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Phosphate-Solubilizing Bacteria in the Rhizosphere of Some Cultivated Legumes from Meknes Region, Morocco

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Authors' contributions

This work was carried out in collaboration between all authors. Author AR designed the study, performed the experiment, accomplished the statistical analysis, and wrote the protocol. Authors LN and JI supervised the study and managed the literature searches. All authors read and approved the manuscript.

Original Research Article

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ABSTRACT

Aims: Through the National Botanical Research Institute's phosphate growth medium (NBRIP) and 16S rDNA sequence analysis were used to isolate and identify the bacterial groups that actively solubilized phosphates *in vitro* from rhizosphere soil for three cultvited leguminous in agricultural soils from Meknes region.
Study Design: Rhizosphere soil samples for three cultivated legumes in different sites from Meknes region were collected for the study.
Place and Duration of Study: Department of biology (Soil & Environment Microbiology Unit) Faculty of Sciences, Moulay Ismail University, Meknes, Morocco; between January and July 2014.
Methodology: Out of several hundred colonies that grew on NBRIP medium eight best isolates were selected based on the solubilization of insoluble phosphates in solid medium with solubilizing index (SI) and Phosphate concentration solubilized in liquid medium; The bacterial isolates were identified based on their phenotypic and 16S rDNA genes sequencing.
Results: P solubilization index of these isolates ranged from 2.51 to 6. Drop in pH of the medium ranged from 6.8 to 3.2 with the continuous growth of these isolates for seven days. P-solubilized ranged from 50.95 to 113.11 mg L^{-1}. They were clustered under the

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genera *Enterobacter*, *Pantoea*, *Rhizobium*, *Klebsiella*, *Rahnella*, *Bacillus* and *Burkholderia.*
Conclusion: This research extends the knowledge on Phosphate solubilizing bacteria in the rhizosphere of some cultivated legumes from Meknes region and development of environmentally friendly bio-Phosphate fertilizers.

Keywords: Phosphate solubilization; 16S rDNA; rhizosphere; bio-phosphate fertilizers.

1. INTRODUCTION

Many agricultural soils are generally low in available phosphorus and some of them sorb large amounts of applied phosphate fertilizer [1]. Consequently, efforts have been made to study the role of soil microorganisms in the solubilization of inorganic phosphates to improve phosphorus availability for plant growth [2]. Phosphorus is an essential nutrient for plant growth and development, and besides to nitrogen it is one of the most important elements in crop production [3]. Because the availability of phosphorus to plants is restricted by various factors, it seems reasonable to study microorganisms that are able to solubilize phosphate from soil and promote its uptake by plants [4-6]. During the last 10 years knowledge on phosphate solubilizing microorganisms increased significantly [7-9]. Phosphate in soil mostly exists in insoluble (bound) forms and the concentration of soluble phosphate in soil solution is very low (400–1,200 mg kg^{-1} of soil) [9]. Plants are able to utilize only a small proportion of phosphatic fertilizers that are applied, as much is rapidly converted into insoluble complexes in soil [10,11]. Several strains of bacterial and fungal species have been described and investigated in detail for their phosphate-solubilizing capabilities [12,13]. Application of bacterial inoculants as biofertilizers has been reported to result in improved plant growth and increased yield [14,15].

The maintenance 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^{2+} or Mn^{2+} depending on soil pH and organic matter [16]. 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 [16]. The aim of this study was to isolate phosphobacteria from rhizospheric soil of three cultivated legumes from Meknes region, fababean (*Viciafaba*), chickpea (*Cicer arietinum*) and green peas (*Pisum sativum*), to identify them using molecular tools and to evaluate their phosphate solubilization activity, sidérophores and indoleacetic acid production and N_2 fixation activity.

2. MATERIALS AND METHODS

2.1 Soil Samples and Isolation of PSB

From the rhizosphere of three cultivated legumes, fababean (*Viciafaba*), chickpea (*Cicer arietinum*) and green peas (*Pisum sativum*), in different sites from Meknes region ((33º53'42" North, 5º33'17" West)), soil samples were collected. The samples have been stored at 4ºC. 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 [17] supplemented with tricalcium phosphate as insoluble inorganic phosphate source, and

incubated at 27ºC for 72-120 h. Colonies showing clear zone of P-solubilization were counted as PSB [18]. Different types of single, well separated colonies, from each sample site, which grew on plates showing clear zones were picked and restreaked on to fresh NBRIP solid medium. This procedure was repeated until pure culture with high P solubilization was obtained. Once purified, each isolate was stored as a glycerol 40% stock at -80ºC.

2.2 Determination of Phosphate Solubilization Index (SI)

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 27ºC. 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 [19].

2.3 Quantitative Estimation of Phosphate Solubilization

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 was served as a control. The flasks were incubated on rotary shaker (180 rpm) at 30ºC. After incubation for seven days, daily the growth medium was centrifuged at 10,000 rpm for 20 min. Supernatant was decanted and autoclaved at 121ºC 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 pH and the soluble P released into the solution. P was measured with molybdenum blue method as described by Murphy and Riley (1962) [20]. 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.

2.4 Determination of siderophore production and N_2fixation activity

Siderophore production was determined as described by Schwyn and Neilands [21] using blue indicator dye, cromeazurol S. Bacterial isolates exhibiting an orange halo after 5 days of incubation at 28 ± 2ºC were considered positive for the production of siderophores. N_2 fixing activity was tested by using the Nfb semi-solid medium [22]. The formation of a characteristic veil-like pellicle near the surface of the semi-solid Nfb media indicated N_2 fixing activity.

2.5 IndoleaceticAcid (IAA) Production

Indoleacetic acid (IAA) production was detected by the method described by Bric et al. (1991) [23]. L-tryptophan (5 mM) agar plates containing nitrocellulose discs were spot inoculated with bacterial cultures (10 µl of approximately 10^8 CFU ml^{-1}) and incubated at 28ºC for 72 h. Nitrocellulose discs were transferred to test tubes and impregnated with Salkowsky reagent [24]. Appearance of pink colour after 30 min to 3 h of incubation indicated IAA production.

2.6 Morphological and Biochemical Identification of PSB

Morphological and biochemical identification of PSB were characterized for colony morphology, Gram staining and biochemical analysis [25]. Isolates were also tested for catalase [26] and oxidase [27].

2.7 Genotypic Identification

The identification of PSB was done on the basis of 16S rDNA gene sequencing. The genomic DNA of PSB isolates was extracted by the Kit of the platform « GenElute Bacterial Genomic DNA kit » (Sigma-Aldrich Corp., St. Louis, MO) according to the manufacturer's protocol. The primers fD1 (5'AGAGTTTGATCCTGGCTCAG-3') and rP2 (3'ACGGCTACCTTGTTACGACTT-5') were used for amplification of 16S rDNA gene [28]. The total PCR reaction mixture was 25.0 µl in each tube comprising 2 µl dNTPs (10 mM), 0.125 µl each primer (100 mM), 2.5µl PCR buffer (10x), 0.2 Taq DNA polymerase (5U/µl), and 5µl genomic DNA (30 ng/µl). The thermocycling conditions involved an initial denaturation at 96ºC for 4 min, followed by 35 cycles of 96ºC for 10 Sec, 52ºC for 40 Sec, and 72ºC for 2 min and final extension at 72ºC for 4 min. Successful amplification of a 1500 bp DNA fragment was confirmed by running 5µl of the PCR product on a 1% agarose gel and sequenced by ABI 3130 XL (Applied Biosystems, CA, USA). Sequence data were aligned and compared with available standard sequences of bacterial lineage in the National Center for Biotechnology Information GenBank (http://www.ncbi.nlm.nih.gov/) using BLAST.

3. RESULTS AND DISCUSSION

Since the knowledge on the diversity of phosphate solubilizing bacteria (PSB) in Morocco soils is lagging, an attempt to isolate and identify PSB through biochemical and molecular methods was made. PSB are important components of soil and directly or indirectly influence the soil's health through their useful activities. It is known that rhizosphere microorganisms mediate many soil processes such as decomposition, nutrient mineralization and nitrogen fixation [29]. Solublization index based on colony diameter and holozone for each PSB isolate is presented in Table 1. Results showed that among PSB, P4 was most efficient phosphate solubilizer on NBRIP plates with SI = 6. In all of the cases it gradually increased. Also these isolates have shown different abilities to solubilize $Ca_3(PO_4)_2$ in liquid medium with concentrations of phosphorus (P) element dissolved ranged between 50.95 and 113.11 mg L^{-1}, PE25 was a best solubilizing strain in liquid medium. These results are in accordance to Chabot et al. (1993) [30], Nahas (1996) [2] and Leandro et al. [31].

All phosphate solubilizing bacteria in this study lowered the pH of NBRIP liquid medium as compared to uninoculated sterile medium control incubated for seven days under conditions as inoculated. Drop in pH by PSB ranged from 4.1 to 3.2 at the end of incubation period (Fig. 1). Similar results were observed by Hwangbo et al. [32] and Pérez et al. [33].The analytical method to determine the solubilization of calcium phosphate used in the present study showed the relation between pH and amount of solubilized P. The decreased in pH of the culture medium is probably caused by production of organic acids by the bacteria [31]. These isolates presumably identified as PSB further characterized by a series of biochemical reactions, were Gram- negative except the strain PC16, with positive for catalase activity and negative for oxidase activity except two strains T4 and P16 which have been a positive for oxidase activity, All strains have been negative for H_2S production, gelatin, starch and lipid hydrolysis (Table 2).

Table 1. Siderophore, IAA production, N2 fixation activity and Solubilization index of the PSB isolates

PSB isolates	N_2 fixation activity	IAA production	siderophore production	solubilization index (SI)		
				10 days	20 days	30 days
P4	-	+	+	4,89	5,63	6,00
P16	+	+	+	0,63	1,32	2,91
P27	-	+	+	1,17	1,63	2,51
T4	+	+	-	2,67	3,76	3,99
T13	+	+	-	0,94	2,43	3,30
T15	-	+	+	5,39	5,40	5,78
PE12	-	+	+	4,38	5,85	5,94
PE25	-	+	+	4,06	5,35	5,84

Table 2. Morphological and Biochemical characteristics of the isolated strains

Biochemical reactions	PSB isolates							
	P4	P16	P27	T4	T13	T15	PE12	PE25
Colony morphologya	E, OW, V	E, W, Cy	E, W, Cy	E, OW, Cy	E, OW, Cy	I, W, Cy	E, Y, Cy	E, Y, Cy
Gram staining	-	-	+	-	-	-	-	-
Motility	-	+	+	-	+	+	+	+
Catalase	+	+	+	+	+	+	+	+
Oxidase	+	-	+	-	-	-	-	-
Indole production	+	-	-	-	-	-	-	-
Methyl red	+	-	-	+	-	+	-	-
Voges-Proskauer	+	-	-	+	+	+	+	+
Citrate (Simmons)	-	-	-	+	+	+	+	+
Carbon source utilization								
Sucrose	+	+	+	+	+	+	+	+
Fructose	+	+	+	+	+	+	+	+
Glucose	+	-	+	+	+	+	+	+
Glycerol	+	+	+	+	+	+	+	+
Maltose	+	+	-	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+
Inositol	-	+	+	-	+	+	+	+
Dulicitol	+	+	+	+	+	+	-	-

Lactose	+	-	+	+	+	+	-	-
Melibiose	+	+	+	+	+	+	+	-
D-Raffinose	+	+	+	+	+	+	+	+
Sorbitol	+	+	+	+	+	+	+	-
H2S production	+	-	-	-	-	-	-	-
Gelatin hydrolysis	-	+	-	-	-	-	-	-
Starch hydrolysis	-	-	-	-	-	-	-	-
Lipid hydrolysis	-	-	-	-	-	-	-	-

+, tested positive/utilized as substrate; -, tested negative/not utilized as substrate. aColony morphology in YMA medium: E/I: entire/irregular edge; Y/W/OW: yellow/white/off-white; V/Cy: viscous/creamy

Table 3. Identification of PSB isolates by 16S rDNA sequencing

Isolate	Length of 16S rDNA gene sequenced (bp)	GenBank accession no.	Most closely related organism	
			Species (strain)	% Gene identity
P4	1503	U29386	*Rhizobium leguminosarum* USDA 2370	99,58%
PE12	1559	EF688012	*Pantoea vagans* LMG 24199	99,13%
P16	1507	U96939	*Burkholderia graminis* C4D1M	99,15%
PE25	1562	EU216735	*Pantoea brenneri* LMG 5343	90,99%
P27	1558	AF234854	*Bacillus safensis*FO-036b	99,92%
T4	2037	AJ233426	*Rahnella aquatilis* CIP 78.65	98,44%
T13	1524	AJ783916	*Klebsiella variicola* F2R9	98,53%
T15	1567	EU629164	*Enterobacter cowanii* CIP 107300	97,37%

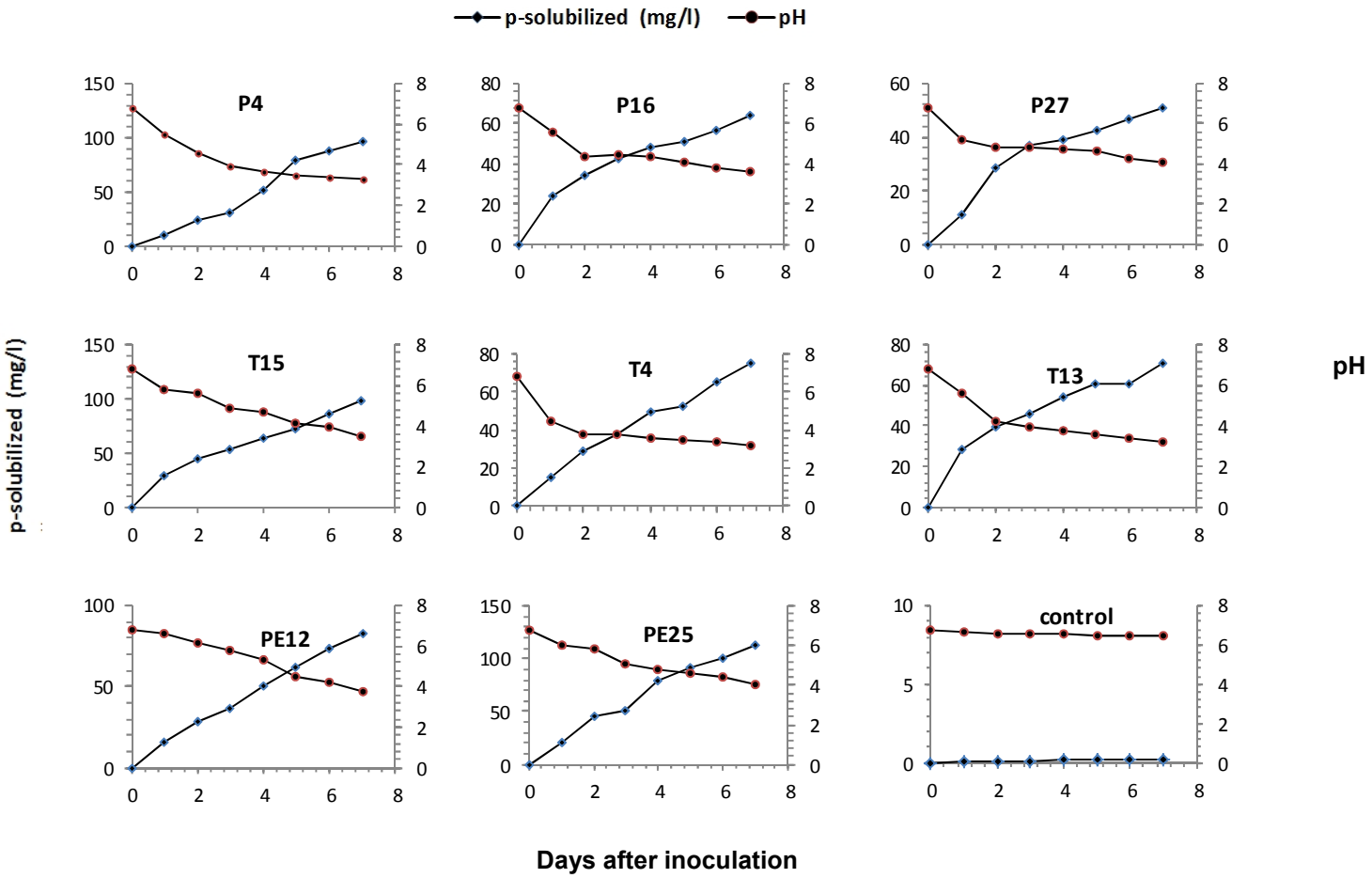

Fig. 1. Changes the Concentration of P element dissolved and the pH value of the culture medium with time of inoculation in National Botanical Research Institute's phophate (NBRIP) medium

PSB isolated strains had potential for other beneficial characteristics and positive effects for the leguminous crops. The capacity to synthesize IAA is widespread among soil and plant-associated bacteria. The indigenous PSB strains isolated from cultivated legumes were able to produce IAA, which are known to have prominent effects on plant growth and development [33]. Other researchers found similar results that isolated strains have potential for IAA production and N_2 fixing ability [34]. All PSB isolated strains were also able to produce siderophores except two strains (T4 and T13). Thereby, only tree isolated strains (P16, T4 and T13) grow in nitrogen free media showed the potentiality of nitrogen fixation (Table 1). PSB can have a direct consequence on plant growth other than the mechanism of phosphate solubilization like, production of phytohormones (IAA), biological N_2 fixation, enhance the availability of other trace elements, increased iron nutrition through iron-chelating siderophores and volatile compounds that affects the plant signaling pathways [35,36].

16S rDNA sequence analysis placed most of the isolates to the family Enterobacteriaceae from various soils, and their P solubilizing activities has also been reported earlier [37-40].The similarity among the sequences studied through GenBank access was between 98,44 and 99,92 % (Table 3 above).

Several species of phosphobacteria isolated from rhizospheric soil known to support plant growth by synthesizing auxins, mainly indole-3-acetic acid, solubilizing insoluble phosphates, producing siderophores or fixing atmospheric nitrogen [41-43]. 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 as well as field conditions before they are recommended as biofertilizers.

4. CONCLUSION

It is concluded from the present study that PSB showed variation in their biochemical charateristics. Present study also showed that *Pantoea brenneri* (PE25), *Enterobacter cowanii* (T15) and *Rhizobium leguminosarum* (P4) are the most efficient strains on the basis of their P solubilizing activity, nitrogen fixation, produce IAA and siderophore. Further research should be continued with such efficient PSB isolates. These may be used for inoculum production and their inoculation effect on the plant growth be studied *in vivo*.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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