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# Phosphate solubilizing bacteria from the acidic soils of Eastern Himalayan region and their antagonistic effect on fungal pathogens

Bhabatarini Panda [a,b,*], H. Rahman [a], Jagabandhu Panda [c]

[a] ICAR Research Complex for NEH Region, Sikkim Centre, Tadong, Gangtok, India
[b] Post Graduate Department of Botany, Utkal University, Bhubaneswar, Odisha, India
[c] College of Post-Graduate Studies, Central Agricultural University, Umroi Road, Umiam, Meghalaya, India

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ABSTRACT

This study tries to identify the native population of phosphate solubilizing bacteria (PSB) in the rhizospheres of maize, rice, ginger and large cardamom grown in different regions of Sikkim (India). Out of 104 rhizospheric samples collected from three agroclimatic zones of the state, 42 number of PSBs were isolated. However, only 26 best performing PSB based upon their solubilization in solid media are reported in this paper. The highest number of PSB with greatest variants were found in the rhizosphere of rice.The population density of PSB varies between 2–36 $\times$ $10^6$ cfu $g^{-1}$. Based upon the morphological and 16S rRNA sequencing, the isolates were clustered under the genera *Bacillus*, *Pseudomonas*, *Micrococcus*, *Staphylococcus*, *Microbacterium* and *Delftia*. Phosphate solubilization efficiency of these isolates varied between 46 to 160% in solid medium and in the liquid Pikovskaya's medium phosphate solubilization capacity varied between 30.2 to 203.7 mg $L^{-1}$. Though all of the isolates were able to solubilize aluminum and iron phosphate significantly however, they solubilized $Ca_3(PO_4)_2$ to a greater extent than the other two with $AlPO_4$ exhibiting comparatively poor solubilization among the three phosphate sources. Further, the *in vitro* antagonistic effect of the PSB isolates were studied with seven locally available fungal pathogens and it revealed that the *Bacillus amyloliquefaciens* PSB isolates showed higher antifungal activity against soil and root borne pathogens (*Rhizoctonia* sp., *Pythium* sp., *Fusarium* sp.) than the leaf pathogens.



## 1. Introduction

Phosphorus (P) is an essential component in plant metabolisms starting from cell division to regulation of metabolic pathways and growth processes, the deficiency of which is believed to be one of the major biophysical constraints for crop yield. However, of the total soil phosphate, only 1–5% are in a soluble, plant available form and the rest became unavailable due to its fixation in soil (Molla and Chowdary, 1984). The unavailability of phosphorous in acidic soil is more severe due to precipitation of soluble phosphates with highly reactive cations, such as $Fe^{+3}$ and $Al^{+3}$ (Del Campillo et al., 1999; Vassilev and Vassileva, 2003, Liu et al., 2014). Out of these two cations, the precipitation induced by aluminum is the major cause of P fixation in acidic soils. (Holford, 1983). Soluble phosphate ions can also be adsorbed by oxides of these metals or on clay mineral surfaces (Paul, 2006) further reducing the availability. Even, a large percentage of phosphate fertilizers applied to the soil also precipitate in their insoluble forms, increasing the fertilizer application requirements and the negative environmental impact (Gyaneshwar et al., 2002, Acevedo et al., 2014). Therefore, the release of these insoluble and fixed forms of phosphorus is an important aspect not only to increasing soil phosphorus availability and thus fertility of soil but also to reduce negative environmental impact.

Insoluble phosphate can be solubilized in acidic soils by microbial activity (Bashan et al., 2013). In fact, a group of soil bacteria called phosphate solubilizing bacteria (PSB) have the ability to dissolve part of the fixed phosphorus and make it available to the crop by secreting low molecular weight organic acids and enzymes (Kucey et al., 1989; Rodríguez and Fraga, 1999; Tilak et al., 2005; Khan et al., 2009).

Numerous studies have already been reported world wide signifying the importance of PSB, its application as biofertilizer, identifying the various strains of PSB and their efficacy in phosphate solubilization and their availability in natural soils under different cropping systems, etc. (Chen et al., 2006; Fankem et al.,

* Correspondence to: Post Graduate Department of Botany, Utkal University, Bhubaneswar, Odisha 751004, India.
*E-mail address:* bhabatarini_panda@rediffmail.com (B. Panda).

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2006; Khan et al., 2007; Jorquera et al., 2008; Chang and Yang, 2009; Dastager et al., 2010, Acevedo et al., 2014; Liu et al., 2014). Few studies have been reported on isolation of PSB from acidic soils and their P-solubilization capacity with varying degree. The identification of those isolates also suggested that there are various different species of PSB found in different locations (Panhwar et al., 2014; Wang et al., 2014). One of such study has been reported from the soils of middle Himalayan region (Gusain et al., 2015).

The scientific literature is full of information on various aspects of PSB, yet a very little information is available on the ability of bacteria to solubilize phosphate under acidic soils in tropical region with suboptimal temperature conditions. Further, many beneficial microbes may be local isolates which are native to the location and cropping systems and they will be more suitable for adaptation to the local climatic environment (Cho and Tiedje, 2000).

Himalayan region is one of the major biodiversity hotspots in our planet and it is home to many species of plant, animal and microbes. Sikkim being situated in the Eastern Himalayan Range with suboptimal temperature and acidic soil due to high rainfall, it is postulated that there may be a possibility of the presence of various strains of plant growth promoting rhizobacteria (PGPR) which can sustain both the acidic soil and suboptimal temperature conditions and having substantial aluminium phosphate and iron phosphate solubilization capability. The present study was, therefore, aimed at looking for the occurrence of PSB in the rhizospheric soil of the major crops of Sikkim i.e., maize (*Zea mays*), rice (*Oryza sativa*), ginger (*Zingiber officinale*) and large cardamom (*Amomum subulatum*) grown in different agro climatic regions prevailing in the state. It may further help in identifying the dominant types of bacteria involved in P-solubilization in these climatic and soil conditions in Himalayan Region and selecting a superior PSB strains for further field application.

The antifungal activity of the isolated PSB on different locally available disease causing fungal strains are also studied so that those strains may also be used further as biocontrol agents.

## 2. Materials and methods

### 2.1. Sample collection

Sikkim covers a total land area of 7096 sq. Km. between 27°04′–28°07′N and 88°00′E–88°56′E. The thumb-shaped state is characterized by wholly mountainous terrain with the elevation ranging from 280 m to 8585 m. Owing to its location in the Himalayas, Sikkim is geographically diverse and it has four agroclimatic zones *viz.*, sub-tropical high humid (approximate altitude $< 1000$ m, annual mean air temperature $> 20°$ C), temperate humid (approximate altitude 1000–2000 m, annual mean air temperature 10–20 °C), sub-alpine low humid (approximate altitude 2000–3000 m, annual mean air temperature $< 10$ °C) and alpine dry zone (approximate altitude $> 3000$ m, average air temperature $< 10$ °C). The present investigation was carried out in three agroclimatic zones of Sikkim (as the alpine dry zone does not contain any crop vegetation under study). A total of 104 soil samples were collected from the rhizosphere of maize (17 samples), rice (38 samples), ginger (24 samples) and large cardamom (25 samples) crops grown in different regions of Sikkim. The rhizospheric soil samples of approximately 40–50 g were obtained from different crop fields and stored in plastic bags at low (4 °C) temperature till the bacterial isolation.

### 2.2. Isolation and identification of PSB

Ten grams of soil sample were suspended in 90 ml of sterile distilled water to obtain $10^{-1}$ dilution. Serial dilutions were prepared by mixing 1 ml of the suspension made into 9 ml of the sterile distilled water, until the $10^{-7}$ dilution was obtained. Three aliquots of 0.1 ml from each dilution was plated on Pikovskaya's solid medium (10 g $C_6H_{12}O_6$, 5 g $Ca_3(PO_4)_2$, 0.5 g $(NH_4)_2SO_4$, 0.2 g $K_2SO_4$, 0.1 g $MgSO4 \cdot 7H2O$, 0.5 g yeast extract, 0.002 g $MnSO_4 \cdot H_2O$ and 0.002 g $FeSO_4$ in 1 L distill water; Pikovskaya, 1948) and NBRI-BPB solid medium (10 g $C_6H_{12}O_6$, 5 g $Ca_3(PO_4)_2$, 5 g $MgCl_2$, 0.25 g MgSO4.7H2O, 0.2 g KCl, 0.1 g $(NH_4)_2SO_4$, and 0.025 g bromophenol blue in 1 L distill water; Nautiyal, 1999) with 2% agar powder. The plates were incubated at $30 \pm 1$ °C for 5 days. The bacterial colonies showing halo zone on Pikovskaya's plate or yellow colored halo zones around the colonies on NBRI-BPB plates were selected as phosphate solubilizers. The colonies were purified and maintained on Pikovskaya's slants for further study. All the isolates were classified on the basis of Grams staining, presence or absence of spore and colony characteristics such as the size, color, shape and texture of each isolate. *Pseudomonas striata,* a standard PSB obtained from the Division of Microbiology, Indian Agricultural Research Institute, New Delhi, India, was used as a reference strain.

### 2.3. Extraction of bacterial genomic DNA

DNA was extracted from overnight cultures grown on nutrient agar at 37 °C using QIAmp DNA mini kit (Qiagen, Duesseldorf, Germany) as per manufacturer's instructions. The concentration of genomic DNA was determined using Nanodrop 2000c (Thermofischer Scientific Inc., Waltham, MA, USA) and stored at $-20$ °C until further use.

### 2.4. PCR amplification and sequencing of 16S rRNA gene

A 16S rRNA gene of about 918 bp long was PCR amplified using the universal primers: 27f (5′-AGA GTT TGA TCC TGG CTC AG-3′) and 907r (5′-CCC CGT CAATTC ATT TGA GTT T-3′) thus generating a PCR product corresponding to nucleotide positions 8 to 926 of the *Escherichia coli* 16S rDNA sequence (Weisburg et al., 1991).

A 15-μl reaction mixture was prepared in a 0.2-ml PCR tube using 0.5 μM of each of the above primer, PCR master mix 2 × (Taq-DNA polymerase in reaction buffer, 2.5 mM $MgCl_2$, 200 μM of dNTP mix) (Promega, Mannheim, Germany), and 50 ng of DNA. The reaction was performed in a thermocycler (Eppendorf, Germany) with an initial 5 minute denaturation at 95 °C, followed by 30 cycles of 1 minute denaturation at 95 °C, 1 min annealing at 55 °C, 1 min extension at 72 °C, and a final extension step of 5 min at 72 °C. Ten microliters of amplified PCR products were resolved in 1.2% agarose gel and stained with ethidium bromide (1 μg/ml). The PCR products were visualized by a gel documentation system (Syngene, UK) after electrophoresis. The amplicons were purified using a QiaAmp PCR purification kit (Qiagen, Düsseldorf, Germany) as described in the manufacturer's protocol. Purified amplicons were commercially sequenced at Eurofins Genomics, Bangalore, India.

To avoid contamination, the solutions were prepared with sterile water (Sigma-Aldrich, Vienna, Austria) and the steps were performed with aerosol-resistant tips in a clean PCR/UV Work Station. Two negative controls were carried out through the whole procedure for each set of reactions, in which instead of sample material, water was used to exclude the possibility of false-positive PCR results through cross contamination.