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Solubilization of phosphate by the *Bacillus* under salt stress and in the presence of osmoprotectant compounds

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The *Bacillus* by their intrinsic properties have several mechanisms of procuring beneficial effects to plants. They are among the most successful bacterial communities in the solubilization of phosphates. Ten *Bacillus* strains were isolated from the rhizosphere of wheat from three areas in arid and semi-arid regions in Algeria. These strains were tested for their ability to solubilize phosphates on liquid and solid media: LB (Luria-Bertani), NBRIP (National Botanical Research Institute Phosphate) and PVK (Pikovskaya) supplemented with $Ca_3(PO_4)_2$ (5 g/l). The highest levels of soluble phosphates were obtained on PVK and NBRIP liquid (177.68 and 173.28 µg/ml for D13 and D1 strains, respectively). Phosphates solubilization is always associated with a decrease in the pH of the medium. An inverse relationship (r = -0.4224) existed between the levels of soluble phosphate and the decrease of pH in medium. To test the solubilization capacity of the *Bacillus* strains under salt stress, concentrations of NaCl (0, 2, 4, 6 and 8%) were added to the NBRIP medium. The solubilization capacity was negatively affected by the increase of salinity. However, the response of the *Bacillus* to salt was strain dependent. Strains D1 and D13, which produced the best rate of solubilization were used to study the ability of osmoprotectants (glycine-betaine, proline) to restore the ability to solubilize phosphate at high salt concentrations. However, these strains do not seem to accumulate these types of osmoprotectants.

Key words: *Bacillus*, phosphate solubilization, NaCl, osmoprotectant.

INTRODUCTION

Improving soil fertility is one of the most common strategies to increase agricultural production. Phosphorus is an essential macronutrient for the growth and development of the plants after nitrogen, but it is considered to be one of the elements that limits plant growth. Chemical fertilizers are the main strategy for the availability of phosphorus in agricultural soils, but about 75 to 90% of phosphate fertilizers added are precipitated by iron, aluminium and calcium complexes present in soils (Gyaneshwar et al., 2002; Turan et al., 2006). Agricultural soils of arid and semi-arid regions are mainly limestone. With high levels of exchangeable calcium, soluble phosphate reacts with $CaCO_3$, precipitates and forms minerals of insoluble Ca-P (Lindsay et al., 1989). The accumulation of this form is a crucial problem in agriculture. The use of rhizospheric microorganisms is the best biological means to enhance solubilisation of phosphate in the soil and to provide sufficient quantities for plant nutrition (Pradhan and Sukla, 2005; Singh et al., 2011). The ability of some microorganisms to convert the insoluble phosphate to an accessible form is an important trait for plant growth promoting rhizobacteria (PGPR) to

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Table 1. Composition of the media.

Media component	Amount (g/l)		
	PVK	NBRIP	LB
Glucose	10	20	10
$Ca_3(PO_4)_2$	5	5	5
$(NH_4)2SO_4$	0.5	0.1	—
NaCl	0.2	—	5
$MgSO_4$-$7H_2O$	0.1	0.25	—
$MgCl_2$-$6H_2O$	—	10	—
KCl	0.2	0.2	—
Yeast extract	0.5	0.1	5
$MnSO_4$-H_2O	0.002	—	—
$FeSO_4$-$7H_2O$	0.002	—	—
Tryptone	—	—	10
Agar*	15	15	15
pH	7.0±0.2	7.0±0.2	7.0±0.2

PVK, Pikovskaya phosphate medium; NBRIP, National Botanical Research Institute; phosphate medium; LB Luria-Bertani medium modified (added with glucose); *added to solid media.

increase plant performance. Phosphate-solubilizing rhizospheric bacteria could be a promising source as a biofertilizer in agriculture (Kucey et al., 1989). The population of microorganisms solubilizing phosphates varies depending on the type of soil. They can exist in fertile or deficient soils (Oehl et al., 2001), and a large proportion are in the rhizosphere (Kucey, 1983), which contains 5 to 20 times more phosphate-solubilizing bacteria than non-rhizospheric soil (Bowen and Rovira, 1999). Many microbial species are able to solubilize phosphates, among them, the *Bacillus* show an important activity for this element. These bacteria have soil as principal habitat and are able to resist adverse environmental conditions.

The capacity of the phosphate solubilizing bacteria is often tested on medium containing calcium phosphate as the sole source of phosphates. Many media are recommended for this type of study such as Pikovskaya medium (Pikovskaya, 1948), the PVK bromophenol blue (BBP) and National Botanical Research Institute Phosphate (NBRIP) medium (Nautiyal, 1999). All media after 1948 are media derived from PVK by changing the source of carbon and/or nitrogen and/or phosphate or salts. The mechanism of phosphate solubilization is triggered by the release of H^+ ions, the production of organic acids and chelating substances. The production of organic acids is the main mechanism. Several studies have associated the phosphate solubilization to the medium pH decrease (Hinsinger, 2001).

The performance of microorganisms solubilizing phosphates is strongly influenced by environmental conditions. The phosphate solubilizing activity is often decreased in saline soils. Most soils of arid and semi-arid regions are saline (Kumar and Narula, 1999). High salinity suppresses the phosphate uptake by plants roots and reduces the available phosphorus (Grattan and Grieve, 1999). The ability of microorganisms to adapt to changes in the external environment is therefore of fundamental importance for their growth and survival. The bacteria have developed a number of adaptation strategies against fluctuating environmental parameters. In saline environments, microorganisms need to balance the osmotic pressure between intra and extracellular. Osmotic adjustment is achieved by increasing solute concentration inside the cell by the accumulation of organic and inorganic solutes (Ventosa et al., 1998). The *Bacillus* species respond to elevated ionic strength media by synthesing or accumulating any variety of osmolytes including proline, glutamic acid, various ectoines and glycine betaine. Among these organic molecules, amino acids are substrates of choice accumulated to face the stress. Proline and glycine-betaine are the most commonly used solutes (Kempf and Bremer, 1998). However, if these compounds also influence the effectiveness of soil inoculants, our knowledge is still limited and scarcely documented. Salt tolerant rhizobacteria with potential of phosphates solubilization play a prominent role in maintenance of soil fertility and plant productivity in saline soils. Therefore, it is becoming important to study the osmoprotective effect of glycine betaine and proline on ability to solubilize phosphates under high salt concentrations.

The objective of this work was to identify a sensitive and effective medium as a tool for screening *Bacillus* solubilizing phosphates, to determine the effect of salt on the solubilization capacity and finally to restore the activity of solubilization by the contribution of the osmoprotectant molecules: proline and glycine-betaine.

MATERIALS AND METHODS

Isolation

Bacillus strains were collected from the rhizosphere of wheat from three areas in arid and semi-arid regions in Algeria. Soil adhering strongly to the roots of wheat was extracted, added to 10 ml sterile distilled water and shaken for 30 min. The processed soil sample was serially diluted, spread plated on nutrient agar (NA) at 30°C/48 h. The colonies were repeated on culturing several times to obtain pure cultures. The genus *Bacillus* strains were verified by the Gram stain and the presence of the endospore. Pure cultures were kept at 4°C on NA slants. From a collection, ten strains were chosen in this study for their ability to solubilize phosphates.

Phosphate solubilization

Culture media

Three media were tested for their efficiency to solubilize phosphate and prepared according to the composition in Table 1: the Pikovskaya medium (PVK) (Pikovskaya, 1948), the National Botanical Research Institutes's growth medium (NBRIP) (Nautiyal, 1999) and modified LB (Luria-Bertani) medium. The PVK and the NBRIP showed similarity in their chemical composition. They contained

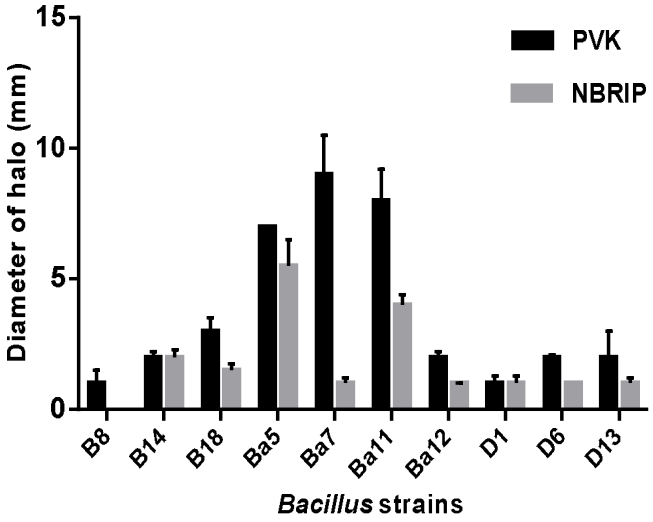

Figure 1. Phosphate solubilization by *Bacillus* strains expressed by clear zone around the colonies on solid media: PVK Pikovskaya phosphate medium; NBRIP National Bota-nical Research Institute phosphate medium. The LB Luria-Bertani medium was not represented because clear zone was not observed around colonies.

the same basic components. The PVK contained more micro-nutrients. However, the LB medium was a standard for the isolation of the *Bacillus* and added with the same source of carbon which is glucose, as the PVK and the NBRIP. The corresponding solid media were obtained after addition of agar (15 g/L).

Solubilization test in solid media

Phosphate solubilization ability of isolated bacteria was tested by the method described by Nautiyal (1999). 10 µl of the culture of each strain were deposited in spot on the surface of the solid media in Petri dishes. The solubilization capacity was assessed by the transparent area formed around the colony. After 10 days of incubation at 30°C, the total diameter (diameter of the halo + diameter of the colony) and the diameter of each colony were measured. The diameter of the solubilization halo, for each isolate**,** was determined by subtracting the diameter of the colony from the total diameter.

Solubilization test in liquid media

For a quantitative estimation of the solubilized phosphate, 100 µl of a young culture of each isolate were inoculated in tubes containing 10 ml of NBRIP, PVK and LB liquid medium. The inoculated media were incubated at 30°C/10 days. The cultures were centrifuged at 3000 rpm for 20 min. The amount of soluble phosphate in the supernatant was measured by the colorimetric method of Olsen and Sommers (1982).

In a 50 ml flask, 1 ml of supernatant, 10 ml of ammonium molybdate (12 mM) and 1 ml of $SnCl_2$ (5 mM) were added and the volume was adjusted to 50 ml with distilled water. The intensity of blue color, measured at 620 nm, was directly proportional to the concentration of soluble phosphoate (P_2O_5) in the sample. A standard calibration curve was made with KH_2PO_4 solution. The pH of the filtrate was determined in each case.

Phosphate solubilization under salt stress

To estimate the effect of salt on the ability of phosphate solubilization of the different isolates, the NBRIP broth (chosen for its less complexity) supplemented with increasing concentrations of NaCl (0, 2, 4, 6 and 8%) was tested. Media inoculated with 100 µl of a young culture of each isolate were incubated at 30°C/5 days. Cultures were centrifuged at 3000 rpm for 20 min. The soluble phosphate was determined by the colorimetric method described previously and the pH of supernatant was registered.

Effect of osmoprotectants

The objective of this experiment was to determine the effect of the osmoprotectants; proline and glycine betaine on the restoration of phosphate solubilization activity of bacterial strains in the presence of salt. Two strains (D1 and D13) solubilizing the highest rates were used for this test. The NBRIP medium at different NaCl concentrations (0, 2, 4, 6 and 8%) was supplemented with proline and glycine betaine at 1 mM. The tubes inoculated with 100 µl of culture were incubated at 30°C/5 days. The soluble phosphate was determined by the method described previously. The pH was measured in each case.

Statistical analysis

All the experiments were performed in triplicate and the average values with ±SD were reported in figures and tables. Analysis of variance (ANOVA) of the data was used for each experiment to compare the mean values using Tukey test at 5% probability level. The significance of correlation of the data was done by regression analysis. All the statistical calculations were performed using Assistat 7.5 beta software.

RESULTS

Solubilization test in solid media

The results of the efficiency of phosphate solubilization of *Bacillus* strains differed depending on the media (Figure 1). Many strains showed a clear halo of solubilization on PVK and NBRIP media. PVK medium allowed the observation of a transparent halo around all strains, where strain BA7 had the largest diameter (9 mm). Eight strains showed clear halos on NBRIP medium whose maximum diameter (5 mm) was observed for the strain BA5. However, on LB medium no strain had any halo of transparency (Table 2).

Solubilization test in liquid media

The analysis of variance showed significant differences in the efficacy of culture media and the ability of strains to solubilize phosphate (Table 3). All *Bacillus* strains solubilized phosphate in the three liquid media but at different rates, depending on the nature of the culture medium (Figure 2). According to the data of Table 2, the PVK and NBRIP media were more effective for solubilization than LB medium. In the PVK medium, the maximum concen-

Table 2. Phosphate solubilization in liquid and solid media and pH in culture media.

Media	P_2O_5 (µg/ml)	Diameter of halo (mm)	Correlation S/L	pH[3]	Correlation pH/P_2O_5 (L)
	Liquid (L)[1]	Solid (S)[2]			
PVK	100.58[a]	3.7 (09)	0.0424[ns]	5.17[b]	-0.4113*
NBRIP	94.80[a]	1.65 (08)	0.3683*	5.17[b]	-0.4224*
LB	54.07[b]	00	/	5.54[a]	0.0983[ns]

Mean amounts of P_2O_5[1]; mean of diameter of clear halo () number of strains having clear halo[2] and pH values in culture media[3]. Means sharing same letter do not differ significantly according to the Tukey's test (p> 0.05). Regression analysis for solubilization in liquid and solid media and for amount of soluble phosphates and pH values. * Significant at a level of 5% (p≤0.05). ns: not significant (p≥0.05).

Table 3. Variance analysis (mean squares) of soluble phosphoate (P_2O_5) and of pH values in three culture media.

Source of variation	Df	P_2O_5(µg/ml)	pH
Media (M)	2	19281.07777**	1.77636**
Strains (S)	9	6828.79001**	0.32886**
MxS	18	2716.69835**	0.19975**
Error	54	729.00420	0.05841

** Significant at a level of 1% of probability (P< 0.01).

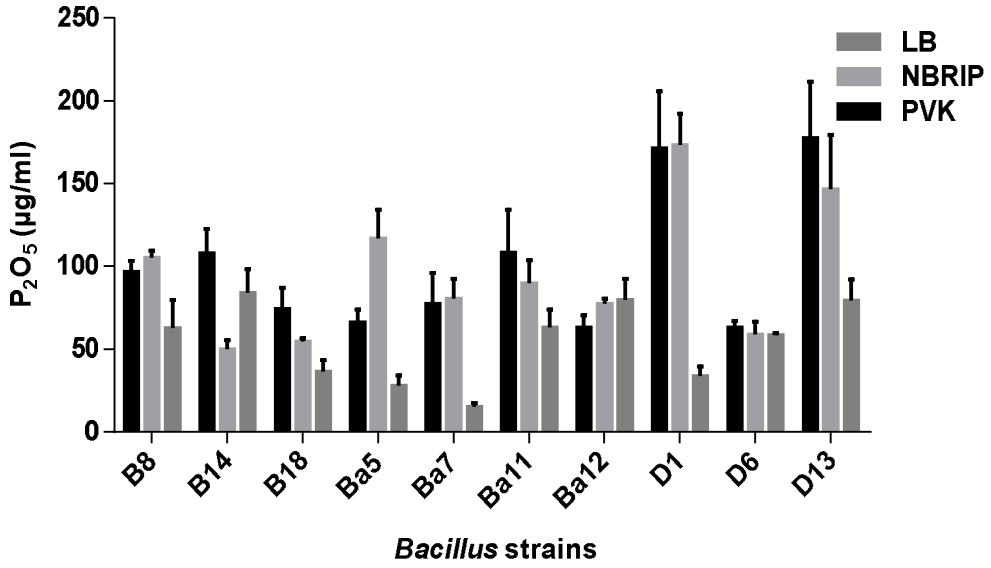

Figure 2. Amounts of solubilized phosphate expressed by phosphate ion (P_2O_5) by *Bacillus* strains in the three liquid media.

tration of soluble phosphate (P_20_5) was observed in strain D13 (177.68 µg/ml) followed by D1 (171.56 µg/ml). The result was reversed in the NBRIP medium. Maximum concentration was observed in D1 (173.28 µg/ml) followed by D13 (146.57 µg/ml). While the results in LB medium showed that the highest rates were observed in strains B14 (83.97 µg/ml), BA12 (79.82 µg/ml) and D13 (79.42 µg/ml). Of all the strains, D13 seemed to be the most effective in all three media.

The correlation analysis between phosphate solubilization on solid and liquid media for all strains showed a significant relationship for NBRIP medium. Strains which solubilized phosphates on solid medium became more efficient on liquid medium. In contrast, no correlation existed between solubilization on PVK media solid and liquid in which strain D13 having a clear zone of 2 mm diameter reached the maximum solubilization rate in liquid medium. However, no relationship existed between the liquid and solid LB medium, the strains showed no transparent halo on solid medium (Table 2).

Solubilization of phosphates was accompanied by a decrease in pH. This decrease was observed for all

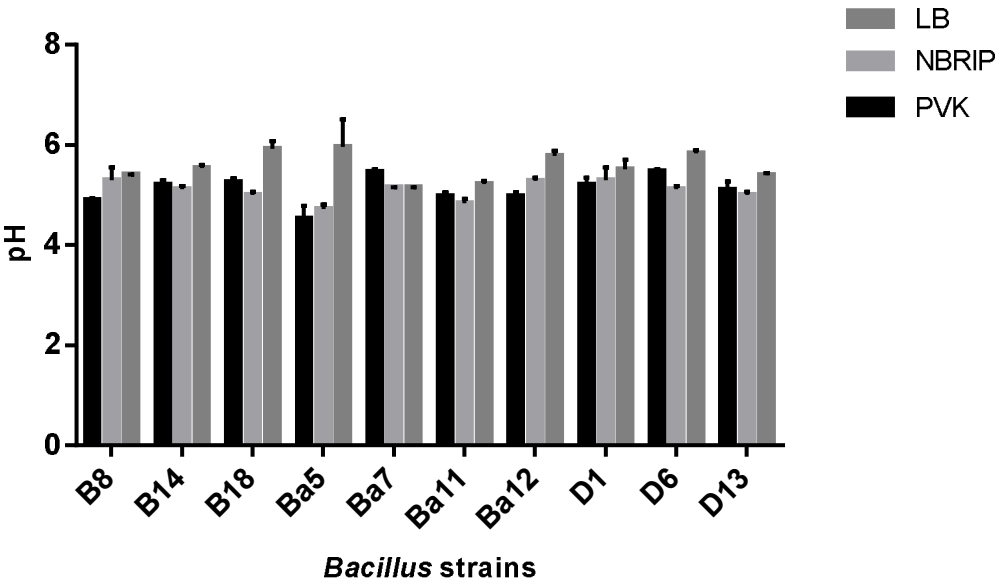

Figure 3. pH values of the three liquid media.

Table 4. Effect of different concentrations of NaCl on phosphate solubilization in NBRIP medium.

Source of variation	Df	P_2O_5 (µg /ml)	pH	r^2
Salinity(S1)	4	9798.11912**	2.09014**	-0.6758**
Strains (S)	9	3193.41812**	1.71681**	
S1xS	36	1240.80964**	0.26392**	
Error	90	63.49535	0.05576	

**Significative at a level of 1% of probability (P< 0.01).

isolates and for the three media (Figure 3). The analysis of variance indicated that the pH of the medium had a significant effect on the solubilization rates of the strains in the three media (Table 3). The results showed that the acidification of PVK and NBRIP media was significantly the same and played a role in the mechanism of phosphate solubilization. The analysis of the results made between the amount of P_2O_5 and the pH of the medium revealed a negative and significant correlation for the PVK and NBRIP media and a non significant correlation for the LB medium (Table 2). Indeed, for all of the strains, correlation analysis showed that, when the pH of the medium was lower, the amount of soluble phosphate was greater.

Phosphate solubilization under salt stress

All strains were tested in NBRIP medium added with increasing concentrations of NaCl (0, 2, 4, 6, and 8%) at 30°C/5 days. The analysis of variance showed a significant effect of salt and that the strains differed in their ability to solubilize phosphate (Table 4). All strains showed a decreased rate of solubilization at the highest concentration of NaCl (8%) (Table 5). In the absence (0%) of NaCl, D1 and D13 strains solubilized the maximum when compared with other strains with 121.84 and 112.83 µg/ml of P_2O_5, respectively. However, some strains showed a high solubilization capacity for higher NaCl concentrations like the case with the B8, BA5 and BA11 that solubilize more at 2% of NaCl (58.77, 78.73 and 88.50 µg/ml, respectively). We observed the same results with strains B14, B18, BA7 and BA12 at 4% of NaCl (31.94, 78.54 75.80 and 35.26 µg/ml, respectively). Phosphate solubilization in liquid medium was always accompanied by a decrease in the medium pH. The analysis of variance showed a significant effect ($P < 0.01$) in decrease of the pH medium for all strains (Table 4).

pH values increased with salt concentration (Table 6). A negative significant correlation ($r = -0.6758$) was observed between the solubilization of phosphate and decreasing pH of the medium (Table 4).

Effect of osmoprotectant

According to the results of halotolerance of *Bacillus* strains,

Table 5. Amounts of solubilized phosphate under different concentrations of NaCl.

NaCl (%)	Strain									
	B8	**B14**	**B18**	**Ba5**	**Ba7**	**Ba11**	**Ba12**	**D1**	**D6**	**D13**
0	44.08±12.43^{aCDE}	24.37±2,67^{abE}	50.08±0.61^{bcCD}	64.75±4.06^{aBC}	51.91±4.02^{bC}	76.00±2.84^{abB}	29.28±1.45^{aDE}	121.84±2.83^{aA}	26.10±5.90^{aE}	112.83 ±4.63^{aA}
2	58.77±9.07^{aCD}	26.15±1.88^{abE}	65.02±4.67^{abBCD}	78.73±5.67^{aABC}	57.38±2.79^{bD}	88.50±1.86^{aA}	31.78±2.97^{aE}	86.68±3.06^{bA}	20.99±1.05^{abF}	81.89 ±3.47^{bAB}
4	51.47±5.40^{aBC}	31.94±2.44^{aCD}	78.54±16.93^{aA}	65.99±5.99^{aAB}	75.80±8.57^{aA}	65.89±3.18^{bAB}	35.26±0.92^{aCD}	27.28±2.75^{cD}	20.39±1.15^{abD}	37.08 ±1.81^{cCD}
6	20.59±2.37^{bBC}	9.69±0.31^{abBC}	37.95±4.31^{cAB}	26.63±0.49^{bABC}	45.67±1.31^{bA}	17.97±2.76^{cBC}	32.96±2.15^{aABC}	18.37±1.91^{cBC}	16.54±3.21^{abC}	31.38±5.07^{cABC}
8	12.08±1.87^{bC}	8.17±1.43^{bC}	38.35±1.91^{cAB}	22.72±0.23^{bABC}	43.50±1.10^{bA}	12.88±1.30^{cC}	22.00±5.57^{aBC}	11.27±1.74^{cC}	6.81±1.23^{bC}	10.14 ±5.19^{dC}

Means amounts of P_2O_5 ± the SD. Means sharing same letter do not differ significantly according to the Tukey test ($p>0.05$)

D1 and D13 showed the best rate of solubilization and a predisposition to resistance to salinity at 2% of NaCl. While, a decrease in amount of soluble phosphate was noted from 4% of NaCl. These strains were used to test the effect of exogenous osmoprotectants on the restoration of phosphate solubilization activity.

Phosphates solubilization of D1 and D13 strains on NBRIP medium was significantly affected by the addition of NaCl. The addition of osmoprotectants (proline and glycine betaine at 1 mM) in the NBRIP medium failed to significantly reduce the inhibitory effect of the salt (Figure 4). The analysis of variance showed that proline and glycine betaine had no osmoprotection effect on the two strains (Table 7). The decrease in pH upon addition of proline or betaine was not significant. No correlation was observed between the pH of the medium and solubilizing capacity of phosphate in the presence of salt and osmoprotectants (Table 7).

DISCUSSION

The *Bacillus* strains were isolated from the rhizosphere of wheat. Several studies indicate that the *Bacillus* are dominant in salty areas and are abundant in the rhizosphere of wheat (Milus and Rothrock, 1993; Maplestone and Campbell, 1989). The *Bacillus* are among the most successful bacterial communities in solubilization of phosphate (Illmer and Schinner, 1995; Wani et al., 2007). The comparative study on the three culture media used for phosphate solubilization by *Bacillus* strains showed that PVK and NBRIP solid and liquid media are more efficient and had a similar solubilization capacity. Comparative studies using both NBRIP and PVK media have shown similar results on solid medium; but the NBRIP broth was three times more effective as compared to the liquid PVK. Therefore, according to Nautiyal et al. (2000), the liquid NBRIP is ranked best medium for the identification of most microorganisms solubilizing phosphate. LB medium proved to be less effective, this is due probably to its composition and richness in nutrients (Nautiyal, 1999). However, several media are used for the study of the phosphate solubilization and the choice of the medium depends on the isolated bacteria species (Banik and Dey, 1982).

The lack of correlation between the liquid and solid media (LB and PVK) indicated that there is a difference in the ability to solubilize phosphate. One isolate solubilizing phosphate in liquid medium could not produce a clear zone on solid medium. This suggests that the solubilization on solid medium is not a reliable technique as compared to liquid medium (Nautiyal, 1999). Solid media are less sensitive than liquid media in the detection of the solubilization capacity. Although, most strains of *Bacillus* showed no solubilization on solid media, they effectively solubilize phosphate in liquid medium. This can be explained by the low diffusion of the acids produced by bacteria in the solid medium during growth. The substrates in the solid medium are limited. The use of substrates from the solid support will vary from one isolate to another and depends on the enzyme system (Brock et *al*., 1994). By contrast, a significant relationship existed between phosphate solubilization on solid medium and liquid NBRIP. Strains forming a halo on NBRIP solid dissolve better in liquid medium (Edi-Premono et al., 1996; Kumar and Narula, 1999).

During the phosphate solubilization, bacteria acidify the medium with the production of organic acids and/or by the secretion of H^+. Therefore, inorganic phosphate can be released by the substitution of protons or complexing Ca^{2+} (Illmer and Schinner, 1992; Whitelaw et al, 1999; Whitelaw, 2000). The type of produced acid depends on the microorganisms. The decrease in pH obtained from these results is probably associated with the excretion of H^+ or the production of organic acids by *Bacillus* strains. The acidification of the medium is important in the solubilization of phosphate. The decrease in pH was observed in all

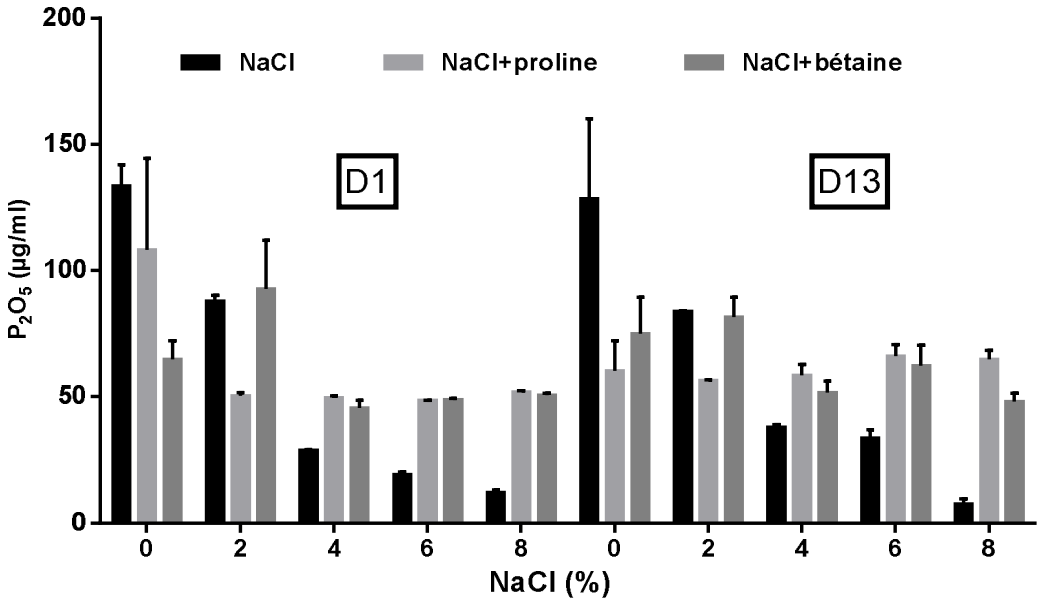

Figure 4. Phosphate solubilization by D1 and D13 in the presence of proline and glycine betaine under different concentrations of NaCl.

three liquid media, PVK, NBRIP and LB. The most significant decrease is obtained in the PVK and NBRIP. This was a significant correlation between pH and solubility in PVK and NBRIP media, and our results are similar to those of Kumar and Narula (1999) and Whitelaw (2000). The lack of correlation between the concentration of soluble phosphate and pH in LB suggests that phosphate solubilization is probably not only due to the excretion of H^+ and organic acid production (Altomare et al., 1999). It is probable that there is another mechanism of phosphate solubilization (Kim et al., 1997). In addition, the LB medium contains a high concentration of yeast extract which may act as a buffer (Nautiyal, 1999).

The halotolerance of *Bacillus* strains grown on NBRIP medium was not similar. Our study allowed us to observe for all strains a capacity of phosphate solubilization at low, and even up to 8% of NaCl. However, some strains have improved solubilization in the presence of salt. Similar results showed that the improvement in the solubilization of phosphate is made in the presence of 1% NaCl (Kim et al., 1997; Kang et al., 2002; Silini-Cherif et al., 2012). At 2.5% of salt, *Bacillus* sp. solubilized at a highest rate (Banerjee et al., 2010). In addition, the work of Srividya et al. (2009) show phosphate solubilization up to 2% NaCl in *Aspergillus niger* F7 and Rosado et al. (1998) attest that the solubilization may be made in the presence of 10% NaCl, but the activity declines after increasing the concentration of NaCl. This can be explained either thus: salt adversely affects growth and cell proliferation resulting in a loss of solubilization efficiency or chloride ions (Cl-) sequester or neutralize protons or acids produced in the media. Whilst the highest rates were recorded for strains D1 and D13 at 0 and 2% NaCl, the rates of solubilized phosphate were decreased when the concentration of salt increased. A similar study on a number of fifty-seven isolates, eighteen strains were active in the presence of 5% NaCl, while two isolates lose this capacity in the presence of NaCl (Johri et al., 1999). Diverse microorganisms are able to change phosphorus forms however their transforming ability may associate with the ecological conditions including soil characteristics and vegetation. It has been found that the performance of phosphorus-solubilizing microorganisms is affected by environmental conditions (Zhu et al., 2011). However, salt tolerance of *Bacillus* strains is a character to each strain. Indeed, environmental sampling stress (salinity, high pH) promotes the growth of bacteria showing better capacity of solubilization. It is agreed that the stress induced the activity of bacterial species (Banarjee et al., 2010; Choure et al., 2012).

Bacteria must constantly adapt their physiology to changes in physico-chemical factors of the environment. Adaptive mechanisms carried out include the accumulation of osmoprotectants. Most *Bacillus* species have the ability to accumulate proline (Whatmore et al., 1990) or glycine betaine (Kappes et al., 1996) under stressful conditions. The functions of compatibles solutes have been firmly established for *Bacillus* and the molecular details of the uptake and synthesis of these compounds have already been studied in quite some details (Bremer, 2002), but can the accumulation of these compatible solutes or their synthesis also play a role in improved ability of phosphates solubilization of these bacteria? The results of this study indicated that the solubilization capacity of the two strains D1 and D13 cannot be restored or enhanced by exogenous osmoprotectant molecules. This

Table 6. pH values in NBRIP medium added different concentrations of NaCl.

NaCl (%)	Strain									
	B8	B14	B18	Ba5	Ba7	Ba11	Ba12	D1	D6	D13
0	5.56±0.18^{bcBC}	6.23±0.35^{abA}	5.56±0.29^{bBC}	4.63±0.66^{bE}	5.17±0.09^{bCDE}	5.30±0.56^{bCD}	6.01±0.07^{aAB}	4.91±0.17^{bDE}	5.95±0.09^{aAB}	4.69±0.26^{bDE}
2	5.40±0.31^{cABC}	6.02±0.05^{abA}	5.90±0.16^{abA}	4.73±0.12^{bD}	5.15±0.06^{bCD}	5.27±0.61^{bBCD}	5.82±0.08^{aAB}	5.72±0.04^{aABC}	5.99±0.03^{aA}	4.72±0.73^{bD}
4	5.48±0.13^{cBC}	5.89±0.10^{bAB}	6.32±0.04^{aA}	4.78±0.03^{bD}	5.22±0.11^{abCD}	6.06±0.02^{aAB}	5.55±0.16^{aBC}	6.06±0.06^{aAB}	6.10±0.06^{aAB}	5.70±0.29^{aABC}
6	6.04±0.05^{abA}	6.14±0.07^{abA}	6.13±0.01^{aA}	5.61±0.06^{aAB}	5.14±0.08^{bB}	6.05±0.01^{aA}	5.77±0.18^{aAB}	6.00±0.05^{aA}	6.13±0.12^{aA}	5.64±0.34^{aAB}
8	6.26±0.11^{aAB}	6.41±0.01^{aA}	6.07±0.10^{abAB}	6.07±0.01^{aAB}	5.72±0.38^{aB}	5.99±0.05^{aAB}	5.95±0.51^{aAB}	5.97±0.08^{aAB}	6.04±0.2^{aAB}	5.80±0.2^{aAB}

Means of pH values ± the SD. Means sharing same letter do not differ significantly according to the Tukey test ($p>0.05$).

Table 7. Effect of glycine betaine and proline on phosphate solubilization and on pH value of NBRIP medium at different concentrations of NaCl by D1 and D13.

Source of variation	Df	P_2O_5	pH	Correlation
Salinity (S1)	4	6963.65783**	0.26752*	-0.2900ns
Treatment (T)	2	173.39806 ns	0.17689ns	
S1xT	8	2462.69646**	0.20862ns	
Strains (S)	1	59.95001ns	0.06241*	
S1 x S	4	362.51752 ns	0.01694ns	
T x S	2	23.02018ns	0.00841ns	
S1xTx S	8	259.76008 ns	0.03014ns	
Error	30	228.07387	0.01017	

*Significative at 5%. ns, non-significative at =5%.

is contrary to the nitrogen fixation of *Azospirillum brasilence* which is more sensitive towards salt stress but stimulated by an exogenous support of glycine betaine (Hartmann, 1988). The osmoprotection is dependent on the bacterial species (Yancey et al., 1982). This could be explained by the deficiency of these strains enzyme system required for the transport and accumulation of the osmoprotectants. In addition, bacteria in the presence of salt can accumulate osmolytes to achieve osmotic adjustment and ensure the stabilization of certain proteins active at the expense of other enzyme activities loss, due to their altered electrostatic properties (Arakawa and Timasheff, 1985). Proteomic approaches have shown that stress acclimation is accompanied by profound changes in cellular metabolism (glycolysis especially) (Barth et al., 2000; Marceau et al., 2002; Prasad et al., 2003).

In this study, the strains (D1and D13) isolated from wheat rhizosphere are efficient phosphate solubilizers and are able to perform significantly under unfavorable environmental conditions.

Furthermore, they evidently also have various PGPR activities combined with the phosphate dissolving. It is concluded from the present study that the isolates can be used as bioinoculants which will increase the available phosphorus in soil, help to minimize the phosphate fertilizer application, reduce environmental pollution and promote sustainable agriculture. However, the osmoprotection for improved ability of solubilizing phosphates of *Bacillus* under high salt concentra-

tions is not established. Further studies are needed to explain this mechanism.

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