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Occurrence of Salt, pH, and Temperature-tolerant, Phosphate-solubilizing Bacteria in Alkaline Soils

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Abstract. An ecological survey was conducted to characterize 4800 bacterial strains isolated from the root-free soil, rhizosphere, and rhizoplane of *Prosopis juliflora* growing in alkaline soils. Of the 4800 bacteria, 857 strains were able to solubilize phosphate on plates. The incidence of phosphate-solubilizing bacteria (PSB) in the rhizoplane was highest, followed by rhizosphere and root-free soil. Eighteen bacterial strains out of 857 PSB were able to produce halo at 30°C in a plate assay in the presence of 5% salt (NaCl) and solubilize tricalcium phosphate in National Botanical Research Institute's phosphate growth medium (NBRIP) broth, in the presence of various salts, pHs, and temperatures. Among the various bacteria tested, NBRI4 and NBRI7 did not produced halo in a plate assay at 30°C in the absence of salt. Contrary to indirect measurement of phosphate solubilization by plate assay, the direct measurement of phosphate solubilization in NBRIP broth assay always resulted in reliable results. The phosphate solubilization ability of NBRI4 was higher than in the control in the presence of salts (NaCl, $CaCl_2$, and KCl) at 30°C. Phosphate solubilization further increased in the presence of salts at 37°C as compared with 30°C. At 37°C, $CaCl_2$ reduced phosphate solubilization ability of NBRI4 compared with the control. The results indicated the role of calcium salt in the phosphate solubilization ability of NBRI4.

Phosphorus is second only to nitrogen as a mineral nutrient required by both plants and microorganisms, its major physiological role being, in certain essential steps, the accumulation and release of energy during cellular metabolism [2]. Phosphorus in soils is immobilized or becomes less soluble either by absorption, chemical precipitation, or both. Plants can absorb only inorganic phosphorus, and the concentration of inorganic phosphate in the soil is very low because most of the phosphorus in soils is present in insoluble forms [1]. This, combined with the relative immobility of the ion in the soil, can cause the phosphate supply to be the limiting factor for plant growth [4]. Organic phosphate can constitute 4–90% of the total soil phosphate. Therefore, organic phosphate mineralization is an important soil process because it results in release of inorganic phosphorus to the soil solution for its availability to plants and soil microbes [2, 18]. Microorganisms are known to solubilize insoluble phosphate through the production of organic acids and chelating oxo acids from sugars [3, 8].

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Seed or soil inoculation with phosphate-solubilizing bacteria (PSB) is known to improve solubilization of fixed soil phosphorus and applied phosphates, resulting in higher crop yields [1, 6, 7, 10].

The use of rock phosphate as phosphate fertilizer and its solubilization through microbes have become a valid alternative to expensive chemical fertilizers. In conjugation with PSB, these materials should provide a cheap source of phosphate fertilizer for crop production. Hence, PSB has the potential to improve crop production in this area. The establishment and performance of these microbes are affected severely under stress such as high salt, pH, and temperature prevalent in degraded eco systems such as alkaline soils with a tendency to fix phosphorus [6, 16]. In the alkaline soils of the tropics, salts concentrations may be as high as 2%, pH as high as 10.5, and temperature may range between 35° and 45°C [6, 16], which may result in poor growth and survival of PSB. However, no information is available on the occurrence of PSB in alkaline soils. The objective of the present investigation was to isolate PSB that could solubilize

insoluble phosphate efficiently at higher salt, pH, and temperature from alkaline soils.

Materials and Methods

Bacterial strains were isolated from four different sites by use of root-free soil, soil surrounding the roots of *Prosopis juliflora* (rhizosphere), and on the roots of *P. juliflora* (rhizoplane) growing in alkaline soil (exposed to high salt, pH, and temperature stress) in the range of pH 8.5 to 11.0 from Banthara Research Station, Lucknow. The soil temperature of the sites from which these bacteria were isolated during summer varies from 48°C to 52°C at Banthra village, Lucknow, as described earlier [13]. To isolate the bacteria from rhizosphere, the adhering soil on the root was gently shaken to collect the rhizosphere soil. Roots were thoroughly washed with tap water for 2 min to remove all loosely adhering soil particles, followed by washing with sterile 0.85% (wt/vol) salise Milli Q water (MQW), to isolate rhizoplane bacteria. The roots were then macerated in 0.85% saline MQW with a mortar and pestle. After serial dilution of the root homogenate and soil (10% soil in 0.85% saline MQW), samples were individually plated on Pseudomonas isolation agar, Nutrient agar, and Tryptone-Glucose-Yeast extract (TGY) agar (from HI-Medium Laboratories Pvt. Ltd., Bombay, India). Bacteria representative of the predominant morphological types present on the plates were selected at random and purified on minimal medium, on the basis of AT salts which contained the following ingredients (per liter): glucose, 10.0 g; KH_2PO_4, 10.9 g; $(NH_4)_2SO_4$, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.16 g; $FeSO_4 \cdot 7H_2O$, 0.005 g; $CaCl_2 \cdot 2H_2O$, 0.011 g; and $MnCl_2 \cdot 4H_2O$, 0.002 g [13]. Pikovskaya (PVK) medium contained (per liter): glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $(NH_4)_2SO_4$, 0.5 g; NaCl, 0.2 g; $MgSO_4 \cdot 7H_2O$, 0.1 g; KCl, 0.2 g; yeast extract, 0.5 g; $MnSO_4 \cdot H_2O$, 0.002 g; and $FeSO_4 \cdot 7H_2O$, 0.002 g [15]. National Botanical Research Institute's phosphate growth medium (NBRIP) contained (per liter): glucose, 10 g; $Ca_3(PO_4)_2$, 5 g; $MgCl_2 \cdot 6H_2O$, 5 g; $MgSO_4 \cdot 7H_2O$, 0.25 g; KCl, 0.2 g; and $(NH_4)_2SO_4$, 0.1 g. The pH of the media was adjusted to 7.0 before autoclaving, as described earlier [14].

Forty-eight hundred bacteria (1200 bacteria from each site) were thus isolated and screened for their phosphate-solubilizing ability on Pikovskaya (PVK) medium (from HI-Medium Laboratories Pvt. Ltd.). Four strains per plate were inoculated in triplicate with sterile toothpicks. The halo and colony diameter were measured after 14 days of the incubation of plates at 30°C. Halo size was calculated by subtracting colony diameter from the total diameter. Observations were made in the second week in triplicate, as described earlier [14]. Initially 50 bacterial colonies/plate were screened on a 90-mm plate by incubating the plates for 2 weeks at 30°C, with PVK media. The initial screening was followed by the secondary screening with PSB obtained from the first screening by using 10 PSB/plate containing 5% salt (NaCl). A third and final screening consisted of PSB with two PSB/plate and checked for their phosphate solubilization ability under stressed conditions by varying the salt (NaCl; 0, 2.5, and 5.0%) and pH (7, 8, and 9) of the medium and incubating the plates at 30°C, 37°C, and 45°C as indicated in Table 2. The data represent the means of three replicates.

Quantitative estimation of phosphate solubilization in broth was carried out with Erlenmeyer flasks (150 ml) containing 40 ml of NBRIP medium inoculated with the bacterial strain (400 µl inoculum with approximately 1 to 2×10^9 cfu/ml). Autoclaved, uninoculated medium served as controls. The flasks were incubated at 30°C on a New Brunswick Scientific (?, USA) Innova Model 4230 refrigerated incubator shaker at 180 rpm. The cultures were harvested by centrifugation at 10,000 rpm for 10 min in a Sorvall RC 5C centrifuge (Dupont, ?, USA). Phosphate in culture supernatant was estimated by the Fiske and Subbarow method [5] and expressed as equivalent phosphorus (µg/ml) as described earlier [14]. The data represent the means of three replicates.

Table 1. Numbers of phosphate-solubilizing bacterial isolates from root-free soil, rhizosphere, and rhizoplane of *Prosopis juliflora* growing in alkaline soils

Site		Numbers of bacteria[a]		
		Root-free soil	Rhizosphere	Rhizoplane
1	Total	400	400	400
	Phosphate-dissolving	23 (5.8)[b]	57 (14.3)	84 (21)
2	Total	400	400	400
	Phosphate-dissolving	32 (8)	83 (20.8)	121 (30.3)
3	Total	400	400	400
	Phosphate-dissolving	41 (10.3)	76 (19)	95 (23.8)
4	Total	400	400	400
	Phosphate-dissolving	52 (13)	84 (21)	109 (27.3)

[a] Each value represents the mean of three replicates.
[b] Figures in parentheses refer to % incidence of phosphate-dissolving bacteria.

Results and Discussion

Stress tolerance towards high salt, pH, and temperature may be important in the survival, multiplication, and spread of bacterial strains in alkaline soils. Stress-tolerant bacteria are likely to be found in environments affected by salt, pH, and temperature stress. The 4800 bacterial strains were isolated from four different sites—root-free soil, rhizosphere and rhizoplane of *P. juliflora* growing in alkaline soil. A comparison of numbers of phosphate-solubilizing bacterial isolates out of 4800 bacterial strains withroot-free soil, rhizosphere and rhizoplane of *P. juliflora* growing in alkaline soil, from four different sites is presented in Table 1. Of the 4800 bacteria, 857 strains were able to solubilize phosphate on PVK plates at 30°C. The PSB were readily isolated from the root-free soil, rhizosphere and rhizoplane of *P. juliflora* growing in alkaline soils. The incidence of PSB in rhizoplane was highest in all four sites, followed by rhizosphere and root-free soil (Table 1). The lower occurrence of PBS in the root-free soil compared with rhizosphere and rhizoplane could result from the nutrient-rich rhizosphere effect of *P. juliflora* roots [13]. Soils poor in organic matter are known to be low in microbial activities, except in the rhizosphere of growing plants [18].

The 857 strains were further tested for their phosphate solubilization ability at 30°C in a plate assay in the presence of 5% salt (NaCl). Eighteen bacterial strains out of 857 PSB were able to produce halo in a plate assay in the presence of 5% salt at 30°C. The strains were screened further for their ability to solubilize phosphate

Table 2. Screening of stress-tolerant, phosphate-solubilizing bacteria[a]

Strain	Temp (°C)	Stress: pH 7 Salt: 0%	pH 7 2.5%	pH 7 5.0%	pH 8 0%	pH 8 2.5%	pH 8 5.0%	pH 9 0%	pH 9 2.5%	pH 9 5.0%
NBRI1	30	6	3	2	2	1	0	2	3	0
	37	3	1	0	2	0	0	0	0	0
	45	5	2	0	0	0	0	3	2	0
NBRI2	30	5	3	1	1	2	0	1	2	0
	37	3	2	0	2	2	0	2	3	0
	45	3	2	0	2	0	0	2	3	2
NBRI3	30	4	3	2	2	0	0	1	0	0
	37	2	2	0	3	0	0	3	0	0
	45	2	1	0	2	0	0	2	3	2
NBRI4	30	0	3	3	1	2	2	1	2	3
	37	0	0	1	0	0	0	0	0	0
	45	0	0	0	0	0	0	0	2	2
NBRI5	30	8	6	4	3	4	3	3	4	3
	37	3	0	0	2	2	0	3	0	0
	45	4	3	2	0	3	0	0	2	3
NBRI6	30	9	5	3	3	3	1	3	4	3
	37	3	0	0	2	0	0	2	2	0
	45	4	3	2	0	4	0	0	3	2
NBRI7	30	0	2	4	2	2	2	2	2	2
	37	0	0	2	0	0	0	0	0	0
	45	0	0	0	0	0	0	0	2	2
NBRI8	30	4	1	3	3	2	4	0	1	2
	37	7	2	2	3	2	1	3	1	0
	45	4	2	4	3	3	2	3	2	2
NBRI9	30	3	2	2	4	3	0	2	3	2
	37	6	2	0	2	0	0	4	0	0
	45	2	2	2	2	0	0	2	0	0
NBRI10	30	5	3	3	4	3	0	3	3	2
	37	4	0	0	2	0	0	3	2	0
	45	3	3	2	3	2	0	3	3	0
NBRI11	30	2	0	0	2	0	0	0	0	0
	37	0	0	0	3	0	0	3	2	0
	45	0	3	2	2	3	0	0	0	0
NBRI12	30	6	3	3	3	3	2	2	0	0
	37	4	3	1	2	0	0	3	2	2
	45	0	3	2	4	2	0	0	2	2
NBRI13	30	5	3	4	3	5	3	3	3	3
	37	2	1	0	2	3	0	4	2	0
	45	0	2	0	4	3	0	0	3	0
NBRI14	30	4	3	4	3	4	2	3	2	3
	37	2	2	0	2	0	0	3	2	0
	45	0	2	0	4	3	0	0	3	0
NBRI15	30	2	2	2	3	3	2	3	3	3
	37	3	3	3	4	2	3	3	3	2
	45	2	3	5	0	2	0	2	3	4
NBRI16	30	4	3	4	2	3	2	3	3	2
	37	3	3	2	4	2	3	3	2	0
	45	5	2	2	3	3	0	3	3	0
NBRI17	30	3	2	2	3	2	2	1	2	2
	37	1	2	3	3	3	3	3	3	3
	45	2	2	2	0	2	0	2	3	3
NBRI18	30	3	2	2	2	2	2	2	2	2
	37	1	3	3	3	4	3	2	2	2
	45	2	4	2	2	2	0	2	1	3

[a] Each value represents the mean of three replicates.

Table 3. Tricalcium phosphate solubilization by bacterial isolates in broth using National Botanical Research Institute's phosphate growth medium (NBRIP)[a]

Strain	Phosphate solubilized (µg/ml) Days: 1	3	5	7	10
NBRI1	110	200	240	190	100
NBRI2	90	175	180	70	60
NBRI3	260	280	510	50	30
NBRI4	15	60	98	90	50
NBRI5	90	155	240	260	55
NBRI6	120	150	330	310	130
NBRI7	10	65	160	180	142
NBRI8	218	230	330	300	240
NBRI9	210	250	425	240	125
NBRI10	140	205	255	310	205
NBRI11	5	18	38	36	35
NBRI12	235	250	410	440	205
NBRI13	125	160	200	270	110
NBRI14	182	284	305	265	145
NBRI15	240	380	390	410	435
NBRI16	245	300	325	280	235
NBRI17	4	28	40	40	42
NBRI18	30	38	42	98	180

[a] Each value represents the mean of three replicates.

under stress conditions such as high salt, pH, and temperature in a plate assay. The appraisal of the relative efficiency of isolates in solubilizing phosphate was effected by varying the salt (NaCl; 0, 2.5, and 5.0%) and pH (7, 8, and 9) of the medium and incubating the plates at 30°C, 37°C, and 45°C (Table 2). The strains seemed generally well adapted to the environments from which they had been isolated, i.e., hot, dry, or salt-affected ecosystems. All of the strains demonstrated diverse levels of phosphate solubilization in the presence of high salt, pH, and temperature (Table 2). It seemed, therefore, that the strains isolated from alkaline soils have the genetic potential to solubilize phosphates at high salt, pH, and temperature.

The 18 PSB strains were further tested for their ability to solubilize tricalcium phosphate in NBRIP broth (Table 3). Among the various bacteria tested, NBRI4 and NBRI7 did not produce halo in a plate assay in the absence of salt at 30°C (Table 2). Contrary to indirect measurement of phosphate solubilization by plate assay, the direct measurement of phosphate solubilization in NBRIP broth assay always resulted in reliable results. Strains varied with respect to levels and time of optimal level of phosphate solubilization achieved. However, by the fifth or seventh day, optimal phosphate solubilization levels were attained by all the strains (Table 3). It has been reported that many isolates that did not show any clear zone on agar plates solubilized insoluble inorganic

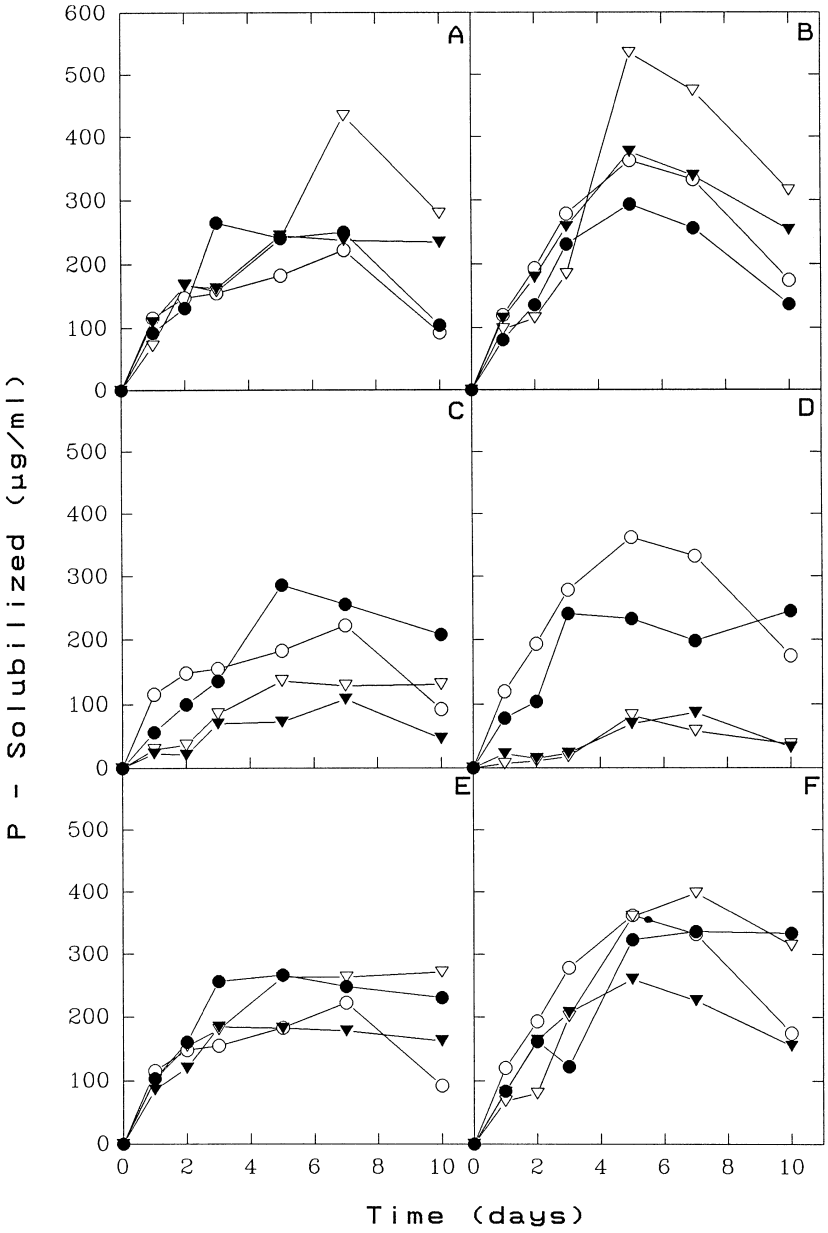

Fig. 1. Effect of NaCl (A and B), $CaCl_2$ (C and D), and KCl (E and F) supplements at 30°C and 37°C respectively, on the phosphate solubilization ability of NBRI4 grown in National Botanical Research Institute's phosphate growth medium (NBRIP) both containing 0 (○), 0.5 (●), 1.0 (▽), and 2.5 (▼) mg of salt/ml.

phosphates in liquid medium [11, 12]. Thus, the existing plate assay fails where the halo is inconspicuous or absent. This may be because of the varying diffusion rates of different organic acids secreted by an organism [9]. We have recently suggested that microbes from soil may be screened in NBRIP broth assay for the identification of most efficient phosphate solubilizers [14]. The present data confirmed our observation that the criterion for isolation of phosphate solubilizers based on the formation of a visible halo/zone on agar plates is not an infallible technique [14].

Phosphate solubilization ability of NBRI4 was higher than that of the control the presence of salt at 30°C (Table 3). To determine the effect of increasing amounts of various salts [NaCl, $CaCl_2$, and KCl] on the phosphate solubilization ability of NBRI4, we grew the strain in NBRIP both containing 0, 0.5, 1.0, and 2.5 mg of salts/ml. Data presented in Fig. 1 depict the phosphate solubilization ability of NBRI4 by NaCl, $CaCl_2$, and KCl at 30°C (Figs. 1A, 1C, and 1E) and 37°C (Figs. 1B, 1D and 1F). Phosphate solubilization increased in the presence of NaCl (Figs. 1A and 1B) and KCl (Figs. 1E and 1F) at 37°C compared with 30°C. On the contrary, except in the presence 0.5% $CaCl_2$ at 30°C (Fig. 1C) the

phosphate solubilization ability of NBRI4 was inhibited by 1.0 and 2.5% $CaCl_2$ at both 30°C and 37°C (Figs. 1C and 1D). There was a large decrease in the solubilization of phosphate at 1.0 and 2.5% $CaCl_2$ at 37°C compared with 30°C (Figs. 1C and 1D). At 37°C, 32, 77, and 76% inhibition of phosphate solubilization ability of NBRI4 was observed at 0.5, 1.0, and 2.5% $CaCl_2$ respectively compared with the absence of $CaCl_2$ (Fig. 1D). This indicated a role of calcium salt in the phosphate solubilization ability of NBRI4. The data are in agreement with the previous studies of Halder and associates [7], who reported a role of calcium in the dissolution of phosphate from phosphate rocks; Wilson and Ellis [17] suggested calcium as an important factor controlling the rate and extent of dissolution of rock phosphate.

Our earlier observation that, contrary to indirect measurement of phosphate solubilization by plate assay, the direct measurement of phosphate solubilization in broth assay always resulted in reliable results was further substantiated by the fact that NBRI4, which did not produce a halo in a plate assay at 30°C and 37°C in the absence of salt (Table 2), solubilized tricalcium phosphate in NBRIP broth at 30°C and 37°C in the presence of NaCl, $CaCl_2$, and KCl (Fig. 1). Thus, the strain NBRI4, which was otherwise indistinguishable from other strains in its ability to solubilize phosphate on a plate assay, was easily identifiable as an efficient strain in an NBRIP broth assay (Table 3), which could solubilize phosphate under stress (Fig. 1). It was noteworthy that up to 195% and 148% induction of the phosphate solubilization ability of NBRI4 was observed at 30°C (Fig. 1A) and 37°C (Fig. 1B) in the presence of 1.0% NaCl, compared with the control, respectively. To our knowledge, this is the first report of a PSB demonstrating salt- and temperature-induced phosphate solubilization ability. The trait of enhanced phosphate solubilization in the presence of salt and high temperature might be of some significance for the survival of phosphate-solubilizing bacteria in alkaline soils.

The results suggest that the bacterial strains isolated from alkaline soils have been able to evolve with the ability to solubilize phosphate in high salt, pH, and temperature conditions. The strains should serve as an excellent model to study the physiological, biochemical, and molecular mechanism(s) of phosphate solubilization under stressed ecosystems. Since the conditions in soil are much more complex than those in vitro, further study of the environmental factors affecting phosphate solubilization in alkaline soils should suggest the basis for obtaining inoculants that are able to give greater phosphate solubilization for crops of economic or agricultural importance, in tropical and subtropical areas.

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