RESEARCH

PHOSPHATE SOLUBILIZERS FROM THE RHIZOSPHERE OF
Piper nigrum L. IN KARNATAKA, INDIA

Usha Seshachala¹, and Padmavathi Tallapragada²*

Black pepper (Piper nigrum L.) is a climbing vine known for its pungent fruit used as a spice worldwide. The aim of this study was to evaluate the available P content in the native soils where pepper is grown as a crop plant. The native population of phosphate solubilising microbes (PSM) was studied from the rhizosphere of P. nigrum plants grown in the Western Ghats of Karnataka, India. A variety of phosphate solubilising bacteria and fungi were isolated from the rhizosphere soil samples. Phosphate solubilising capacity of different isolates was studied on Pikovskaya’s medium. The isolates were tested for their phosphate solubilising capacity in vitro with three different phosphate sources, tricalcium phosphate (TCP), potassium dihydrogen phosphate (KHP), and rock phosphate (RP) in the concentrations 2.5, 5.0 and 7.5 g L⁻¹. The three phosphate sources were solubilised by the isolates in varying proportions. The dominant PSM flora obtained from the samples included Bacillus and Aspergillus. The study showed that PSM utilised the three phosphate sources TCP, KHP, and RP with considerable variability. The phosphatase activity of the isolates showed that the predominant microorganisms were Bacillus subtilis (5.33 U mL⁻¹) and Aspergillus (11.5 U mL⁻¹). The predominant organisms were identified up to molecular level.

Key words: Aspergillus, Bacillus, phosphate solubilising microbes, 16s rDNA analysis.

¹Dr. MGR University, Department of Microbiology, Maduravoyal, Chennai, 400095 Tamil Nadu, India.
²Jain University, Department of Microbiology, Jayanagar Bangalore 560011, Karnataka, India.
*Corresponding author (vam2010tpraviju@gmail.com).
Received: 5 November 2011.
Accepted: 21 June 2012.
Phosphate solubilising microbes are a group of common soil organisms in the rhizosphere of several plants. Secretion of organic acids and phosphatases to solubilise insoluble phosphate to soluble forms are common in this group. Although several PSBs occur in the soil their numbers are not adequate to compete with other bacteria commonly established in the rhizosphere. Since the population of inorganic PSM in the soil is comparatively low, the number of PSM in the rhizospheric soil is more important than in the non-rhizospheric soil (Chailharn et al., 2008).

The availability of P for plant uptake is also determined by the amount of bioactivity in the soil. Phosphate solubilising microorganisms acting in unison with the plant roots are responsible for solubilising phosphatic minerals (Levyal and Janer, 2001). In natural systems PSM consist of a broad class of bacteria and fungi that interact in the soil, especially in the extreme micro environments found around the plant roots, the rhizosphere. While most PSM obtain a great deal of their energy needs from the plant root exudates, some derive their nutrient requirements directly from rock minerals (Taalab and Badr, 2007). These bacteria and fungi have a tremendous potential in being used as bio fertilizers and thus being currently promoted widely in agriculture. It has been reported that the highest numbers of PSM depends on the cultural activities and the different soil properties like physical and chemical properties and the content of organic matter and soil P (Yahya and Azawi, 1998).

The present study aims at isolation of phosphate solubilising microorganisms- and fungi from the rhizospheric soils of pepper in Karnataka, India. Our experiment focused at testing the solubility of various sources of inorganic phosphates by the PSM of pepper rhizosphere. The different phosphates tested included tricalcium phosphate, potassium dihydrogen phosphate, and rock phosphate at three different concentrations - 2.5, 5.0 and 7.5 g L⁻¹. A comparative analysis of the solubility of the three phosphates by the PSM in the laboratory media was sought and the predominant PSM being identified up to molecular level.

MATERIALS AND METHODS

Soil analysis
Rhizospheric soil samples from four different pepper cultivating regions of Karnataka, India, were collected from six different regions in Murunadu (12°18’ N, 75°45’ E), and these soils were mixed together and triplicates were taken for further analysis. Similar procedure was followed from the other two different pepper plantations from Birur (13°53’ N, 75°58’ E), and one home stead farm from Bangalore (12°58’ N, 77°48’ E), co-cultivated with coconut (Cocos nucifera L.) trees, and these soils were analysed for their physico-chemical properties. The pepper plants were uprooted and the rhizospheric soil around roots was collected. Pepper plants selected were mature, 3-yr of age, and at fruiting stage for soil sampling to obtain variability in microorganisms. Soil samples were dried, crushed and passed through a 2 mm sieve to represent one composite sample (Sharma et al., 2007; Turan et al., 2007). The dried and homogenised soil was tested for their organic C and P content and pH in the concentration of 10 g in 100 mL of distilled water. Organic C was analysed using wet digestion method, P by using titrimetric method (Olsen and Sommers, 1982).

Murunadu is a hilly region of Karnataka with an annual rainfall of over 3000 to 3800 mm where pepper plants are grown in natural conditions on sloppy terrains, rich organic soils under the cover of tree plantation crops like coconut, coffee (Coffeea arabica L.), areca (Areca catechu L.), tea (Camellia sinensis L. Kuntze), sesbania (Sesbania grandiflora [L.] Pers.) (Hamza et al., 2007). Birur is located in Malnad region of Karnataka, and receives normal average rainfall of 2725.5 mm and has red loamy soil. Bangalore soil is red loamy and receives an average rainfall of 150 to 200 mm and pepper plants are artificially irrigated.

Isolation of phosphate solubilizers
Phosphate solubilising fungi and bacteria were isolated from serial dilution technique. The soil was suspended in 9 mL of saline blank and serial dilutions of this stock were done to obtain dilutions of 10⁻⁶. Each dilution was pour plated on nutrient agar and Pikovskaya’s medium (PK medium) simultaneously (Chen et al., 2002). Plates were incubated at 28-30 °C for 3-4 d for bacterial growth and 5-7 d for fungal growth. Pure cultures were identified on the basis of their morphological and cultural characteristics (Pikovskaya, 1948; Seshadri and Ignacimuthu, 2002).

The colonies surrounded with halo zones around it were picked and transferred thrice by streak plate method on to new Pikovskaya’s medium to obtain pure cultures.

Detection of phosphate solubilisation efficiency
The isolates were spot inoculated on Pikovskaya’s medium for detection of their phosphate solubilising capacity for three different phosphates tricalcium phosphate (TCP), potassium dihydrogen phosphate (KHP) and American rock phosphate (RP) and incubated at 28 °C for 3 and 7 d for bacteria and fungi respectively. The halo zones around the colonies were measured in mm and the solubilising efficiency was calculated according to the formula (Abou El Yazeid et al., 2007):

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PSE = \frac{\text{diameter of zone/diameter of colony}}{100}
\]

Phosphate solubilisation on Pikovskaya’s solid medium was examined by growing the different isolates on PK media substituted with TCP, KHP, or RP. The solubilisation ability of different isolates for the three different phosphates was noted at three different concentrations - TCP, KHP and RP, used in different concentrations of 2.5, 5.0, and 7.5 g L⁻¹ (Seshadri and Ignacimuthu, 2002;
Ponmurugan and Gopi, 2006a; Nopparat et al., 2007). The phosphatase activity of the isolates was detected by using p-nitrophenol as substrate by colorimetric estimation (Ponmurugan and Gopi, 2006b).

**Identification of PSM**

Pure cultures were used for the identification of isolated organisms. The isolates sub cultured on the slants were identified morphologically and culturally using staining and cultural characteristics. Identification of the bacterial culture to its nearest species, 16s rDNA sequence data was obtained using genomic DNA. The 1.4 kb rDNA was amplified with the help of high fidelity (polymerase chain reaction) PCR polymerase. The PCR product thus obtained was sequenced bi directionally with the aid of forward, reverse and internal primers. A sample of 1 µL of genomic DNA was taken and was amplified with 400 ng of 16 s forward primer with the sequence of 5’-AGAGTRTGATCMGYCTWAC-3’ and 400 ng of reverse primer reading 5’-CGYTAMCTTWTTACGRCT-3’ and Taq polymerase enzyme (Sundara et al., 2002). The sequence data obtained was aligned and analysed to its nearest neighbours (Weisburg et al., 1991; Espinosa-Victoria et al., 2009).

The phylogenetic tree was built using System Software Aligner and the distance matrix was generated using the Jukes-Counter corrected distance model. The distance matrix was generated only with alignment model positions and the alignment inserts were ignored. The minimum position for alignment comparisons was 200 bases. The phylogenetic tree was constructed with an alphabet size of 4 and length size 1000 (Bruno et al., 2000). The fungal identification was carried out using the genomic DNA extracted from the pure culture. The internal transcribed spacer (ITS) region of the rDNA was amplified with the help of universal primers ITS 4 & 5 and then sequenced. The editing of the crude sequence was done using the manual mode and it was aligned with the reference sequence obtained from National Center for Biotechnology Information (NCBI) database (Rodríguez et al., 2006).

**Statistical analysis and experimental design**

All the investigations were conducted in triplicates. Viable count and primary isolation was done in triplicate. The phosphate solubilisation was conducted in Petri dishes in triplicate on PK media with a single organism inoculated in the centre as a point and from each replicate; three readings of the zone of inhibition were recorded in millimetres. Results were presented as the mean zone of inhibition for each organism. The phosphatase assay was carried out in Erlenmeyer flasks independently in triplicates, each flask containing 100 mL of the broth medium. Data were subjected to ANOVA with significance of ± 0.5. Most efficient phosphate solublising bacteria and fungi were identified up to molecular level.

**RESULTS**

The soil analysis result shows a variable phosphate content in all soil samples (Table 1). The physicochemical analysis of the soil samples indicated low P content in correlation with the acidity of the soils in case of Murunadu and Birur soil samples, whereas Bangalore showed a higher pH and a lower P content. The first three soil samples tested showed a low pH ranging from 5.4 to 6.0 and showed the presence of high organic C content varying between 4.1-6.8%. The rhizospheric soil samples collected from Murunadu, Birur, and Bangalore districts of Karnataka, India, range in the texture from black soil, clayey, to red loamy soil in Bangalore region. The soils being mainly fertilised with organic compost and fish meal (Birur) showed the presence of high organic C content but extremely low in phosphates (3-4 kg ac⁻¹). Pepper plants in these areas are co-cultivated with several species of tree plantation crops like coconut, coffee, areca, tea, and sesbania. It has been reported that these hilly regions where pepper cultivation is extensive, have a sloppy terrain resulting in soil losing its nutrients and being low in phosphates (Hamza et al., 2007).

**Phosphate solubilising microorganisms (PSM)**

Rhizospheric soils of pepper have yielded the presence of several bacterial and fungal forms that were capable of phosphate solubilisation in form of TCP, KHP and RP (Table 2) used in different concentrations of 2.5, 5.0 and 7.5 g L⁻¹. The primary isolation of PSM on Pikovskaya’s medium using soil dilution method indicated the presence of several species of bacteria – *Bacillus*, *Arthrobacter*, *Pseudomonas*, *Streptomyces*, *Actinomycetes*, and *Nocardia*. Thereafter the isolated bacteria were supplied

| Sample No | Geographical location from collected | Soil type | Soil pH | Organic carbon | Phosphate content | Available potash |
|-----------|-------------------------------------|-----------|--------|---------------|-----------------|-----------------|
| 1         | Murunadu, Karnataka, 12°18’ N, 75°45’ E | Black clayey soil | 5.4   | 1.4           | 7.4            | 291             |
| 2         | Birur, Karnataka 13°53’ N, 75°58’ E | Red loamy soil | 5.7   | 4.1           | 7.4            | 617             |
| 3         | Birur, Karnataka 13°53’ N, 75°58’ E | Red loamy soil | 6.0   | 3.7           | 9.9            | 948             |
| 4         | Bangalore, Karnataka 12°58’ N, 77°48’ E | Red soil    | 8.0   | 6.8           | 7.4            | 1007            |
with different phosphates TCP, KHP and RP (Table 2) at 2.5, 5.0 and 7.5 g L\(^{-1}\). Amongst the isolated bacteria the most predominant forms with maximum phosphate solubilisation capacity were various species of Bacillus. The predominant PSF included Aspergillus species. The results (Table 2) indicated greater numbers of these forms in the rhizospheric zone showing maximum clearance of phosphate on Pikovskaya’s medium. The maximum zone of clearance was shown by B. subtilis (28 mm) and a minimum zone was given by the Actinomycetes isolate.

Solubilisation efficiency of TCP, KHP and RP by different bacteria and fungi are indicated in Figures 1 and 2. TCP was found to be solubilised greatest by Bacillus subtilis followed by Bacillus species str 2 at a concentration of 2.5 g L\(^{-1}\), but at 5.0 g L\(^{-1}\) it was seen that B. subtilis showed the maximum zone. Though the optimum concentration for solubilisation of KHP by the bacterial isolates was at 7.5 g L\(^{-1}\), the solubilisation of TCP and KHP was much reduced at 2.5 g L\(^{-1}\). The solubilisation of RP was rather too negligible or not solubilised by the bacterial isolates.

Among the different species of Aspergillus isolated, A. niger showed optimum zone with TCP and KHP, but reduced activity for RP. The other isolates showed variations in their phosphate solubilisation efficiency at the concentrations of 2.5 and 5.0 as well as at 7.5 g L\(^{-1}\) of different phosphates (Table 3).

The dominant phosphate solubilizers were identified as B. subtilis (86 mm) among bacteria and A. niger (26.3 mm) among fungal isolates. Both strains were able to solubilise TCP, KHP, and RP efficiently at an optimum concentration of 5.0 g L\(^{-1}\). The phosphatase activity of the isolates showed that the predominant isolates B. subtilis and A. niger had the maximum activity for phosphate solubilisation (Figure 3).

**Fungal identification**

The six strains of Aspergillus used in the study of phosphate solubilisation were identified based on morphological and cultural features (Ponmurugan and Gopi, 2006a). They were identified as A. flavus, A. terreus, A. clavatus, A. flavus str. 2, A. fumigatus and A. niger.

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**Table 2. Phosphate solubilising bacteria from Piper nigrum rhizosphere on Pikovskaya’s medium with 5.0 g L\(^{-1}\) of tricalcium phosphate (TCP).**

| Sample N° | Soil sample  | Organisms isolated | Colony count (in cfu g\(^{-1}\)) | Zone (mm) |
|-----------|--------------|-------------------|-------------------------------|----------|
| 1         | Murunadu, Karnataka | Bacillus spp. | 3 x 10^4                     | 8        |
|           |              | Arthrobacter     | 3 x 10^4                     | 0        |
|           |              | Pseudomonas      | 6 x 10^3                     | 2        |
| 2         | Birur 1, Karnataka | Streptomyces    | 5 x 10^4                     | 5        |
|           |              | Bacillus subtilis| 3 x 10^3                     | 28       |
| 3         | Birur 2, Karnataka | Actinomycetes   | 4 x 10^3                     | 10       |
| 4         | Bangalore, Karnataka | Bacillus spp.   | 4 x 10^3                     | 10       |
|           |              | Actinomycetes   | 2 x 10^4                     | 5        |
|           |              | Nocardia         | 6 x 10^6                     | 6        |

**Table 3. Phosphate solubilising fungi from Piper nigrum rhizosphere on Pikovskaya’s medium with 5.0 g L\(^{-1}\) of tricalcium phosphate (TCP).**

| Sample N° | Soil sample  | Organisms isolated | Colony count (in cfu g\(^{-1}\)) | Zone (mm) |
|-----------|--------------|-------------------|-------------------------------|----------|
| 1         | Murunadu, Karnataka | Aspergillus niger | 4 x 10^5                     | 6        |
|           |              | Aspergillus sps 1 | 2 x 10^6                     | 10       |
| 3         | Birur 2, Karnataka | A. terreus       | 4 x 10^5                     | 4        |
| 4         | Bangalore, Karnataka | A. flavus       | 5 x 10^4                     | 7        |
The strain of \textit{A. Niger}, which showed a maximum zone of clearance with different phosphates, was identified using genomic DNA extract from the pure culture. It possessed 100\% ITS sequence similarity with seven \textit{Aspergillus} species: \textit{Aspergillus vadensis} Samson R.P. de Vries, Frisvad (2005), \textit{A. tubingensis} (Mossery) Kozak (1934), \textit{A. foetidus} Thom & Raper (1945), \textit{A. costaricaensis} Samson & Frisvad (2004), \textit{A. niger} var. \textit{niger} Tiegh. (1867), \textit{A. awamori} Nakaz. (1915), and \textit{A. niger} var. \textit{phoenicis} (Corda) Al-Musallam (1980).

Table 4 gives the alignment sequence data of \textit{A. niger} which showed 100\% similarity with \textit{A. niger} var. \textit{niger} Tiegh. (1867) and \textit{A. awamori} Nakaz. (1915).

Amongst the different bacterial forms solubilising phosphates, the predominant form \textit{Bacillus} was identified to its nearest species based on 16s rDNA sequence data (Table 5).

**Bacterial identification**

Analysis of ~1.4 kb rDNA fragment of the bacterium was done using high ~ fidelity PCR polymerase amplification. Sequence data showed the identification of the bacterium \textit{B. subtilis}; RB14; \textit{FJ}263381. The closest neighbour homologue was \textit{Bacillus amyloliquefaciens}; 7-70; fJ378040. The alignment view and distance matrix (Table 5) for the organism \textit{B. subtilis} showed 0.961 s.ab score with \textit{B. subtilis}; 3EC2A10 of NCBI Acc. N° EU304917 (Hamaki et al., 2005; Fankem et al., 2006; Tallapragada and Seshachala, 2012). Phylogenetic tree was generated using Juke’s Cantor distance model (Figure 4) (Bruno et al., 2000; Espinosa-Victoria et al., 2009).


discussion

Results are supported by similar observations made in a study which clearly indicates high efficiency of phosphate solubilisation by \textit{B. subtilis}. Accordingly it was found that \textit{A. niger} being the most efficient with an activity of 11.5 Um L$^{-1}$ and giving the greatest amongst bacteria with an activity of 5.33 Um L$^{-1}$ at pH 10.

The dominant phosphate solubilizers were identified using molecular techniques up to species level. The 16s rDNA sequencing resulted in the identification of the bacterium \textit{Bacillus amyloliquefaciens} using high ~ fidelity PCR polymerase amplification. Analysis of ~1.4 kb rDNA fragment of the bacterium was done using high ~ fidelity PCR polymerase amplification. Sequence data showed the identification of the bacterium \textit{B. subtilis}; RB14; \textit{FJ}263381. The closest neighbour homologue was \textit{Bacillus amyloliquefaciens}; 7-70; fJ378040. The alignment view and distance matrix (Table 5) for the organism \textit{B. subtilis} showed 0.961 s.ab score with \textit{B. subtilis}; 3EC2A10 of NCBI Acc. N° EU304917 (Hamaki et al., 2005; Fankem et al., 2006; Tallapragada and Seshachala, 2012). Phylogenetic tree was generated using Juke’s Cantor distance model (Figure 4) (Bruno et al., 2000; Espinosa-Victoria et al., 2009).

**DISCUSSION**

The present study indicates that soils with high organic content indicates that soils with high organic content showed a greater number and variety of rhizospheric soil bacteria and fungi capable of phosphate solubilisation. The greater numbers of PSB have been more efficient in solubilising phosphates than bacterial isolates. The intracellular phosphate activity of these organisms showed a higher value too, \textit{A. Niger} being the most efficient with an activity of 11.5 Um L$^{-1}$ and giving the greatest amongst bacteria with an activity of 5.33 Um L$^{-1}$ at pH 10.

The predominant phosphate solubilizers were identified using molecular techniques up to species level. The 16s rDNA sequencing resulted in the identification of the bacterium \textit{B. subtilis}; \textit{FJ}263381. Phylogenetic tree was generated using Juke’s Cantor distance model (Figure 4) (Bruno et al., 2000; Espinosa-Victoria et al., 2009).
The present study indicates the presence of phosphate solubilising organisms in the rhizospheric soils of pepper plants in the growing regions of Karnataka, India. The study clearly shows the ability of these rhizobacteria and fungi in the solubilisation of inorganic phosphates and the role played by these organisms in enhancing the fertility of the soil. The phosphate solubilising ability of *Bacillus subtilis* subtilis and *Aspergillus niger* can be exploited further by using them as P fertilizers in the field of agriculture and crop plantation after further field studies done to support these findings.

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