The ability of two fungi to dissolve hardly soluble phosphates in solution

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ABSTRACT

Low phosphorus availability in soil is one of the major causes that hamper crop yield. The main objectives of this work were to isolate efficient phosphate-solubilising strains from semi-arid soil and to check their efficiency to solubilise different inorganic phosphorus forms (viz., dicalcium phosphate (DCP), tricalcium phosphate and Udaipur rock phosphate) at different temperatures. Initially, 40 strains were isolated from rhizosphere soil of \textit{Sesamum indicum} grown in semi-arid region of Rajasthan, India, of which 24 isolates showed phosphate-solubilising ability. Further, screening of these isolates for their phosphate solubilisation efficiency in solid media and broth led to the selection of two most competent isolates viz. SI32 and SI39. Both the isolates were effective in solubilising phosphorus at wide range of temperatures with different inorganic insoluble phosphorus sources. Amongst various insoluble phosphate sources tested, DCP was solubilised the most at all the temperatures but the performance was especially good in the range of 25–35°C.

Introduction

Phosphorus (P) is the second major nutrient after nitrogen (N) that limits plant growth and yields (Gyaneshwar et al. 2002). Microorganisms form a central part of the ecosystem and play an important role in nutrient cycling. Many soil bacteria, actinomycetes and fungi have the ability to change the insoluble P into plant available P by acidification, chelation, exchange reactions, etc. (Oberson et al. 2001; Egamberdiyeva et al. 2003; Hamdali et al. 2008). The population of phosphate-solubilising microorganisms varies from soil to soil and ranges from less than $10^2$ to $3 \times 10^6$ cfu g$^{-1}$ of soil (Piex et al. 2001). Phosphate solubilising fungi (PSF) are able to reach greater distance and show good attachment to insobulised P particles as a result of its hyphal structure compared to bacteria and actinomycetes, which do not form hyphae. In general, fungi are good acid producers and consequently show greater phosphate solubilisation activity than bacteria (Kucey 1983; Jain et al. 2012; Xiao et al. 2015). Phosphate solubilisation was reported to be weak in saline, alkaline and acidic soils that often led to P deficiency in plants (Johri et al. 1999).

Banasthali, located in south east region of Rajasthan, India has a semi-arid climate where the temperature generally varies from 5°C in winter to 50°C in summers. The soil of this region is alkaline, calcareous and low in nutrients. Most of the available P is present in calcium bound form. The main constraints in agricultural production in this region are high temperatures in summers and low P availability. \textit{Sesamum indicum} commonly known as “til”, is an ancient oil crop. India is the largest producer of \textit{S. indicum} in the world and it constitutes an important crop in the semi-arid regions. The objective of present investigation was to isolate PSF from the rhizosphere of \textit{S. indicum} and to characterise these with respect to different calcium bound P forms and temperatures, so that these can be used for crop improvement in future in semi-arid regions.

Materials and methods

Sampling

The PSF were isolated from rhizosphere soil of \textit{S. indicum} plants grown in Krishi Vigyan Kendra farm, Banasthali (latitude 26°23'24.0879" N and longitude 75°52'5.3357" E). For the collection of rhizosphere soil, plants were uprooted and the loosely adhering soil was removed by mechanical shaking. Ten grams of rhizosphere soil was then suspended in 100 ml of
sterile saline (0.5% NaCl) and shaken for 6 h on a rotary shaker to separate the microorganisms from the sample. All sampling procedures were performed in triplicate.

**Isolation and screening of phosphate-solubilising fungi**

The samples were serially diluted to $10^{-7}$ and spread on potato dextrose agar (PDA) plates. The plates were incubated at 30°C for 5 days or until the colonies developed. Single colonies from different areas were transferred to fresh medium and purified. The pure strains were maintained on PDA slants at 4°C.

To check the phosphate-solubilising activity, the cultures were spot inoculated on Pikovskaya agar (PA) plates containing 0.5% tricalcium phosphate (TCP) as an insoluble phosphorus source. After 6 days of incubation at 30°C, the plates were examined for the presence of clear zones around colonies. The experiment was performed in triplicate.

**Determination of TCP solubilisation efficiency on PA plates**

The phosphate solubilising efficiency (SE) was calculated according to Nguyen et al. (1992) by following formula:

$$
\% \text{Solubilization Efficiency (SE)} = \frac{\text{Diameter of solubilization zone (S)}}{\text{Diameter of the colony}} \times 100
$$

**Micro-determination of phosphorus**

Phosphate solubilisation assay were carried out in shake flasks in triplicate with 100 ml Pikovskaya broth containing 5% TCP as sole phosphate source. Two mycelial discs (10 mm) from 4-day old actively growing colonies on Pikovskaya medium, were added for each isolate. Flasks were incubated at 130 rpm at 28°C for 12 days. Autoclaved, uninoculated medium served as control. A 2 ml sample was withdrawn from each flask every 48 h, centrifuged at 11,000 rpm for 15 min and the supernatant was collected. This supernatant was further assayed for soluble phosphate. The amount of phosphate released into the culture supernatant was used as the criteria for choosing the most efficient PSF. The isolates showing the highest insoluble phosphate-solubilising activity viz. SI32 and SI39 were selected for further studies.

**Effect of temperature and phosphate sources**

To study the efficiency of SI32 and SI39 strains to solubilise various types of phosphate sources, 0.2% TCP in 100 ml Pikovskaya broth was replaced with dicalcium phosphate (DCP) or Udaipur rock phosphate (URP; $P_2O_5$ content = 34%, mesh size = 74 μm) and inoculated with 1 ml spore suspension containing $5 \times 10^5$cfu ml$^{-1}$. The triplicate flasks were incubated at varying temperatures (i.e. 15°C, 25°C, 35°C and 45 ± 2°C) on an orbital incubator shaker for 12 days at 130 rpm. The samples were collected every 48 h. The uninoculated autoclaved medium with different phosphate substrates was incubated under similar conditions to serve as control.

**Analytical method**

Soluble phosphate in the supernatant was determined by using the molybdenum blue method (Murphy & Riley 1962). Uninoculated flasks were used as control for molybdenum blue method. The pH was recorded with a pH metre equipped with a glass electrode.

**Statistical analysis**

All experiments were performed in triplicate. Means ± standard deviation (SD) were computed. The correlation coefficient ($r$) between soluble P and pH value was calculated using SPSS software. The data obtained through the experiments were subjected to analysis of variance (ANOVA) by using SPSS software, version 16.0, and comparison of means was made using Duncan’s multiple range test at $p < 0.05$ levels. ANOVA was performed between two classes that are incubation time and soluble P.

**Results and discussion**

Initially, 40 isolates were selected, which were further screened for phosphate solubilisation potential on PA plates. It resulted into 24 positive strains for solubilisation of TCP. These isolates showed different levels of phosphate solubilisation from inconspicuous to a substantial. Thirteen isolates produced clear zones showing higher solubilisation efficiency, 10 developed
translucent and the remaining isolates had inconspicuous zones. Of the 13, 11 made larger clear zones, while the remaining 2 made smaller clear zones in comparison to the colony. Eight isolates that made translucent zones had bigger zones in comparison to the colony (Table 1). In the present study, the variable potential of phosphate solubilisation based on solubilisation efficiency may be the result of differences in type and amount of organic acids produced by different fungi, and also the diffusion rate as earlier reported by Yadav et al. (2011).

All 24 isolates were identified to genus level on the basis of colony morphology and microscopic characteristics. Sixteen isolates belonged to *Aspergillus*, five to *Penicillium* and three were from mycelia sterilia group. In the present study, *Aspergillus* spp. were found to be the most frequently occurring PSF. This may be due to the efficiency of *Aspergillus* spp. in root colonisation as reported by Nenwani et al. (2010) and Elias et al. (2016).

**Micro-determination of phosphorus**

Phosphate solubilising activity ranged from 69 to 1114 mg l⁻¹ in Pikovskaya broth. Maximum and mean phosphate solubilisation over 12 days is summarised in Table 1. The highest average TCP solubilisation was shown by SI32 strain (575 mg l⁻¹), followed by SI39 (502 mg l⁻¹). Similar to our results, a large variation in ability of isolates to solubilise P have been reported with values ranging from 210–840 µg PO₄³⁻ ml⁻¹ to 5.4–1097.0 µg PO₄³⁻ ml⁻¹ by Barroso et al. (2006) and Surange (1985), respectively. A direct correlation between P solubilisation in agar plates and broth media was seen in 13 strains, that is, SI1, SI6, SI16, SI17, SI18, SI21, SI23, SI24, SI26, SI28, SI35, SI38 and SI39. Such positive correlations were commonly observed in earlier papers (Mehta & Nautiyal 2001; Jain et al. 2014). In contrast, six strains (SI5, SI12, SI13, SI22, SI32 and SI37) showed better P solubilising activity in liquid medium compared with solid medium, and the remaining five strains (SI8, SI11, SI19, SI20 and SI34) had better activity on solid medium. The possible reason for these anomalous behaviours on liquid and solid media could be attributed to nutrient availability, varying diffusion rates of different organic acids secreted by fungi, and growth requirement of fungi (Jain et al. 2014). The results concluded that for the selection of efficient PSF isolates, the screening should be done on both solid and liquid media (Johri et al. 1999; Alam et al. 2002; Elias et al. 2016). Two P-

| S. No | Fungal strain | Type of fungi | Maximum value of soluble P⁺⁻ (mg l⁻¹) | Solubilisation efficiency (%) + type of zone | Average soluble P in 12 days (mg l⁻¹) |
|-------|---------------|---------------|----------------------------------------|------------------------------------------|-------------------------------------|
| 1     | SI1           | *Aspergillus* sp. (niger group) | 569±0.00                  | 8                                          | 129.69+C                         |
| 2     | SI5           | *Aspergillus* sp. (niger group) | 539±0.00                  | 8                                          | 97.33+T                          |
| 3     | SI6           | Penicillium sp.     | 586±0.01                  | 10                                         | 121.18-C                         |
| 4     | SI8           | *Aspergillus* sp. (niger group) | 378±0.02                  | 10                                         | 97.18+C                          |
| 5     | SI11          | *Aspergillus* sp.     | 101±0.00                  | 4                                          | 109.18+C                         |
| 6     | SI12          | *Aspergillus* sp.     | 538±0.07                  | 6                                          | 65.15+I                          |
| 7     | SI13          | *Aspergillus* sp. (niger group) | 539±0.05                  | 6                                          | 93.83+T                          |
| 8     | SI16          | *Aspergillus* sp. (niger group) | 578±0.13                  | 6                                          | 101.34+C                         |
| 9     | SI17          | *Aspergillus* sp. (niger group) | 626±0.04                  | 6                                          | 107.64+C                         |
| 10    | SI18          | *Aspergillus* sp. (niger group) | 695±0.02                  | 8                                          | 109.15+C                         |
| 11    | SI19          | NI               | 75±0.00                   | 6                                          | 105.61+T                         |
| 12    | SI20          | *Aspergillus* sp.     | 69±0.00                   | 6                                          | 130.4+C                          |
| 13    | SI21          | Penicillium sp.     | 524±0.00                  | 12                                         | 123.26+C                         |
| 14    | SI22          | *Aspergillus* sp. (niger group) | 415±0.00                  | 12                                         | 109.68+C                         |
| 15    | SI23          | NI               | 728±0.01                  | 10                                         | 158.76+C                         |
| 16    | SI24          | *Aspergillus* sp. (niger group) | 543±0.00                  | 12                                         | 97.20+C                          |
| 17    | SI26          | NI               | 76±0.01                   | 4                                          | 109.09+C                         |
| 18    | SI28          | Penicillium sp.     | 546±0.00                  | 10                                         | 110.99+C                         |
| 19    | SI32          | *Aspergillus* sp. (niger group) | 1114±80.05                | 12                                         | 107.04+C                         |
| 20    | SI34          | Penicillium       | 195±0.09                  | 10                                         | 165.56+C                         |
| 21    | SI35          | *Aspergillus* sp. (niger group) | 773±0.13                  | 8                                          | 114.57+C                         |
| 22    | SI37          | *Aspergillus* sp. (niger group) | 568±0.00                  | 10                                         | 102.78+C                         |
| 23    | SI38          | *Aspergillus* sp. (niger group) | 751±0.01                  | 10                                         | 107.02+C                         |
| 24    | SI39          | Penicillium sp.     | 711±0.02                  | 10                                         | 122.41+C                         |

a) The value of soluble P is a means ± standard error (n = 3).
b) Means followed by same letter within the column were not significantly different, as determined by Duncan’s multiple range test.
c) NI = not identified; C = clear; T = translucent; I = inconspicuous.
solubilising strains viz., SI32 and SI39 were selected for further study on the basis of better phosphate-solubilising potential.

**Effect of temperature and phosphate sources**

Three forms of most commonly found insoluble phosphates viz. DCP, TCP and URP were used to evaluate the solubilisation potential of selected fungal strains in Pikovskaya broth at different temperatures. The results are depicted in Table 2 and Figure 1. Phosphate solubilisation by these fungal isolates was compared with the uninoculated control. Inoculation of the liquid medium with these isolates significantly ($p < 0.05$) altered the amount of soluble P as well as the pH, while the uninoculated control remained almost unchanged throughout the experiment except for URP at 45°C. In general, phosphate solubilisation was negatively correlated with pH. There are several reports where such a correlation was documented (Whitelaw et al. 1999; Pandey et al. 2008; Jain et al. 2012; Xiao et al. 2013a). This drop in pH indicated the production of organic acids in medium (Pradhan & Sukla 2005; Jain et al. 2012; Saxena et al. 2013). In general, isolate SI32 was a significantly better solubiliser than isolate SI39. Three solubilisation patterns were observed that were followed by these two isolates (Figure 1). In the first pattern, a gradual increase in phosphate solubilisation after a sharp increase was observed, in second one, soluble P reached a peak before decreasing and then increased for a second time, while in the third case it reached a peak and then became constant or decreased slightly. These patterns are similar to results reported by Nenwani et al. (2010), Mahamuni et al. (2012) and Jain et al. (2012).

**Correlation between pH and soluble P**

In the case of DCP, at 15°C both isolates showed the first pattern. The correlation coefficients ($r$) for soluble P versus pH at 15°C were $-0.96$ and $-0.82$, respectively, for isolates SI32 and SI39 when data from the entire incubation period was included. The second solubilisation pattern was followed by the isolate SI32 at 25°C, 35°C and 45°C, while SI39 only followed this pattern at 25°C and 45°C only. In these cases, soluble P showed a strong negative correlation with pH, especially for the period leading up to the peak. After this point the correlation decreased. At 35°C, isolate SI39 did not show any significant correlation between pH and soluble P. From these results, it can be inferred that in case of isolate SI32 at 25°C, 35°C and 45°C, before attaining the peak, the major phosphate solubilisation mechanism was acid production but after the peak was obtained, another mechanism was involved. In contrast, acidification in SI39 at 35°C was not the reason for phosphate solubilisation.

In the case of TCP, at extreme temperatures, that is, 15°C and 45°C, the SI32 isolate followed the first solubilisation pattern, whereas at 25°C it followed the third solubilisation pattern. These patterns indicated that acidification was the main mechanism of TCP solubilisation at lower temperatures but at higher temperatures after peak formation another phosphate solubilisation mechanism was used by fungi that are showing some inhibiting and promoting

| Phosphate source | Temperature (°C) | Average soluble P (mg l$^{-1}$) | pH | Correlation between soluble P and pH |
|------------------|-----------------|---------------------------------|----|------------------------------------|
|                  | SI32 | SI39 | SI32 | SI39 | SI32 | SI39 | SI32 | SI39 |
| DCP              | 15   | 332$^{ab}$ | 218$^a$ | 2.06 | 3.02 | -0.96 | -0.82 |
|                  | 25   | 354$^b$ | 368$^b$ | 2.22 | 2.73 | -0.75 (−0.94) | -0.72 (−0.94) |
|                  | 35   | 289$^b$ | 347$^{ab}$ | 5.86 | 2.82 | -0.30 (−0.54) | 0.25 (−0.44) |
|                  | 45   | 335$^{ab}$ | 177$^d$ | 2.58 | 5.08 | -0.72 (−0.99) | -0.81 (−0.70) |
| TCP              | 15   | 215$^{ab}$ | 186$^a$ | 2.10 | 2.84 | -0.92 | -0.80 |
|                  | 25   | 309$^b$ | 298$^{ab}$ | 2.37 | 2.63 | -0.53 | -0.52 (−0.94) |
|                  | 35   | 296$^{ab}$ | 306$^a$ | 5.56 | 2.55 | 0.42 (−0.99) | -0.03 (0.08) |
|                  | 45   | 160$^b$ | 57$^d$ | 5.89 | 6.81 | -0.96 | 0.34 (−0.065) |
| URP              | 15   | 24$^c$ | 32$^c$ | 3.10 | 3.95 | -0.63 | -0.54 |
|                  | 25   | 101$^{ab}$ | 62$^{ab}$ | 3.10 | 3.74 | -0.75 (−0.79) | -0.35 (−0.91) |
|                  | 35   | 149$^a$ | 76$^a$ | 3.04 | 3.65 | 0.04 | 0.61 |

a) Mean values in each column with the same letters do not differ significantly by Duncan’s multiple range test at $p ≤ 0.05$.

b) Values in parentheses are correlation coefficient for the shorter period, that is, from day 0 to the day that the P solubilisation peak was obtained.

c) DCP: dicalcium phosphate; TCP: tricalcium phosphate; URP: Udaipur rock phosphate.
effects. Earlier, Jain et al. (2012) reported the same findings.

It can be concluded from the results that 35°C was the optimum temperature for URP solubilisation by these isolates. Xiao et al. (2013a) reported maximum solubilisation of RP at 25–35°C (90–100 mg l⁻¹). There was very poor or no correlation between pH and soluble P, which indicated that acid production had role in URP solubilisation but it did not completely control the solubilisation. At 45°C, traces of phosphate solubilisation was observed, even less than in the control flasks and very little fungal growth was observed.

In both the fungal strains, the solubilisation of DCP was the highest, closely followed by TCP, while URP was the least solubilised. The possible reason could be that these isolates were isolated from semi-arid region where the main inorganic form of phosphorus is calcium derivatives, hence it is better adapted for their solubilisation in comparison to URP. Also, according to a few reports, URP is less amenable to microbial solubilisation than TCP (Gaur 1990; Seshadri et al. 2004; Pradhan & Sukla 2005; Vyas et al. 2007; Jain et al. 2012; Xiao et al. 2013b) due to the complex mineral composition, particle size in the medium and the presence of strong apatite bond, which reduced phosphate solubilisation.

The present study furnishes useful information about the phosphate-solubilising fungi and their solubilisation mechanisms in vitro. It is expected that these fungal strains could serve as suitable bio-inoculum for P solubilisation in semi-arid conditions. However, further

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**Figure 1.** Dicalcium phosphate (a, b, c and d), tricalcium phosphate (e, f, g and h) and Udaipur rock phosphate solubilisation (i, j and k) (bar graph) and changes in pH (lined graph) of broth by *Aspergillus sp* SI32 (dark coloured bar and lined graph) and *Penicillium sp*. SI39 (grey coloured lined and bar graph) at four different temperature (15°C, 25°C, 35°C and 45°C).
characterisation is needed so that they can be developed as efficient phosphate bio-inoculants.

**Disclosure statement**

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