Full Length Research Paper

Genetic diversity and phosphate solubilizing ability of *Triticum aestivum* rhizobacteria isolated from Meknes region, Morocco

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Received 7 June, 2013; Accepted 22 April, 2014

The objective of this work was to isolate, screen and evaluate in vitro, the phosphate solubilization activity of rhizobacteria isolated from *Triticum aestivum* rhizosphere soil in the Meknes region of Morocco. Five best isolates from 240 ones were selected based on the solubilization of insoluble phosphates (Ca₃(PO₄)₂) in both agar plate and broth assays using National Botanical Research Institute's phosphate (NBRIP) medium. The bacterial isolates were identified based on their phenotypic and 16S rRNA genes sequencing data as *Variovorax paradoxus* (BT1), *Pseudomonas reinekei* (BT4), *Pseudomonas libanensis* (BT10), *Pseudomonas lurida* (BT11) and *Pantoea agglomerans* (BT12). P solubilization index of these isolates ranged from 2.88 to 3.48, amount of phosphate solubilized ranged from 24.23 to 56.95 mg P L⁻¹ and drop in pH of the medium ranged from 7 to 3.2. All the isolated PSB were efficient phosphate solubilizers and can be used as bioinoculants to increase the available phosphorus in the soil for wheat plant growth.

Key words: *Triticum aestivum*, rhizobacteria, rhizosphere, phosphate, solubilizing capacity.

INTRODUCTION

Phosphorus is one of the major plant nutrients limiting plant growth. Most agricultural soils contain large reserves of P, a considerable part of which has accumulated as a consequence of regular applications of chemical fertilizers. A deficiency in soluble P for many agricultural soils is one of the major factors hampering crop production worldwide (Arcand and Schneider, 2006). It is now becoming urgent to reduce the environmental impact of agriculture and, for example by replacing the expensive soluble chemical P fertilizers by novel, cheaper, more ecological but nevertheless efficient P fertilizers (Macias et al., 2003). It is well known that a considerable number of phosphate solubilizing bacteria (PSB) have the ability to solubilize insoluble mineral P by producing various organic acids, siderophores, mineral acids, protons, humic substances, CO₂ and H₂S, and release soluble P (Illmer and Schinner, 1995; Ivanova et al., 2006; Song et al., 2008). Phosphate solubilizing bacteria (PSB) are being used as biofertilizer since 1950s (Kudashev, 1956; Krasilnikov, 1957). The maintenance

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of high level of soil phosphorus has been a major challenge to agricultural scientists, ecologists and farm managers because in most of the soils, phosphate is present in unavailable form due to complex formation with Ca\(^{2+}\), Al\(^{3+}\), Fe\(^{3+}\) or Mn\(^{2+}\) depending on soil pH and organic matter. The main problem of phosphorus in soil is its rapid fixation and the efficiency of P solubilization rarely exceeding 10–20%. The fixed forms of P in acidic soils are aluminium and iron phosphates while in neutral to alkaline soils as calcium phosphates (Kuhad et al., 2011). Release of P by PSB from insoluble and fixed / adsorbed forms is an import aspect regarding P availability in soils. There are strong evidences that soil bacteria are capable of transforming soil P to the forms available to plant. Microbial biomass assimilates soluble P, and prevents it from adsorption or fixation (Khan and Joergeson, 2009). Accordingly, it is reported that the phosphate-solubilizing bacteria (PSB) when applied with PGPR could reduce P fertilizer application by 50% without any significant reduction in crop yields (Jilani et al., 2007; Yazdani et al., 2009). The aim of this study was to isolate and identify efficient phosphate solubilizing bacteria (PSB) from Triticum aestivum rhizosphere soils of different regions of Meknes.

**MATERIALS AND METHODS**

**Soil samples and isolation of PSB**

During the fall of 2012, in Meknes area (33°53'42" North, 5°33'17" West), the soils were classified as alkaline, vertisols, according to the Provincial Directorate of Agriculture of Meknes (http://www.dramt.e-makane.net/site/dpameknes.htm). Soil samples were collected from rhizosphere of wheat variety Marchouch (National Institute of Agronomic Research, Morocco, selected in 1984). The samples were stored at 4°C in sterile containers. Soil samples of each soil were mixed thoroughly. Phosphate solubilizing bacteria were isolated from soil samples by serial dilution using spread plating on NBRIP medium (Nautiyal, 1999) supplemented with tricalcium phosphate as insoluble inorganic phosphate source, and incubated at 27°C for 24-48 h. Colonies showing clear zone of P-solubilization were counted as PSB (Gyaneshwar et al., 1999). Once purified, each isolate was stored as a glycerol 40% stock at -80°C.

**Qualitative evaluation of phosphate solubilization in agar assay**

All bacterial strains were tested by an agar assay using National Botanical Research Institute’s phosphate (NBRIP) medium supplemented with tricalcium phosphate. Each isolate was assayed by spotting 10 μl of cultures on the media plates. The halo and colony diameters were measured after 10, 20 and 30 days of incubation of the plates at 27°C. The ability of the bacteria to solubilize insoluble phosphate was described by the solubilization index (SI) = the ratio of the total diameter (colony + halo zone) to the colony diameter (Edi-Premono et al., 1996).

**Quantitative estimation of phosphate solubilization in broth assay**

The quantitative bioassay was carried out using Erlenmeyer flasks (250ml) containing 50 ml of NBRIP broth medium supplemented with (Ca\(_3\) (PO\(_4\))\(_2\)) and inoculated by 200 μL of bacteria (5 × 10\(^8\) CFU ml\(^{-1}\)). Autoclaved uninoculated NBRIP medium served as control. The flasks were incubated on rotary shaker (180 rpm) at 30°C. After 168, 336 and 504 h of incubation, the growth medium was centrifuged at 10,000 rpm for 20 min. Supernatant was decanted and autoclaved at 121°C for 20 min. Autoclaved samples were then filtered through Whatman paper no. 42 followed by 0.2 μm millipore membrane and were used for the determination of the P and the soluble P released into the solution. P was measured with molybdenum blue method as described by Murphy and Riley (1962). The pH of the supernatant was measured in each case by pH meter (Metrohm 620 pH meter, swiss made). All the data were an average of three replicates.

**Phenotypic characterization**

Morphological and biochemical identification of PSB were characterized for colony morphology, Gram staining and biochemical analysis (Holt et al., 1994). Isolates were also tested for catalase (Graham and Parker, 1964) and oxidase (Kovacs, 1956). The tolerance of strains in extrinsic and intrinsic environmental was tested by the ability of the PSB isolates to grow on media at several values of stresses.

**Salt tolerance test**

PSB were examined for their tolerance of salinity on yeast extract mannitol (YEM) agar plate (Vincent, 1970), medium supplemented with 0.5, 1, 1.5 and 2 gL\(^{-1}\)NaCl (Ben Romdhane et al., 2006).

**Temperature tolerance**

Temperature tolerance was tested by incubating the inoculated plates with PSB at 4, 20, 30, 40 and 50°C for 48 h (Hung et al., 2005).

**pH tolerance test**

The ability of the PSB isolates to grow on media at several pH values was tested by streaking cultures on the YMA plates at different pH values, the pH values were adjusted to 4.8, 5.8, 6.8 and 8.8 with either NaOH or HCl (Kucuk et al., 2006). Isolates were incubated at 27°C for 48 h.

**Intrinsic antibiotic resistance**

The bacteria were spread out on the YMA medium containing antibiotics discs of tetracycline (30 μg), streptomycin (25 μg), kanamycin (30 μg) and ampicillin (10 μg). The control to YMA medium without antibiotics discs. PSB were incubated at 27°C for 24 h.

**Genotypic identification**

The identification of PSB was done on the basis of 16S rRNA gene sequencing. The genomic DNA of PSB isolates was extracted by the kit of the platform “GenElute Bacterial Genomic DNA kit”
Figure 1. Solubilization index (SI) of PSB isolated from the rhizosphere of *Triticum aestivum* after 10, 20 and 30 days of incubation at 27°C.

Statistical analysis

Simple correlation was run to determine correlation coefficients (r) by the method of Ordinary Least Squares (OLS). The result means were depicted diagrammatically using Microsoft Excel version 2010.

RESULTS

Isolation and qualitative evaluation of PSB in agar assay

A total of 240 bacterial strains were isolated out of which 52 strains (21.6%) showed clear zones but after repeated plating on NBRIP solid medium only five strains (2.08% of the total) preserved P-solubilization function. Fluctuations in solubilization index based on colony diameter and halozone for each PSB were observed during the thirty days observation period as presented in Figure 1. Solubilization index showed that among PSB, BT12 was most efficient phosphate solubilizer on NBRIP agar plate with SI = 3.48. BT12 followed by BT11 (3.25) and BT10 (3.18). Moderate solubilization index was observed in BT1 (2.88) and BT4 (2.90) isolated strains (Figure 1).

Quantification of P-solubilizing activity by PSB in broth assay

The PSB strains forming clear zones in NBRIP agar medium were able to release P from tricalcium phosphate in NBRIP broth medium. P release was observed from first week onwards and gradually increased until after 21 days (Figure 2). The final pH of the culture filtrate ranged from 3.7 to 3.1 starting at initial pH of 6.8 - 7.0 after 504 h, indicating acidic nature (Figure 3). PSB strains BT10 (56.95 mg P L⁻¹), BT12 (54.55 mg P L⁻¹) and BT1 (47.5 mg P L⁻¹) released high amount of P on 21 day, while BT4 (37.43 mg P L⁻¹) and BT11 (24.23 mg P L⁻¹) released low amount of P. Maximum P release was seen in BT10 and BT12 strains (56.95 and 54.55 mg P L⁻¹) as compared to all the other PSB strains (24.23 to 47.5 mg P L⁻¹) (Figure 2). The relationship between final culture pH/solubilization index and concentrations of solubilized P in the culture released by PSB was analyzed using the method of ordinary least squares (OLS). From the
**Figure 2.** Change in P-solubilized in NBRIP liquid medium by the PSB strains isolated from the rhizosphere of *Triticum aestivum* after 7, 14 and 21 days of incubation.

**Figure 3.** Change in pH values in NBRIP liquid medium by five selected PSB after 7, 14 and 21 days of incubation.
Figure 4. Correlation analysis between final culture pH and tricalcium phosphate solubilizing ability of phosphate solubilizing bacteria.

statistical analysis, it is clear that no significant positive correlation between the solubilization index (SI) and concentrations of solubilized P was found ($r = 0.188$) (Figure 5). For example, the isolate BT11 had a good SI (3.25) on NBRIP agar medium, but its solubilized P released into the liquid culture was 24.23 mg L$^{-1}$, contrarily, the isolate BT1 had a small SI (2.88) but released the highest amount of solubilized P of 47.50 mg L$^{-1}$. However, significant negative correlation between final culture pH and concentrations of solubilized P was found ($r = -0.99$) (Figure 4). If the final culture pH decreased concentrations of solubilized P increased.

Identification of PSB isolates
PSB selected in the previous steps were all Gram-negative, some differences were observed in the phenotypic study of colonies (Table 1). Others biochemical and physiological characteristics were also studied as shown in Table 1. Nucleotide sequencing of 16S rRNA genes amplified by PCR and sequence comparison with the data available in GenBank using the BLAST (Altschul et al., 1997) algorithm has enabled us to identify the isolates (Table 2). The tolerance of strains in extrinsic and intrinsic environmental stress is shown in Table 3.

DISCUSSION
This study provides the first clear characterization of phosphate solubilizing bacteria in Meknes. Phenotypic and genotypic methods were used to evaluate these PSB isolates. Studies on agar plates revealed that solubilization index (SI) increased with increase in the total diameter (colony + halo zone), these results are in accordance with that of Baig et al. (2010) and Yang et al. (2012). Most of PSB isolated strains in this study lost their ability to form halozone on agar medium on repeated subculturing. This result is in accordance with that of Kucey et al., (1989); Illmer and Schinner (1995) and Rodriguez and Fraga (1999). From the observed results, it is clear that P released by BT12 strain was higher as compared to other PSB strains. All the PSB strains isolated were efficient in solubilizing tricalcium phosphate. From the statistical analysis by method of ordinary least squares (OLS), no significant positive correlation between the solubilization index (SI) and concentrations of solubilized P was found ($r = 0.188$) (Figure 5). These results are in accordance with those obtained by Yang et al. (2012), which reported that no significant positive correlation was found between halo diameter and solubilized P ($r = 0.03$). However others reports show no correspondence between solubilization ability in plate and in liquid culture (Baig et al., 2010). It has been reported that solubilization halo measurements is not always the best way to discriminate those isolates that would eventually have the greatest solubilization potential. Thereby, significant negative correlation between final culture pH and concentrations of solubilized P was found ($r = -0.99$) in our study (Figure 4). On the contrary, Yang et al. (2012) reported no significant negative correlation
Table 1. Phenotypic and biochemical characteristics of the PSB isolates.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Colony morphology</th>
<th>Oxidase</th>
<th>Soluble P</th>
<th>Lactose</th>
<th>Mannitol</th>
<th>Mannose</th>
<th>Arabinose</th>
<th>Maltose</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT1</td>
<td>C, E, R, Y, Cy</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BT4</td>
<td>C, I, R, W, Cy</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>BT10</td>
<td>C, E, R, OW, Cy</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>BT11</td>
<td>C, I, R, W, Cy</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BT12</td>
<td>C, E, R, OW, V</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

+, Tested positive/utilized as substrate; -, tested negative/non-utilized as substrate. *All PSB isolates were shown to be Gram-negative, reacted positive to catalase, glucose utilization, and negative for H2S production and citrate (Simmons) utilization. †Colony morphology in YMA medium: C: circular; E/I: entire/irregular edge; R/Cr: raised/crateriform; Y/W/O: yellow/white/off-white; V/Cy: viscous/creamy.

Table 2. Identification of PSB isolates by 16S rDNA sequencing.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Length of 16S rDNA gene sequenced (bp)</th>
<th>GenBank accession number</th>
<th>Specie (strain)</th>
<th>Accession number</th>
<th>Gene identity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BT1</td>
<td>1524</td>
<td>CP001635.1</td>
<td>Variovorax paradoxus S110</td>
<td>NR_074654</td>
<td>94</td>
</tr>
<tr>
<td>BT4</td>
<td>1492</td>
<td>AM293565.1</td>
<td>Pseudomonas reinekei MT1</td>
<td>NR_042541.1</td>
<td>99</td>
</tr>
<tr>
<td>BT10</td>
<td>760</td>
<td>AF057645.1</td>
<td>Pseudomonas libanensis CIP 105460</td>
<td>NR_024901.1</td>
<td>97</td>
</tr>
<tr>
<td>BT11</td>
<td>514</td>
<td>AJ581999.1</td>
<td>Pseudomonas lurida DSM 15835T</td>
<td>NR_042199.1</td>
<td>98</td>
</tr>
<tr>
<td>BT12</td>
<td>1473</td>
<td>AJ233423.1</td>
<td>Pantoea agglomerans DSM 3493</td>
<td>NR_041978.1</td>
<td>96</td>
</tr>
</tbody>
</table>

between the final culture pH and tricalcium phosphate solubilization activity ($r = -0.15$). Others studies support the idea that there is a strong negative correlation between the concentration of soluble P and the final pH value of the PSB culture (Hwangbo et al., 2003; Perez et al., 2007); these studies are in accordance with our results. The values of P in solution obtained for our isolates are in accordance with those of other studies which
survival of microorganisms in wheat soil. The bacteria (Chaiharn and Lumyong, 2009; Thakuria et al., 2010; Son et al., 2006). The data of this study showed that the isolates studied are globally tolerant of alkalinity and neutrality. They were able to grow at an initial pH of 8.8 and 4.8 (Table 3). The isolates which can survive on a wide pH range are candidates for further strain improvement to highly acidic or alkaline conditions. Extremes of pH can be a major factor limiting microorganisms in soil (Rodriguez and Fraga, 1999). The ability to adapt to temperature stress may be important in the survival of the microorganisms during drought. All the isolates in this study could tolerate temperatures ranging between 20 and 30°C, but some of them were also able to grow at 4 (BT4 and BT12) and at 50°C (BT4) (Table 3). The results of this test are in concordance with previous studies (Chaiharn and Lumyong, 2009; Thakuria et al., 2004). Acid tolerance is important in the growth and survival of microorganisms in wheat soil. The bacteria tested in this study tolerated 2 g L⁻¹ salt concentration (Table 3). These results are in accordance with those obtained by Chaiharn and Lumyong (2009). Regarding the intrinsic resistance, all PSB isolated strains in this study showed a good resistance against different antibiotics (Table 3). In general, the phenotypic study showed physiological and biochemical differences between isolates. Indeed, the studied strains showed a variable resistance against stress factors, namely, temperature, pH, salinity and resistance to antibiotics, which allowed the selection of good candidates as biofertilizers for wheat crops. Five isolates with the greatest solubilization potential were selected to determine their 16S rDNA sequences. When those sequences were compared with the GenBank (NCBI) database, three out of the five isolates belong to the genus Pseudomonas (Table 2). The genus Pseudomonas has been extensively studied as a plant growth promoting rhizobacteria (PGPR) (Walsh et al., 2001), and it is known for having members able to synthesize phytohormones, act as biocontrol agents, and solubilize phosphate (Jha et al., 2009). Nevertheless, Pseudomonas appears as a very promising group for its proven suitability as a potential inoculant. Most of the PSB obtained in our study were affiliated with groups previously identified in other soils. However, novel PSB, Variovorax paradoxus S110, was identified for the first time in this investigation. Application of bacterial inoculants as biofertilizers has been reported to result in improved plant growth and increased yield (Bashan and Holguin, 1998; Vessey, 2003). The results of this study make these isolates attractive as phosphate solubilizers. It requires further in depth studies based on the plant growth promoting activities of these isolates under pot culture of wheat as well as field conditions in Meknes area before recommended as biofertilizers for wheat crops.

### Table 3. Phenotypic characteristics of the strains under environmental stresses.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Growth pH</th>
<th>Growth salinity (g/l)</th>
<th>Growth temperature (°C)</th>
<th>Antibiotic resistance a</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>4.8</td>
<td>5.8</td>
<td>6.8</td>
<td>8.8</td>
</tr>
<tr>
<td>BT1</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BT4</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>BT10</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
<tr>
<td>BT11</td>
<td>+</td>
<td>±</td>
<td>±</td>
<td>±</td>
</tr>
<tr>
<td>BT12</td>
<td>+</td>
<td>+</td>
<td>±</td>
<td>-</td>
</tr>
</tbody>
</table>

+ : Growth, ±: weak growth, -: no growth. aAMP: Ampicillin (10 μg), TE: tetracycline (30 μg), S: Streptomycin (25 μg), K: kanamycin (30 μg).

Conclusions

In our study, the characterization and screening of rhizobacteria from wheat rhizosphere soil helped in the selection of various phosphate-solubilizing bacteria, have the potential to increase the available phosphate in the soil, which in turn will help to minimize the P-fertilizer application, reduce environmental pollution, promote sustainable agriculture and increase yields of wheat in Meknes area and other similar areas. The studied phosphate solubilizing bacteria tolerate high concentrations of NaCl. Hence, these isolates may be the candidates for use in the saline soil. The selected isolates were tolerant to temperatures ranging from 4 and 40°C, and to extreme pH from 4.8 to 8.8. The antibiotic resistance of the isolated strains showed a high level of resistance against streptomycin, kanamycin, ampicillin and tetracycline. The advantage of using native PSB isolated from T. aestivum rhizosphere soil in the Meknes region of Morocco is the ability to more easily adapt and go through succession when inoculated into soil. Further investigation is required on the bacterial strains to exploit these strains as biofertilizers for wheat field soils to enhance the growth of wheat crops.

Conflict of Interests

The author(s) have not declared any conflict of interests.

REFERENCES


