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Research Article

PHOSPHATE SOLUBILIZATION POTENTIAL OF SALINITY TOLERANT AZOTOBACTER SPECIES

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A B S T R A C T

Phosphate solubilizing bacteria plays a central role in maintaining soil nutrient status and in plant growth promotion. The performance of these organisms is affected severely in presence of high salt concentrations and nitrogen limitation. Use of salinity tolerant nitrogen fixing bacteria belonging to genus Azotobacter as bioinoculant can help to overcome this situation. In the present study, sixty three salinity tolerant Azotobacter species were isolated from agricultural soils and were screened for their phosphate solubilization potential in presence of varying salt concentration (0.5 to 5%). Twenty seven isolates showing effective phosphate solubilization ability were screened further by quantitative studies. Three potential isolate were identified at species level by conventional methods. Three isolates AZP-13, AZP-21 and AZP-39 were potent phosphate solubilizers under high salt concentration. The isolates AZP-13 and AZP-21 were identified as Azotobacter chroococcum and AZP-39 was found as Azotobacter vinelandii. The phosphate solubilization ability of these isolates under saline conditions highlighted their significance to exploit them as biofertilizer for saline soils.

INTRODUCTION

Soil is a rich source of major nutrients (nitrogen, phosphorus and potassium) demanded by plants to flourish their growth and development. Soil nitrogen is mainly accumulated due to nitrogen fixing microbes that fix atmospheric nitrogen into a plant usable form. Soil phosphorus is available in bothe organic and inorganic form and their availability to plants is low due to their low solubility in soil solution. The plants can take up phosphorus only in soluble or dissolved form.

The genus Azotobacter is one of the important members of a group of plant growth promoting microbes that are involved in improving plant growth and development. Along with nitrogen fixation (Dixon and Kahn, 2004, Tejera *et al*., 2005), Azotobacter species are producers of phytohormones such as auxins and gibberellins (Hayat *et al*., 2010, Farajzadeh *et al*., 2012) and phosphate solubilizers (Hayat *et al*., 2010, Farajzadeh *et al*., 2012) and also plays important role in control of phytopathogenic fungi (Bhosale *et al*., 2013). Use of Azotobacter species with higher potential to solubilize organic and inorganic phosphates is one of the important strategy for the development of sustainable agriculture. They can provide sufficient nitrogen and soluble phosphorus that contribute for improved crop production (Nosrati *et al*., 2014).

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Use of higher level of chemical fertilizers in agricultural fields to increase the crop yield not only alters the soil characteristics but also affect the performance of the plant beneficial microbes. These microbes are constantly exposed to these altered conditions and hence may not perform well under conditions of biotic and abiotic stress. These conditions may be of high temperatures, acidic or alkaline pH, high salt concentration and microbes may not adapt these conditions within short time that may affect their performance and lead to compromised applications in agriculture.

Azotobacter species are well adapted to the natural climates of their habitat and particularly under harsh environmental conditions. Hence, in the present study, nitrogen fixing Azotobacter species capable of hydrolyzing both organic and inorganic phosphate compounds are isolated and effect of high salt concentrations on their growth and phosphate solubilization potential was assessed.

MATERIALS AND METHODS

Collection of Soil Samples

The agricultural soil sites were selected from different regions of Nanded. From each location, 25 random soil samples were collected at a depth of 15 cm using auger. The samples were thoroughly mixed together in a large container. After removing recognizable stones and debris, the composite soil samples

were air dried and sieved through a 2mm mesh sieve and kept in sealed plastic bags at 4℃ till processed.

Enrichment, Isolation and identification of Azotobacter sp.

For enrichment of *Azotobacter* strains, one gram from each of the soil sample was added into 100 ml Erlenmeyer flasks containing 50 ml of Azotobacter broth (mannitol 20g, K_2HPO_4 0.8 g, KH_2PO_4 0.2 g, $MgSO_4 \cdot 7H_2O$ 0.5 g, $FeSO_4 \cdot 6H_2O$ 0.10 g, $CaCO_3$ 20 g, $NaMoO_4 \cdot 2H_2O$ 0.05 g supplemented with $ZnSO_4.7H_2O$ 10 mg, $MnSO_4.4H_2O$ 1.0 mg and nystatin (100µg/ml) per liter, pH 7.2). The flask was incubated at 28°C for seven days. The enriched culture from was serially diluted till 10^{-8} and 0.1 ml of serially diluted culture (10^{-4} to 10^{-7}) was spread on Azotobacter agar plates. The plates were incubated at 30°C for 48 hrs and well isolated, morphologically distinct colonies were selected as Azotobacter strains.

Physiological and biochemical characteristics were performed according to the criteria given in Bergey's Manual of Systematic Bacteriology (Staley and Brenner, 2005) including cell morphology and motility, cyst formation, pigmentation, enzyme studies (amylase and catalase), nitrate reduction and utilization of carbohydrates (rhamnose, inositol, mannitol, malonate, maltose, benzoate, galactose, raffinose, sorbitol and pimelate).

Salinity tolerance of Azotobacter sp

To study the salinity tolerance of Azotobacter species, 63 morphologically distinct isolates were selected. The 100µl active culture of each selected isolate was inoculated in Azotobacter broth supplemented with varying concentrations of sodium chloride (0.5 to 5.0%). After incubation at 30℃ for 48 hrs, growth was measured in terms of optical density at 600 nm. Broth medium without sodium chloride was served as control. The growth of isolates in presence of salt was compared with control to determine salinity tolerance of isolates (%). Twenty seven salinity tolerant Azotobacter species showing growth at or above 3 % salt concentration were selected to study their phosphate solubilization potential.

Phosphate solubilization

To examine phosphate solubilization potential of selected isolates qualitatively, 10 µl of the individual bacterial suspensions was spotted on the center of Sperber medium plate (10 g glucose, 0.5 g yeast extract, 0.1 g $CaCl_2$, 0.25 g $MgSO_4.7H_2O$, 5 g tricalcium phosphate and 15 g agar per liter, pH: 7.2 (Malboobi *et al*., 2009). The plates were incubated at 28°C and after incubation observed for presence of a clear halo surrounding the bacterial colonies. The diameter of zone of clearance as well as the diameter of colony was measured after 7 days in triplicates.

To determine amount of phosphate solubilized by Azotobacter isolates, Sperber broth with tricalcium phosphate was distributed in Erlenmeyer flasks in 50 ml quantities and inoculated with bacterial suspensions (50µl) individually. The flasks were incubated at 30℃ for seven days under shaking conditions at 120 rpm. After incubation, the broth was centrifuged at 8000 rpm for 10 minutes. The cell free supernatant was used for estimating the amount of soluble phosphate. To 1 ml of the supernatant, in a 50 ml volumetric flask 10 ml of chloromolybdic acid was added and mixed thoroughly and then 0.25 ml. chlorostannous acid was added and mixed. Immediately the volume was made upto 50 ml with distilled water and the contents were mixed thoroughly. Within 15 minutes, the blue colour developed was read on a spectrophotometer at 610 nm using a reagent blank. The amount of phosphorus solubilized was calculated from the standard curve prepared using KH_2PO_4 (10 -100 µg/ml) solution.

RESULTS AND DISCUSSION

Sixty three Azotobacter strains were isolated from soils collected from agricultural fields of Nanded and identified as per the criteria given in Bergeys Manual of Systematic Bacteriology. The selected isolates were screened for salinity tolerance in presence of 0.5 to 5 % sodium chloride. Twenty seven isolates showed salinity tolerance above 3 to 5 %, nineteen isolates tolerated upto 2% sodium chloride, and 10 isolated showed growths in presence of 1 % sodium chloride whereas remaining seven tolerated only 0.5 % of sodium chloride when grown in Azotobacter broth. Twenty seven isolates showing salt tolerance in between 3 to 5% were selected for further studies (Table 1).

Table 1 Salt tolerance of Azotobacter sp isolated from soil

Sr. No	Isolate	Salt concentration	Sr. No	Isolate	Salt concentration
1	AZP-3	3%	15	AZP-28	4.5%
2	AZP-6	3%	16	AZP-30	3.5%
3	AZP-7	3%	17	AZP-31	4%
4	AZP-8	3%	18	AZP-34	4%
5	AZP-10	3.5%	19	AZP-35	4.5%
6	AZP-12	3.5%	20	AZP-39	4%
7	AZP-13	4%	21	AZP-40	3%
8	AZP-16	4%	22	AZP-41	4.5%
9	AZP-17	3%	23	AZP-45	5%
10	AZP-21	3.5%	24	AZP-47	3.5%
11	AZP-22	3.5%	25	AZP-48	3%
12	AZP-23	3.5%	26	AZP-53	4%
13	AZP-24	3.5%	27	AZP-59	4.5%
14	AZP-26	4%			

The performance of nitrogen fixing bacteria is drastically influenced by both biotic and abiotic environmental conditions.. Tolerances to extreme climatic conditions as temperature, salinity, pH are of special interests for bacteria to exploit their potential as biofertilizer. It is known fact that the members of the genus *Azotobacter* produce copious amount of capsular slime which allows these species to adapt the stressful environmental conditions. It is also suggested that the cyst formation ability of these organisms under unfavorable environmental conditions reduces oxygen transferring into the cell and consequently increases the nitrogenase activity (Sabra *et al*., 2000, Nosrati *et al*., 2012, Nosrati *et al*., 2014). Similarly, salt and alkali tolerant strains of Azotobacter chroococcum were isolated from calcareous saline alkali soil and showed good growth and nitrogen fixation at pH 9.6.

Phosphate solubilization was measured for all 27 isolates showing tolerance to high salt concentrations (Table 2). Of the total 27 isolates, 16 isolates solubilized inorganic phosphate in detectable range (10 mm to 20mm) while other 11 isolates didn't showed detectable phosphate solubilization potential when tested in triplicate.

These 16 isolates were subjected for determining the amount of phosphate solubilized in seven days incubation period. All isolates released varying amounts of soluble phosphate in the medium as detected by intensity of blue colour formed during

estimation. Three isolates AZP-13, AZP-21 and AZP-39 showed effective solubilization of phosphate (Table 3) and hence identified further at species level based on the creiteria given in Bergeys Manual of Systematic Bacteriology as mentioned above.

Table 2 Phosphate solubilization potential of Azotobacter sp isolated from soil

Sr. No	Isolate	Diameter of zone of clearance (mm)	Sr. No	Isolate	Diameter of zone of clearance (mm)
1	AZP-3	10	15	AZP-28	13
2	AZP-6	10	16	AZP-30	-
3	AZP-7	-	17	AZP-31	15
4	AZP-8	-	18	AZP-34	-
5	AZP-10	-	19	AZP-35	-
6	AZP-12	11	20	AZP-39	20
7	AZP-13	17	21	AZP-40	16
8	AZP-16	-	22	AZP-41	13
9	AZP-17	13	23	AZP-45	-
10	AZP-21	20	24	AZP-47	11
11	AZP-22	14	25	AZP-48	-
12	AZP-23	-	26	AZP-53	12
13	AZP-24	14	27	AZP-59	-
14	AZP-26	16			

Table 3 Phosphate solubilization potential and changes in pH of medium

Sr. No	Isolate	Amount of available Phosphate (mg/L)	Final pH of medium
1	AZP-3	17.01±0.05	6.81±0.02
2	AZP-6	14.21±0.31	6.98±0.12
3	AZP-12	13.10±0.5	6.99±0.11
4	AZP-13	157.03±0.34	4.19±0.05
5	AZP-17	48.31±0.15	5.78±0.13
6	AZP-21	145.3±019	4.34±0.08
7	AZP-22	98.21±0.12	4.48±0.16
8	AZP-24	137.51±0.05	4.08±0.06
9	AZP-26	80.7±0.12	4.76±0.13
10	AZP-28	59.12±0.13	5.13±0.10
11	AZP-31	85.42±0.03	4.82±0.05
12	AZP-39	179.13±0.08	4.08±0.16
13	AZP-40	103.25±0.07	4.40±0.10
14	AZP-41	92.01±0.013	4.51±0.06
15	AZP-47	80.02±0.04	4.78±0.05
16	AZP-53	14.45±0.12	6.91±0.02

Soil microorganisms play a crucial role in mobilizing phosphorus 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*., 1990). Production of organic acids results in acidification of the microbial cell and its surroundings. As a result, phosphate may be released from a mineral phosphate by proton substitution for Ca^{+2} (Goldstein, A. H. (1994).

CONCLUSION

The isolated Azotobacter species showed salinity tolerance as well as phosphate solubilization potential. These beneficial bacteria can engineer positive interactions in the rhizosphere and stimulate plant growth under saline conditions.

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