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Screening of efficient halotolerant phosphate solubilizing bacteria and their effect on seed germination under saline conditions

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Abstract

Seventy four halotolerant strains were isolated from saline soil and tested further to screen efficient halotolerant phosphate solubilizing microorganisms. For isolation Halobacterium medium and for selection modified Pikovskaya broth under various salt concentrations was used. Total nine strains were selected on the basis of the release of soluble phosphorous in the medium detected by using colorimetric molybdate blue method. The tomato (*Lycopersicon esculentum*) seeds which were inoculated with isolated strains and tested for their ability to germinate in different saline conditions. Out of all nine strains, inoculums of three strains PSB-1, 2 and 6 had significantly increased the germination percentage of the seeds at salt concentration between 0 to 60 mM. The results suggested that the isolated halotolerant PSB may to be used as source to supply phosphorous to the growing plant in saline conditions.

Keywords: Halotolerant bacteria, Halobacterium medium, Phosphate solubilizing bacteria, Pikovskaya medium, *Lycopersicon esculentum*.

Introduction

Soil salinity is the salt content in the soil; the process of increasing the soil content is known as salination. Salt is the natural element of soil and water. Salination can be caused by several natural processes as well as manmade such as irrigation. Expanding problems of soil salinity and water logging have become serious issues of concern as they affect productivity and threaten the very sustainability of agriculture. Saline soil is distributed throughout the world especially in the arid and semiarid regions where agriculture performs under irrigation.[1]The phosphorus deficiency frequently compounds the problems of saline soil of the tropics.[2] High salinity affects plant growth through osmotic effects; toxicity of salt ions; and the changes in the physical and chemical properties of soil.[3] It also suppresses the phosphorus uptake by plant roots and reduces the available phosphorus by sorption processes and low solubility of the Ca-P minerals.[4] Since phosphorus is a critical nutrient limiting plant growth[5], the adverse effects on plant growth in saline soil are multiplied. The yield and profits of agricultural productions in saline soil are reduced drastically. Therefore, the use of chemical fertilizers is the most common approach to improve soil fertility. However, the available phosphorus in the chemical fertilizers is rapidly fixed to the unavailable forms[6, 7] especially in saline soil and accounts for low phosphorus use efficiency. Phosphorous (P) is the major growth-limiting nutrient.

It plays its role in root development, stalk and stem strength, flower and seed formation, maturity and production, crop quality and resistance to plant diseases.[8] There are evidences of occurrence of rhizopheric Phosphorous solubilizing microorganisms (PSM).[9] Soil microorganisms play a key role in soil P dynamics and subsequent availability of phosphorous to plants.[10] Microorganisms involved in phosphorous acquisition include mycorrhizal fungi and PSMs. Strains from bacterial genera *Psedomonas*, *Bacillus*, *Rhizobium* and enterobacteria alongwith *Penicillium* and *Aspergillus* fungi are the most powerful P solubilizers.[11] Phosphate-solubilizing microbes can transform the insoluble phosphorus to soluble forms HPO_4^{-2} and $H_2PO_4^-$ by acidification, chelation, exchange reactions and polymeric substances formation.[12] Therefore, the use of phosphate-solubilizing microbes in agricultural practice would not only offset the high cost of manufacturing phosphatic fertilizers but would also mobilize insoluble phosphorus in the fertilizers and soils to which they are applied. The salinity stress causes less effect on halotolerant bacteria since they have adapted during evolution to tolerate and optimally grow in hyper saline environments.[13] In the present study phosphate solubilizing bacteria were isolated from saline soil and their effect on the germination of *Lycopersicum esculentum* seeds as well as seedling growth in saline conditions.

Materials and Methods

Microorganisms and Culture Media

Phosphate solubilizing halotolerant bacteria were isolated from saline soil sample collected from Hazira coastline of Surat city in Gujarat state of India by serial dilution method.[14] 1 gm of soil sample was dissolved in 10 ml of sterile water and kept in shaking conditions at 120 rpm overnight at room temperature. 0.2 ml of soil suspension was spreaded with glass spreader on Halobacterium medium which contained the following ingredients per liter: $MgSO_4.7H_2O$ 10.0g; Casein hydroxylate 5.0g; KCl 5.0g; KNO_3 1.0g; $CaCl_2.2H_2O$ 0.2g; Disodium citrate 3.0g; NaCl 0.6 M, Yeast extract 1.0g and Agar 15.0g. Bacterial cells were scraped from the plates and diluted to obtain 10^8 CFU ml^{-1} by adjusting their optical density at 600 nm to approximately 1.0 using phosphate buffer (0.1M, pH 7.0).

The modified Pikovskaya (MPVK) medium was used for screening of efficient halotolerant PSB experiment. This medium was first described by Pikovskaya[15] and was modified by Son et al.[16] The medium contain (per litre): 1.0% (wv^{-1}) glucose; 0.05% (wv^{-1}) $(NH_4)_2SO_4$; 0.02% (wv^{-1}) NaCl; 0.01%(wv^{-1})$CaCl_2.2H_2O$; 0.01%(wv^{-1})$MgSO_4.7H_2O$; 0.05% (wv^{-1})$MnSO_4.7H_2O$; 0.05% (wv^{-1}) $FeSO_4.7H_2O$; 0.05% (wv^{-1}); Yeast extract in distilled water (pH 7.5). NaCl and Agar are supplemented as required. The tricalcium phosphate ($Ca_3(PO_4)_2$) at 0.5% (wv^{-1}) was added as source of insoluble phosphate. It was autoclaved separately from other ingredients and then aseptically mixed together.

Screening of Efficient Halotolerant PSB

The screening procedure comprised of three steps each with three replicates. In primary screening step, seventy five halotolerant bacterial strains were screened for their phosphate solubilizing ability on modified Pikovskaya (MPVK) agar medium containing 0.6M NaCl. The plates were incubated at 30ºC for 5 days. Only the bacterial strains that formed the clear halos around their colonies were selected for using in the next step. In the secondary screening step, the quantitative analysis of phosphate solubilization was carried out with Erlenmeyer flasks containing 150 ml of MPVK broth inoculated with individual bacterial strains and incubating them for 5 days at 37ºC with shaking at 120 rpm. The cultures were harvested after centrifugation at 10,000 rpm for 30 minutes. Phosphate solubilising efficiency of PSB isolates was determined by using colorimetric molybdate blue method. In this method 40 ml of the supernantant was reacted with mixed reagent containing 125 ml NH_2SO_4; 37.5 ammonium molybdate (4%), 75 ml ascorbic acid diluted to final volume of 250 ml. 1M solution of potassium dihydrogen phosphate was used as standard. The autoclaved, uninoculated medium served as the negative controls APHA et al.[17]

Germination Studies

Tomato (*Lycopersicon esculentum*) seeds were surface sterilized by immersion in 70% ethanol for 1 min., followed by 30 min. in 1% sodium hypochlorite. Then the seeds were washed three times with sterile distilled water. An inoculum of the efficient halotolerant PSB was prepared. The disinfected seeds were immersed in PSB bacterial inoculum made in nutrient broth at10^8 CFU ml^{-1}. For control the seeds were kept by in phosphate buffer of 0.1M, pH 7.0 without bacterial inoculum for one hour. Germination studies on these halotolerant PSB inoculated and uninoculated seeds were conducted. Two replicates of 25 seeds were germinated in sterilized petridishes containing two sheets of filter papers moistened initially with 5 ml of 0.5 (w/v) sterilized tricalcium phosphate solution supplemented with different concentrations (0, 30, 60, 90 mM) of NaCl at room temperature. Germination was observed daily and germination percentage was calculated by following formula:

Germination percentage: n/N*100

Where, n= total no. of germinated seeds

N= total no. of initiated seeds

Results and Discussion

Isolation and Screening of Efficient Halotolerant PSB

Soil collected from the seashore of Hazira was rich in salts and microorganisms present in the soil were supposed to be well adapted to salinity. In the first step Halotolerant medium was used to isolate the isolate halotolerant group of organisms. Total seventy-five bacterial strains which have the ability to grow in the media having 0.6M NaCl were isolated. In the process of screening halotolerant PSB modified PVK medium supplemented with tricalcium phosphate as the source of insoluble phosphate was used. Nath et al had also compared the tricalcium phosphate solubilizing activity of two different endophytic *Penicillium* species isolated from tea leaves.[18] Qualitative estimation of PSB was performed on Pikovskaya medium agar plates and fifteen organisms producing visible clear zone on the agar plate were selected. Twenty five strains had given clear zones on MPVK agar plates. Quantitative screening of efficient halotolerant PSB was done in MPVK broth and most efficient phosphate solubilizing was selected on the basis of the soluble phosphorous present in the supernatant detected colorimetrically. Out of twenty five, nine bacterial strains were selected as efficient phosphate solubilizing halotolerant strains and results obtained as concentration of soluble phosphorous found in the supernatant were shown in Table 1. These nine PSB strains were used further for studying their effect on tomato seed germination. Preliminary cultural and biochemical analysis of the strains was performed and results for six strains were shown in Table 2. It was seen that majority of the strains were gram positive and with white pigmentation. Literature studies had revealed that gram positive genus *Bacillus* plays very significant role in PSB[13, 19-21] so the probability of presence of Bacillus strains was there. Increased crop yields by inoculation of *Bacillus* spp. either through single inoculation or co-inoculation with the other plant growth-promoting bacteria, were reported by several researchers with several plant species. Different research groups had isolated bacterial strains with phosphate solubilizing ability and other plant growth promoting traits increasing the plant biomass.[22-25].

Table 1: Results for phosphorus solubilizing activity of different PSB isolates

Isolates	PSB-1	PSB-2	**PSB-3**	PSB-4	PSB-5	PSB-6	PSB-7	**PSB-8**	PSB-9
Soluble phosphorus (mg/lit)	0.04	0.25	**0.71**	0.08	0.21	0.02	0.13	**0.64**	0.35

Table 2: Microbiological characteristics of some selected PSB isolates

Characteristics	**Isolates**					
Morphological & Cultural	**PSB-1**	**PSB-2**	**PSB-3**	**PSB-5**	**PSB-8**	**PSB-9**
Size	Large	Large	Large	Small	Small	Small
Shape	Irregular	Irregular	Amoeboid	Round	Round	Irregular
Texture	Alveolate	Smooth	Vesicular	Contoured	Bullet	Alveolate
Edge	Undulate	Undulate	Undulate	Undulate	Undulate	Undulate
Elevation	Flat	Convex	Capitate	Flat	Flat	Flat
Opacity	Opaque	Cretaceous	Cretaceous	Opaque	Opaque	Cretaceous
Pigmentation	White	Nil	White	Nil	Nil	Nil
Consistency	Dry	Dry	Butyrous	Butyrous	Butyrous	Butyrous
Gram staining	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive	Gram positive
Biochemical						

tests						
Ammonia production	-	+	+	+	-	+
Methyl Red	+	+	+	-	-	-
Voges Proskauer	-	-	+	-	-	-
Citrate utilization	-	+	+	-	-	+
Urea Hydrolysis	-	-	-	-	-	-
Triple Sugar Iron	+	-	+		-	+
Sugar fermentation						
Glucose	-	+	-	+	-	A
Sucrose	-	-	-	-	-	A
Lactose	-	-	-	-	-	A
Maltose	-	-	-	-	-	A
Manitol	+	-	-	-	-	A
Xylose	-	+	+	+	-	-

Key: + = Positive; **-** = Negative; A = acid production; G = gas production

Effect of Efficient Haloterant PSB on Promoting Seed Growth under Saline Conditions

The aim of this study was based on the hypothesis that an inoculation of the isolated efficient halotolerant PSB could solubilize the insoluble phosphate and provides sufficient available phosphorous for plants grown under saline conditions which consequently results in enhancement of plant growth. All the nine isolates were tested on tomato seed germination under saline conditions. The bacterial suspension of the selected strains was used to inoculate tomato seeds prior to germinate under saline conditions (0, 30, 60 and 90 mM) and supplemented with 0.5% (w/v) insoluble phosphate. The germination percentage of the seedlings inoculated with the PSB isolates were compared with those of uninoculated seedlings taken as control. The germination percentage was found to be highest in the seeds inoculated with strain PSB-6 (85%) in comparison with the control (62%) at 0 mM NaCl (table 3). Significant increase in germination percentage was observed with strains PSB-1and 2 (72 and 70% respectively) while it was same as in control with other strains. One important observation was that in control the seeds were not able to germinate in the presence of salt in the media while showed germination when treated with bacterial inoculums PSB-1,2,3,5 and 8 in the saline environment. It means that saline conditions were unfavorable for the seed germination in general due to unavailability of important element P but the availability of this important element was restored in the presence of PSBs with which the seeds were treated before germination. It might be due to delayed actions of salt on seed germination by interfering with the uptake of essential nutrients, the direct toxicity effects of salt ions and the prevention of seed water uptake.[26, 27] It is also seen that salinity also causes change in morphology and physiology of plant roots.[28] Phosphorous uptake was seen to be hindered by the plant roots and its translocation from root to shoot in high salinity.[29] One research group had isolated three strains and tested on maize (*Zea mays*) and evaluated the biofertilizer potential activity both individually and in consortia.[30] An enhancement of tomato growth under saline conditions might be the result of increasing available phosphorous from microbial activity which was then taken up by plants for the emergence and development of the seedlings. The results were in same line as obtained by other research groups[31, 32] and suggested the ability of the selected halotolerant PSB to provide available phosphorous for plants grown in saline soils.

Table 3: Effect of PSB isolates inoculum on Seed Germination percentage in saline conditions

Isolated strains	**Germination Percentage under various salt concentrations (mM)**		
	0	**30**	**60**
Control	62	-	-
PSB-1	72	45	-
PSB-2	70	67	-

PSB-3	52	40	6
PSB-4	60	-	-
PSB-5	60	11	-
PSB-6	**85**	-	-
PSB-7	50	-	-
PSB-8	50	20	4
PSB-9	65	-	5

Conclusion

Present study had revealed that saline soil collected from seashore might serve as good source for the halotolerant microbes which when grown on special Pikovskaya media in the presence of tricalcium phosphate as a source for inorganic phosphorous might result in isolation of most efficient halotolerant phosphate solubilizing organisms. These microbes had the ability to solubilize phosphate in the presence of high salt. In our study we had found strains PSB-1, 2, 6 and 9 treated seeds had showed significant increase in germination percentage in the presence of salt in the media depicting their ability of solubilize the phosphate even in the presence of salts and facilitating the seeds to germinate. These strains facilitating the germination and growth of seedlings in the presence of high salt concentrations may serve as good source in alone or in consortia. However, the natural saline soil conditions are quite different then what the organisms have in petridishes with artificial salt and phosphate source therefore further study on the activity of halotolerant PSB in pots for small scale and in field with existing environmental conditions is to conducted.

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