was kept as control. Color change in filter paper from deep yellow to dark brown was visually assessed for production of HCN. Ammonia production was detected according to Cappuccino and Sherman (1992) and Ahmad et al., (2008). In brief, all the isolates were grown separately in different culture tubes containing 10 ml of peptone water. After 4 days of incubation at 30^0C, 1 ml Nessler's reagent was added in each tube to determine the presence ammonia. Brown to yellow colour indicated the ammonia production.

Statistical analysis

The results were graphically presented by using Sigmaplot 11.0 (Systat Software Inc., Chicago, USA). The effect of CFU, pH, and time (individual and combined) on $Ca_3(PO_4)_2$ solubilization was analyzed by analysis of variance test with R software (www.r-project.org).

Results

The phosphate solubilizing bacteria isolated from tomato rhizosphere strains demonstrated a wide range (17-189 µg/ml) of $Ca_3(PO_4)_2$ solubilization after 2 days (Fig.1). Two strains (TRSB10 and TRSB16) with highest phosphate solubilizing efficiency were selected for further characterization. Both bacterial strains showed high efficiency to solubilize $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$ (Fig 2). In the time-course of phosphate solubilization assay, TRSB16 consistently showed high rates of solubilization of $Ca_3(PO_4)_2$ (239 µg/ml), $AlPO_4$ (144 µg/ml), and $FePO_4$ (92 µg/ml). Relatively low solubilization of $Ca_3(PO_4)_2$ (180 µg/ml), $AlPO_4$ (34 µg/ml), and $FePO_4$ (71 µg/ml) was observed in case TRSB10. The level of $Ca_3(PO_4)_2$ solubilization by TRSB16 was highest on the fourth day whereas TRSB10 showed maximum solubilization on the third day. For $AlPO_4$ both strains showed maximum solubility on the fourth day. In case of $FePO_4$, phosphate solubilization by TRSB16 was highest on the fifth day but TRSB10 solubilized highest amount of $FePO_4$ on fourth day. The solubilization of insoluble phosphate was found to be proportional to the number of CFU (Fig. 3). A steady increase in CFU number was observed up to Day 3 after which the microbial population size decreased gradually. The production of organic acid is thought to be responsible for phosphate solubilization which again reduces the pH of the medium. The pH of Pokivskaya's broth decreases sharply after Day 1 which is followed by a gradual decline. This supports the production of organic acids and explains the aforementioned phosphate solubilization patterns. The F values obtained from analysis of variance show that the effect of three factors viz. time, cfu, and pH is different in TRSB 10 and TRSB 16 (Table 1). Time and cfu were found to have high statistically significant impact on $Ca_3(PO_4)_2$ solubilization by both the strains. However, pH has significant effect on TRSB 16 only. These strains showed different levels of phosphate solubilization under various stresses (Fig 4). The production of soluble phosphate by TRSB16 was maximum at pH 10 and in case of TRSB10 it was pH 9. Both strains showed lowest phosphate solubilization at pH 8. At 2.5% salt concentration both TRSB10 and TRSB 16 showed the highest solubilization followed by a sharp decrease at subsequent salt concentrations. Phosphate solubilization was highest by TRSB10 at 45^0 C (165.37 µg/ml), however, for TRSB16 it decreased steadily with the increasing temperature.

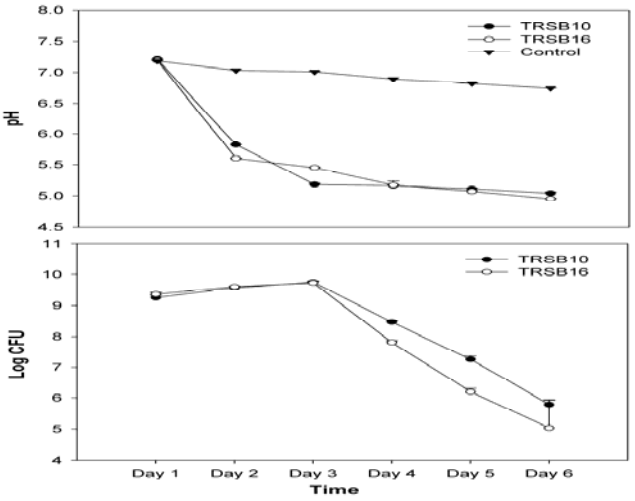

Figure 3. Change in pH and CFU during 6-days of incubation. Each value is the mean of three replicates. Error bars show one standard error of the mean.

Table 2. Morphological, biochemical, and plant growth promoting characteristics of TRSB 10 and TRSB 16.

Characteristics	TRSB 10	TRSB 16
Morphological-		
Gram staining	+	+
Motility	-	+
Pigmentation	-	-
Biochemical-		
Catalase	+	+
Amylase	+	+
Urease	-	+
Oxidase	-	+
Methyl red	-	+
H_2S production	-	-
Gelatin hydrolysis	-	+
Citrate utilization	-	+
Casein hydrolysis	+	+
Esculin hydrolysis	+	-
Starch hydrolysis	+	+
Voges Proskauer test	-	+
Plant growth promoting-		
IAA production	3 µg/ml	20.3 µg/ml
Ammonia production	+	+
Hydrogen cyanide production	-	+
Antibacterial activity-		
Xanthomonus	+	-
Pseudomonas	-	-

However, both strains showed efficient phosphate solubilization at 50^0 C with the amount of solubilized phosphate exceeding 100 µg/ml. With regard to biochemical characteristics, TRSB16 was more consistent than TRSB 10 (Table 2). TRSB16 is highly efficient IAA producer (20.3 µg/ml), however, TRSB10 also showed significant IAA production (3 µg/ml). Although both TRSB10 and TRSB16 showed ammonia production but only TRSB16 was HCN producer. Interestingly, TRSB10 was found to possess antimicrobial activity. Based on their morphological and biochemical characteristics TRSB10 and TRSB16 were identified as *Arthrobacter* sp and *Bacillus* sp respectively (Institute of Microbial Technology, Chandigarh, India).

Discussion

Phosphate-solubilizing bacteria are known to improve solubilization of fixed soil phosphorus and applied phosphates, resulting in higher crop yields. Phosphate solubilizing microbes have been routinely isolated from rhizospheric soil of various plants such as rice (Chaiharn and Lumyong, 2009), wheat (Ahmad et al., 2008), soybean (Son et al., 2006), mustard (Chandra et al., 2007), aubergine (Ponmurugan and Gopi, 2006), and chili (Ponmurugan and Gopi, 2006). However, the prevalence of PSB in tomato rhizosphere is not well-investigated in alluvial soil. Alluvial soils occupy approximately 75 million ha in India constituting the largest soil group in India. Our study shows that tomato rhizosphere comprises of abundant PSB with a range of P solubilizing efficiency. Ability to solubilize various insoluble phosphates is always desirable to be a competent PGPR. These two isolated strains were found highly efficient solubilizers of three common insoluble phosphates. Solubilization of $Ca_3(PO_4)_2$ was found in similar range as reported by Chen et al. (2007), Pandey et al. (2006), Johri et al. (1999). However, $AlPO_4$ and $FePO_4$ solubilization

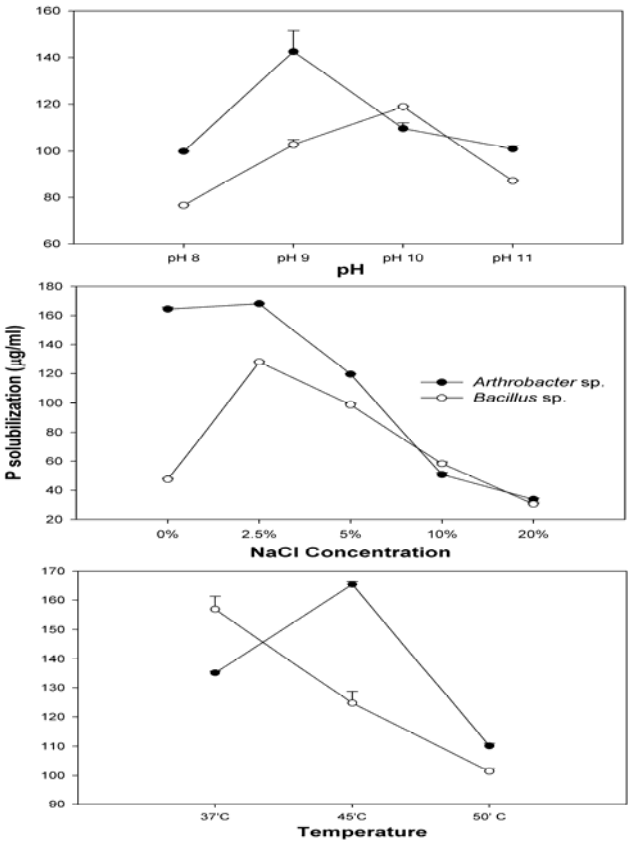

Fig 4. Stress induced solubilization of tricalcium phosphate in Pikovskya's broth. Each value is the mean of three replicates. Error bars show one standard error of the mean.

rate was found to be higher than the reported values by Henri et al. (2008) and Sulbaran et al. (2009). Phosphate solubilizing rhizobacteria are always confronted with various environmental stresses. The ability to withstand the adverse environmental conditions such as high salinity, high/low pH, and high temperature is significant not only for rhizobacterial survival in tropical agricultural soils but also to be used as biofertilizer. Stress induced phosphate solubilization has been studied by several researchers (Gaind and Gaur, 1991; Johri et al., 1999; Nautiyal et al., 2000; Son et al., 2006). Phosphate solubilization rate of these two strains under stressed condition was significantly higher than the reported values by Gaind and Gaur (1991), Johri et al. (1999), Nautiyal et al. (2000) and was in the similar range as reported by Son et al. (2006) for *Pantoea agglomerans* R-42. Plant growth promoting rhizobacteria can enhance plant growth directly by secreting plant growth promoting substances and making available some nutrients present in the environment and indirectly by anticipating or minimizing the influence of soil-borne phytopathogens (Ahmad et al., 2008). Auxin is the most effective plant growth hormone and among different auxins IAA is the commonest one, which is mainly produced by tryptophan dependent pathway. Rhizobacterial IAA production plays a significant role in the host plant's growth. Indole acetic acid production in microbial has been investigated by several researchers (Ahmad et al., 2008; Ghosh et al., 2008; Gulati et al., 2009). Both the strains especially TRSB16 are highly efficient IAA producer.

Production of ammonia is an important attribute of PGPR that influences plant growth indirectly (Wani et al., 2007). Production of this secondary metabolite was found in both bacterial strains. Hydrogen cyanide is a secondary metabolite implicated in plant protection. Thus the ability to produce HCN is a desired quality of plant growth promoting rhizobacteria. By synthesizing HCN some rhizobacteria inhibit plant disease development thus strengthening the host's disease resistance mechanism (Schippers et al., 1990). The presence of HCN in the soil can also act as an efficient biological weed control measure by inhibiting seed germination and seedling vigor. The strain TRSB16 was found to be a strong HCN producer.

Tomato (*Lycopersicon esculentum*) is the second most important vegetable crop after potato. World's total production was approximately 126.2 million tons in 2007 (FAOSTAT, 2007) and India is the fourth largest producer. Therefore, tomato production plays a key role in Indian agriculture. Application of P fertilizer is important for tomato as P limitation can result in stunted growth. To achieve maximum yield, every year large quantity of P fertilizer is applied, majority of which becomes immobilized and unavailable to plants. Phosphate solubilizing bacteria isolated from tomato rhizosphere can play a critical role by making P available to tomato plants. Furthermore, the PSB with PGPR functions such as IAA, HCN, ammonia production and biocontrol activities may have profound impact on tomato plants growth. In this study, we have shown that TRSB10 and TRSB16, isolated from tomato rhizosphere are efficient phosphate solubilizers, which have various PGPR activities and are also able to perform significantly under unfavorable environmental conditions. These two strains show potential as plant growth beneficial inoculants in alkaline soil regions. Further studies on the rhizocompetence of these two strains are recommended.

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