Journal of Agriculture in the Tropics and Subtropics
Volume 103, No. 1, 2002, pp. 81 - 87

Phosphate solubilization by soil isolates of *Azotobacter chroococcum* and their survival at different temperatures

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Keywords: *Azotobacter chroococcum*, phosphate solubilization, survival, temperature

Abstract
Six phosphate solubilizing isolates of *A. chroococcum* were isolated from the rhizosphere soil of mustard crop and were evaluated for their growth, indole-acetic acid production and phosphate solubilization activity at three different temperatures (30°C, 37°C and 42°C). Our results indicate that all the six isolates of *A. chroococcum* showed solubilization of tricalciumphosphate (TCP) and rock phosphate (RP) and led to the acidification of the medium at all the three temperatures. Maximum solubilization was observed by isolates PS 2 and PS 6 at 37°C and 42°C respectively.

1 Introduction

Phosphorus is one of the major and essential plant nutrients. Application of phosphate fertilizer is therefore essential for the optimum crop yield. The problem of P-fertilizer is becoming difficult because of its higher cost of production. Among the factors which control the availability of inorganic P, soil pH is of primary importance. Although the total P in the soils is high, only a part is available to the plant. Thus the release of insoluble and fixed forms of P is an important aspect of increasing soil P availability.

Soil microorganisms play a very significant role in mobilizing P for the use of plants by bringing about changes in pH of the soil microenvironment and producing chelating substances which lead to native as well as added insoluble phosphates (HALDER *et al.*, 1991). A large fraction of soil microbial population can dissolve insoluble inorganic phosphates that occur in soil (NAHAS *et al.*, 1994). Various bacteria and fungi are reported to solubilize different types of insoluble phosphates (GAIND and GAUR, 1991; SINGH *et al.*, 1984). A large number of phosphate solubilizing bacteria (PSB) have been isolated from the rhizosphere of various crop plants. The higher production of P-solubilizers in the rhizosphere is of great relevance to plants especially in P-deficient soils as it helps in mobilization of insoluble P. Keeping the above points in mind, *Azotobacter* strains were isolated from rhizosphere of mustard crop and were screened for their growth at high temperature, nitrogen fixation and P-solubilization with the aim of using it as an inoculant for various crops.

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2 Materials and methods

2.1 Isolation, maintenance and characterization of PSB

Phosphate solubilizing bacteria (PSB) were isolated from rhizosphere soil of mustard crop by enrichment culture technique (GAIND and GAUR, 1991). 10 g of soil were added into 100 ml of Jensen's medium (JM) amended with 2 per cent tricalcium phosphate (TCP) and incubated at 30°C for a week. Three successive transfers were made into fresh JM+TCP medium at weekly intervals and plated on pikovskaya (PIKOVSKAYA, 1948) and Jensen medium containing tricalcium phosphate. Isolated colonies showing the zone of clearance were picked, purified and maintained on JM slants. These isolates were characterized by performing certain biochemical tests according to the Bergey's manual of determinative bacteriology (TCHAN, 1984).

2.2 Preparation of inoculum

100 ml of JM+TCP broth was inoculated with loopful of respective isolates and incubated for 4 days at 30°C. The inoculum was further distributed into three flasks for each culture and incubated at three different temperatures, i.e. 30°, 37° and 42°C to check their survival at these temperatures.

2.3 Determination of IAA

Determination of IAA production by phosphate solubilizing bacteria after 4 days was done by growing the cultures in JM containing 100 mg L^{-1} tryptophan at 30°C. Cells were centrifuged and IAA was determined in the supernatant by Salkowski's method (TANG and BONNER, 1974).

2.4 Determination of viable counts

Cultures grown at different temperatures were serially diluted and plated on JM for total counts. The experiment was done in triplicate.

2.5 Determination of pH and soluble P

Isolates were grown in JM+TCP, JM+RP and incubated on shaker for 7 days at three different temperatures i.e. 30°, 37° and 42°C. Cells were centrifuged, pH and soluble P were determined in various supernantants by the method of JOHN (1970).

3 Results and Discussion

Nineteen isolates were isolated from the rhizosphere soil of mustard crop of Haryana Agriculture University Farm of Hisar, Haryana, India. All the isolates were identified as *Azotobacter*.

All except one isolate belong to the species *A. chroococcum* (Table 1). All identified isolates were tested on Jensen's and pikovskya's medium containing tricalcium phosphate and their zone of clearance was measured and solubilization index was calculated. Out of all the 19 isolates, 6 isolates showed a big zone of clearance (Table 2) and were chosen for further studies.

Table 1: Characterization and identification of *Azotobacter* sp. for P-Solubilization

Isolates No.	JM+TCP	N-fixation	JM+Butanol	JM+strach	JM+Mannitol	Identified as
1.	+	+	++	+	+++	*A. chroococcum*
2.	+	+	+++	++	+++	-do-
3.	+	+	+++	±	+++	-do-
6.	+	+	+++	++	+++	-do-
8.	+	+	+++	++	+++	-do-
10.	+	+	+++	++	+++	-do-
11.	+	+	++	++	+++	-do-
12.	+	+	+++	+	+++	-do-
13.	+	+	+++	±	+++	-do-
14.	+	+	+++	++	+++	-do-
15.	+	+	++	++	+++	-do-
16.	+	+	++	++	+++	-do-
17.	+	+	-	-	-	?
18.	+	+	-	-	-	?
19.	+	+	+++	++	+++	*A. chroococcum*
20	+	+	+++	++	+++	-do-
21.	+	+	++	++	+++	-do-
24.	+	+	++	++	+++	-do-
27.	+	+	-	-	-	?

* All the isolate were identified according to the Bergey's manual of determinative bacteriology (TCHAN, 1984) and were found to be *A. chroococcum* isolates.

Table 2: Detection of phosphate solubilization on plates

Isolate No.	Diameter of the colony (B)		Diameter of the zone (A)		Solubilization index (A/B)	
	JM+TCP	PSK+TCP	JM+TCP	PSK+TCP	JM+TCP	PSK+TCP
PS 2	0.8	0.8	1.0	1.3	1.25	1.63
PS 6	0.8	0.9	1.0	1.1	1.25	1.22
PS 11	0.6	1.1	1.1	1.3	1.83	1.18
PS 12	0.6	0.8	0.7	0.8	1.16	1.00
PS 13	0.1	0.2	0.9	0.8	9.0	4.00
PS 21	0.2	0.3	0.6	0.8	3.0	2.66

Solubilization Index = $\frac{A}{B}$ (KUMAR and NARULA, 1999)

A = Diameter of zone; B = Diameter of colony; JM = Jensen medium;TCP = Tricalcium phosphate; PSK = Pikovskaya medium

Efficiency of different P-solubilizing isolates of *A. chroococcum* was studied at different temperatures of incubation. Phosphate solubilization was maximum in *A. chroococcum* isolate PS 2 followed by PS 6 and PS 12 (Table 3 and 4) as observed by decrease in pH, it might be due to acid production and total soluble P. Solubilization of insoluble P by microorganisms is mainly caused by production of organic acids and chelating substances (SINGH *et al.*, 1980; MISHRA *et al.*, 1983; HALDER *et al.*, 1991; SURANGE and KUMAR, 1993). The solubilized phosphate may react with Ca and Mg present in rock phosphate as soon as the pH of the medium increases. Many soil bacteria like *Pseudomonas*, *Bacillus subtilis*, *Bacillus polymyxa*, *Bradyrhizobium* etc. are reported to dissolve and solubilize different types of insoluble phosphates (SINGH *et al.*, 1980; HALDER *et al.*, 1991).

Table 3: pH measurement of the supernantant at various temperatures

Isolate No.	pH(TCP [a])		pH (RP [b])	
	30°C	37°C	42°C	30°C
PS 2	4.70	4.31	4.83	5.60
PS 6	4.77	4.84	4.84	5.85
PS 11	4.87	4.93	4.90	5.79
PS 12	4.62	4.62	6.04	6.30
PS 13	5.07	4.55	4.80	6.66
PS 21	5.44	5.09	6.71	6.34

[a] TCP = Tricalcium phosphate (2%)
[b] RP = Rock phosphate (1%)

Table 4: TCP and RP solubilization by chosen soil isolates of A. chroococcum

Isolate No.	µg P solubilized (TCP [a])		µg P solubilized (RP [b])	
	30°C	37°C	42°C	30°C
PS 2	2.081	3.125	2.437	0.943
PS 6	2.575	2.312	3.031	1.000
PS 11	2.612	2.525	1.581	1.032
PS 12	2.275	2.687	2.668	0.737
PS 13	1.856	2.581	2.668	0.900
PS 21	1.793	1.993	1.443	1.025

[a] TCP = Tricalcium phosphate; [b] RP = Rock phosphate
Mean of two values.

Growth in terms of viable count was also monitored at all the above temperatures. Viable count was maximum in isolate PS 11, PS 12, PS 13 and PS 21 (10^{11}) at 30°C followed by PS 2 and PS 6 (10^8). There was decline in viable count at 42°C. Maximum growth at 42°C was 10^7 in isolate PS 21 followed by PS 12, PS 6, PS 13 and PS 11 (Table 5).

Table 5: Total viable counts of PS *Azotoabacter chroococcum* at different temperatures

Isolate No.	30°C	37°C	42°C
PS 2	$1.7x10^{10}$	$6.8x10^{9}$	$9x10^{4}$
PS 6	$6.0x10^{10}$	$4.7x10^{9}$	$2x10^{6}$
PS 11	$2.4x10^{12}$	$5.9x10^{8}$	$6.7x10^{7}$
PS 12	$1.8x10^{12}$	$1.2x10^{9}$	$9x10^{6}$
PS 13	$2.6x10^{12}$	$1.0x10^{7}$	$8x10^{4}$
PS 21	$8.5x10^{12}$	$5.2x10^{9}$	$9x10^{7}$

Jensen's medium plates were used for determination of total viable counts.

Various reports show that plant growth promoting effects of phosphate solubilizing bacteria may also be related to their ability to synthesize plant growth regulating substances (AZCON *et al.*, 1978); SATTER and GAUR, 1987). All the isolates were tested for IAA production under cultural conditions. Results (Table 6) show that the maximum IAA production was by *A. chroococcum* isolate PS 21 (22.96 µm) followed by PS 13, PS 2, PS 12, PS 11 and PS 6 (180, 12.10, 4.70, 4.36, 1.48). Nearly all the phosphate solubilizing isolates of *A. chroococcum* were able to produce IAA in liquid culture containing tryptophan. The results demonstrate that the capability of phosphate solubilizing *Azotobacter* producing biologically active indole auxins in vitro is affected by pH, environmental factors and sugar concentration (HALDER *et al.*, 1991; LEINHOS, 1994). It is suggested that the plant growth that results from inoculation of these bacteria is caused primarily by growth regulators. This results in an increased plant size, phosphate uptake and nitrogen fixation.

Table 6: In vitro - IAA production by P-solubilizing *Azotobacter* isolates

Isolate No.	µM IAA
PS 2	12.10
PS 6	1.48
PS 11	4.36
PS 12	4.70
PS 13	18.90
PS 21	22.96

The rhizosphere of crops was found to give a greater number of phosphate solubilizing bacteria (PSB) (GAIND, 1987; GAIND and GAUR, 1991; KUNDU and GAUR, 1980) and larger population of these bacteria was found in plants like broadbean (MAHMOUD *et al.*, 1973), wheat (KUMAR and NARULA, 1999) and barley.
The large number of PSB might be due to the favourable influence of root exudates which has amino acids, organic acids, sugars and growth promoting substances.

A thermotolerant, P-solubilizer and IAA producing *A. chroococcum* which is not easily available might prove to be efficient to be a better biofertilizer for various cereal crops.

4 Summary

Phosphate solubilizing isolates of *Azotobacter chroococcum* were isolated and were tested for their solubilization of inorganic phosphates (Tricalcium phosphate, Rock phosphate), IAA production and growth at different temperatures. Out of 19 isolates isolated, six isolates showed a big zone of clearance on Pikovskaya and Jensen medium plates containing tricalcium phosphate (TCP). *A. chroococcum* PS 2 solubilized maximum phosphate (TCP, RP) followed by PS 6 and PS 12. Solubilization was maximum at 37°C (from 1.993 to 3.125 µg P solubilized). These isolates could grow at all the three temperatures studied, but there was decline in the count of all the six isolates except PS 2 at 42°C. IAA production was also maximum in PS 21 (22.96 µm) followed by PS 13 (18.90 µm) and PS 2 (12.10 µm).

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