British Microbiology Research Journal
6(5): 247-254, 2015, Article no.BMRJ.2015.077
ISSN: 2231-0886

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Isolation and Characterization of Phosphate Solubilizing Bacteria from the Rhizosphere of Faba Bean (*Vicia faba* L.) in 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 statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors LN and JI managed the analyses of the study. Author LN managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/BMRJ/2015/14379
Editor(s):
(1) Lingan Rajendran, Tamil Nadu Agricultural University, India.
Reviewers:
(1) Anonymous, Nigeria.
(2) G. Rama Krishnaiah, Biotechnology, IES Gate Academy, Chennai, India.
(3) Anonymous, Venezuela.
Complete Peer review History: http://www.sciencedomain.org/review-history.php?iid=831&id=8&aid=7911

Original Research Article

Received 28th December 2014
Accepted 13th January 2015
Published 28th January 2015

ABSTRACT

Aims: The objective of this research was to isolate phosphate solubilizing bacteria (PSB) from faba bean rhizosphere in Meknes region and evaluate *in vitro* their potential for P solubilization as well as their response to the effect of temperature, NaCl, pH and antibiotics.
Study Design: Rhizosphere soil and root nodules from faba bean crop 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 February and August 2014.
Methodology: Four strains, which retained their P-solubilizing ability after repeated subculturing, from a total of seventy eight isolates were purified, characterized and identified by 16S rRNA gene sequencing.
Results: The isolates were closely related to *Pseudomonas cedrina* (PT19), *Rahnella aquatilis HX2* (P24), *Rhizobium nepotum* (BSP30) and *Rhizobium tibeticum* (Rh8). P solubilization index of

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these isolates ranged from 1, 57-2, 88 in tricalcium phosphate amended National Botanical Research Institute's Phosphate (NBRIP) agar medium. Amount of phosphate solubilized ranged from 62.45 to 119.95 mg P L^{-1} and drop in pH of the medium ranged from 7 to 3.5. Besides, these strains exhibited resistance to several antibiotics.

Conclusion: The ability of the PSB to solubilize insoluble P and their adaptation to environmental conditions add value to these strains, which could lead to improved inoculants to increase the available phosphorus in the soil for faba bean plant growth and to promote sustainable agriculture.

Keywords: Phosphate solubilizing bacteria; phosphate; rhizosphere soil; root nodule; faba bean.

1. INTRODUCTION

Phosphorus (P) is a vital plant nutrient, available to plant roots only in soluble forms that are often in short supply in the soil. In fact, P can be tightly bound with soil cations, particularly calcium, iron, or aluminum, leading to precipitation of P in the soil. It has a critical role in plant metabolism and other activities such as cell division, development, photosynthesis, breakdown of sugar, nutrient uptake, nuclear transport within the cell, plant disease resistance and regulation of metabolic pathways [1]. Therefore, despite P being widely and abundantly distributed in the soil in both its inorganic and organic forms, it is not easily accessible for plant growth. Thus, phosphate solubilizing bacteria (PSB) play an important role in reducing P deficiency in soil through transforming insoluble phosphate to available, soluble phosphate [2]. Genera with the ability to solublize phosphorus include *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Micrococcus*, *Flavobacterium*, *Erwinia* [3], *Serratia*, *Ralstonia*, and *Pantoea* [4], *Ewingella*, *Enterobacter* [5] and *Photorhabdus* [6]. Although several mechanisms may be involved, the main one is through the production of organic acids [7]. It is assumed that these organic acids solubilize insoluble forms of phosphate to usable forms which increase the potential availability of phosphate for plants [7]. Microorganisms isolated from rhizospheric soil may be better adapted to crop plants and provide better growth and disease control than organisms isolated from other sources such as composts or harsh environments as the formers have been already closely associated with the plant system and adapted to the local environment as well [8,9]. Moreover, some of these microorganisms can induce resistance in plants against some pathogenic bacteria, fungi and viruses, a phenomenon termed induced systemic resistance (ISR) [10]. In recent years, beneficial rhizospheric microorganisms have gained special attention due to their potential to enhance plant growth by a variety of mechanisms such as phosphate solubilization [11,12]. The application of bacterial inoculants as biofertilizers results in improved plant growth and productivity [1,10].

Faba bean (*Vicia faba* L.) is a legume which forms an important part of the diet of many people worldwide, is considered major legume crop in Morocco and has been extensively cultivated as green manure as well as for feed and food. There is extensively used in co-cropping and intercropping systems with different cultures where it's believed to enhance soil fertility through their symbiotic potentialities. This crop is usually well nodulated in Moroccan soils; however, there are no studies about phosphate solubilization bacteria isolated from its rhizosphere soil and root nodules. In this study a special interest has been focused to isolate and characterize bacterial strains with phosphate solubilization activity with the aim to select efficient strains for subsequent inoculation to increase the available phosphorus in the soil for faba bean plant growth.

2. MATERIALS AND METHODS

2.1 Isolation of Phosphate Solubilizing Bacteria

From the rhizosphere of *V. faba* in different sites from Meknes region (33°53'42" North, 5°33'17" West), rhizosphere soil and root nodules were sampled. To isolate rhizosphere bacteria, soil adherent to roots was collected. One g of each sample soil was transferred in Erlenmeyer containing 10 ml of buffer (0,8% NaCl; 0,02% KCl; 0,14% Na_2HPO_4; 0,024% KH_2PO_4) [13]. The flasks were incubated on rotary shaker (180 rpm) at 30°C. After one h of incubation, phosphate solubilizing bacteria were isolated from soil samples by serial dilution using spread plating on NBRIP medium [13] supplemented with tri-calcium phosphate as insoluble inorganic phosphate source, and incubated at 27°C for 72-

120 h. For isolating bacteria from nodules, two undamaged, healthy root nodules of similar size and root location were sampled from the lateral roots of unsterilized faba bean plants were chosen at random from the original sample. Nodules were rinsed in running tap water and commercial detergent to remove soil particles, then shaken on a wrist-action shaker for 3 min in Mercury(II) chloride ($HgCl_2$) (0.1%) and then rinsed 10 times with sterile distilled water. Surface-sterilized root nodules were suspended in sterile distilled water and crushed individually in a sterile mortar and pestle [14]. The macerate was plated into Yeast Mannitol Agar (YMA) and restreaked into fresh NBRIP agar medium. Colonies showing clear zone of P-solubilization were counted as PSB [15]. Once purified, each isolate was stored as a glycerol 40% stock at -80°C.

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 tri-calcium phosphate (5 gL^{-1}). Each isolate was assayed by spotting 10 µl of cultures (5 × 10^8 CFU ml^{-1}) on the media plates. The halo and colony diameters were measured after 3, 5 and 7 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 [16].

2.3 Quantification of Inorganic Phosphate Solubilization

The quantitative bioassay was carried out using Erlenmeyer flasks (250 ml) 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 incubation for seven days, daily the growth medium was centrifuged at 10,000 rpm for 20 min. Supernatant was decanted and 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 [17]. The pH of the supernatant was measured in each case by pH meter (Metrohm 620 pH meter). All the data were an average of three replicates.

2.4 Morphological and Biochemical Identification of Strains

Identification based on morphological, physiological and biochemical characteristics of selected bacterial isolates were carried as described by Holt et al. [18].

2.5 Bacterial Extrinsic and Intrinsic Stress Resistance

The tolerance of strains to extrinsic and intrinsic environmental stress was tested by the ability of the PSB isolates to grow on media at several types and values of stresses. Growth in Lysogeny broth (LB) agar at different temperatures (from 10 to 50°C), pH (from 4.8 to 8.8) and salinities (from 0.5 to 2 g L^{-1} NaCl) was examined. Additionally, bacterial resistance to different antibiotics was evaluated by saturated disc diffusion technique in Petri dishes containing LB solid medium. The antibiotics studied were tetracycline (30 µg), streptomycin (25 µg), kanamycin (30 µg), and ampicillin (10 µg). Bacteria were grown in LB liquid medium for 3 days with constant stirring. After the incubation, 100 µL of each bacterial culture was spread into Petri dishes containing the LB solid medium. Subsequently, using sterile forceps, three discs saturated with different antibiotics were added to each plate. Each strain was tested in triplicate (3 plates/strain). The discs were lightly pressed and kept equidistant from one another to prevent the inhibition zones from overlapping. The plates were inverted and incubated for 24 h at 28°C. After this period, the diameter of the growth inhibition halo (a translucent area around the disc) was measured.

2.6 Molecular Identification Based on 16S rRNA Gene Sequence

Genetic characterization based on 16S rRNA gene sequence was also done. Briefly, DNA extraction from bacterial strains on liquid culture using the kit "Gen Elute Bacterial Genomic DNA kit" from SIGMA, Aldrich according to the protocol provided and PCR amplification of 16S rDNA gene was carried out by using the primers: FD1(5′ AGAGTTTGATCCTGGCTCAG 3′) and rp2 (5' ACGGCTACCTTGTTACGACTT 3') [19]. Sequencing was performed on the 515 bp to 907 bp region of the 16S rRNA gene using the 3130XL Dye Terminator Cycle Sequencing (DTCS) Quick Start kit (Applied Biosystems) according to manufacturer instructions. The

optimal thermocycling conditions for the cycle sequencing reaction were as follows: 25 cycles of 96°C for 1 min, 96°C for 10s, 50°C for 5s, and 60°C for 4 min, followed by a 4°C infinite hold. The Sephadex G50 superfine (Sigma Aldrich) was used to remove unincorporated dye terminators from the cycle sequencing reaction, according to manufacturer's instructions with an additional 300 µl wash of the column with distilled H_2O and centrifugation at 1500×g for 3 min prior applying the sample to the column. The sequences obtained were compared with sequences in the GenBank database from the National Centre for Biotechnology Information (NCBI) using blastn program (http://blast.ncbi.nlm.nih.gov/) to obtain most similar accessions and their similarities.

3. RESULTS AND DISCUSSION

A total of seventy eight bacteria strains were isolated on NBRIP plates from the rhizosphere of faba bean collected from Meknes region in Morocco. Most of PSB isolated in this study lost their ability to solubilize phosphate this fact was confirmed via repeated subculturing. However, just four strains were retained due to their P-solubilizing ability as reflected by formation of a translucent to clear halo around their colonies in the NBRIP agar after 72-120 h of growth at 27°C (Fig. 1). This result was in accordance with Illmer et al. [20] and Rodriguez et al. [3]. Solubilization index of each isolated PSB was based on colony diameter and Halo-zone; Solubilization indexes were presented in Table 1. Results showed that among the isolated PSB's, Rh8 was the most efficient on P- solubilizing in the NBRIP plates with SI=2.88 followed by P24 with SI=1.85. Our results were in accordance with those of Baig et al. [21] and Yang et al. [22] which showed that the halo zone increased with the increase of the colony diameter. The amount of the P-solubilized by these isolates varied from 62.45 to 119.95 µg.ml-1 on the NBRIP broth at 27°C for seven days (Table 1). However, in this assay, the maximum P solubilization achieved with Rh8 (119.95 mg/l) was followed by BSP30 (84.44 mg/l). The results of this analysis were in accordance with the previously reported studies [4,23,12,24]. Drop in pH had been showed by PSB and ranged from 4.1 to 3.5 at the end of incubation period (Table 1). A clear relationship could be established between bacterial growth, supernatant acidification and P solubilization from $Ca_3(PO_4)_2$. The growth of these microorganisms was accompanied with the increase of the amount of the solubilized P and with the production and the release of the organic acids and/or chelating agents and protons [3,20]. We are currently attempting to identify the metabolites excreted by our isolates involved in P solubilization from $Ca_3(PO_4)_2$.

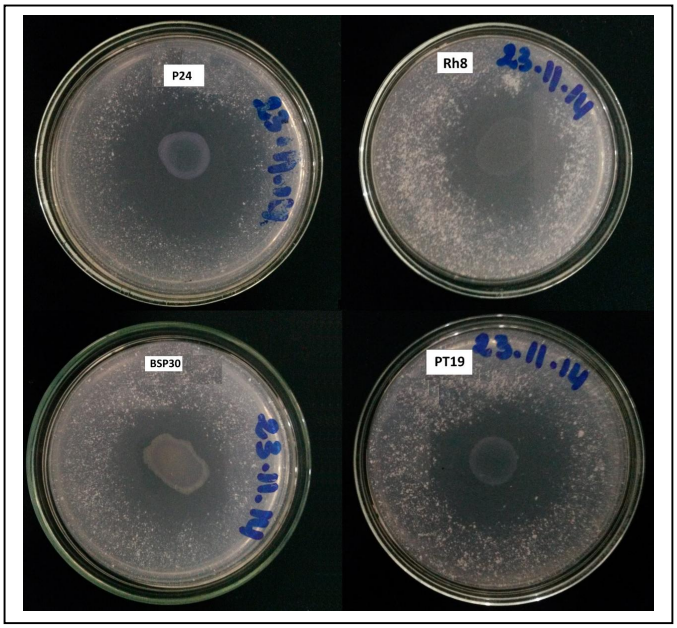

Fig. 1. Phosphate solubilization halo produced by bacterial isolates in NBRIP agar plates

Table 1. Tricalcium phosphate solubilization by bacterial isolates in agar and broth using NBRIP medium

Isolate name	Agar (solubilization index (SI))			Broth (P-solubilised (µg /ml))			pH in broth medium*		
	3 days	5 days	7 days	3rd day	5th day	7th day	3rd day	5th day	7th day
PT19	1,23	1,18	1,73	41,20	58,40	62,45	3,8	3,6	4,1
P24	1,33	1,28	1,85	48,98	56,89	68,33	3,9	3,7	3,8
BSP30	1, 12	1,25	1,57	29,12	47,87	84,44	4,5	4,1	3,6
Rh8	1,34	2,11	2,88	64,94	80,96	119,95	4,8	4,6	3,5

**Initial pH = 6.8*

These isolates presumably identified as PSB were further biochemically characterized. So, all PSB isolates were Gram negative unicellular rods. Bacterial colonies were circular (PT19) and noncyclical (P24, BSP30 and Rh8). The color colony was white for the strain PT19 and off-white for the remained strains (P24, BSP30 and Rh8). All PSBs were Oxidase positive. The isolates P24 and BSP30 were catalase positive however the strains PT19 and Rh8 were negative. All the strains were non Inositol fermenters (Table 2). All PSBs were indole positive except the strain P24 and methyl red negative except the strains BSP30 and Rh8. All strains didn't produce H2S, didn't use the simmon citrate, gelatin and starch except the strains P24 and Rh8, and didn't hydrolyze lipid (Table 2).

Furthermore, the BLAST results of the 16S rRNA gene sequences allowed to classify the isolated strains from various soils into the family of *Enterobacteriaceae*, *Pseudomonadaceae* and *Rhizobiaceae*. The four evaluated strains were aligned against sequences available from GenBank data; the four PSBs PT19, P24, BSP30 and Rh8 matched to *Pseudomonas cedrina*, *Rahnella aquatilis HX2*, *Rhizobium nepotum* and *Rhizobium tibeticum* respectively with 99% of similarity percentage through GenBank data base as represented in Table 4. All of the genera identified in this study have been associated with plant rhizosphere and their phosphate-solubilizing activities has also been reported earlier [3,25,26].

Phosphate solubilizing microorganisms were isolated from the rhizosphere of different crops. The growth and the role of these isolates are mainly affected by different stress such as salt, pH and temperature [27]. The present data showed that the isolates studied were globally tolerant to alkalinity, acidity, salinity, temperature and antibiotics. All of the present isolates tolerated temperature ranging between 10 and 40°C, moreover both of them (P24 and Rh8) were also able to grow at 50°C (Table 3). The adaptation to temperature stress may present an important characteristic of the microorganisms which could survive even during the drought season. These results were in concordance with previous studies [28,29].

Our isolates were able to grow and survive on a wide pH range (4.8 – 8.8) (Table 3). However, pH extremes could be a major factor limiting the microorganisms' growth [3]. These results indicated that our PSBs could be applied on the acidic and/or alkaline soils. Moreover, in this study, all the isolates grew at higher NaCl concentrations (2 g L^{-1}); these results were in accordance with those obtained by Chaiharn and Lumyong [29]. This tolerance could be explained by the synthesis of protective factors and the adaptation to the current environmental conditions [30].

Further to the loss of agricultural production, particularly due to different abiotic stresses mainly salinity, the microorganism tolerance to these various Stress is very important trait because it could present a good alternative in order to fight against this problem. Moreover, microorganisms surviving at extreme environmental conditions have been found suitable for use in different agricultural practices [31,32]. It was suggested that the anti-stress effect of rhizobacteria on the plants is an important mechanism for their interaction and mutual resistance to unfavorable conditions [33].

Antagonism between microorganisms is a parameter capable of influencing population dynamics in the soil. The investigated strains were classified based on their Inhibition distances (ID). Generally, PSB isolated strains in this study showed an intrinsic resistance against different antibiotics tested (Table 3). However, PT19 was the best intrinsic resistance strain showed a resistance for all antibiotics tested.

Also, obtaining a specific set of resistance genes is likely to give some advantages to a population of PSB as a way to establish suitable inoculants for the plant growth.

Table 2. Morphological, physiological and biochemical characteristics of phosphate solubilizing bacteria strains in the study

Characteristics	PSB isolates[a]			
	PT19	P24	BSP30	Rh8
Colony morphology[b]	C, I, R, W, Cy	E,R, OW, Cy	E, R,OW, V	E, R,OW, V
Catalase	-	+	+	-
Oxidase	+	+	+	+
Motility	+	-	-	-
Urease	+	+	+	+
Indole production	+	-	+	+
Methylred	-	-	+	+
Citrate (Simmons)	-	-	-	-
Carbon source utilization				
D-Glucose	+	+	+	+
Fructose	+	+	+	+
D-Mannitol	+	+	+	+
D-Mannose	+	+	+	+
Inositol	-	-	-	-
D-Sorbitol	+	+	+	+
L-Rhamnose	+	+	+	+
D-Sacarose	+	+	+	+
D-Melibiose	+	+	+	+
L-Arabinos	+	+	+	+
D-Maltose	+	+	+	+
Gelatin hydrolysis	-	-	-	-
Starch hydrolysis	-	+	-	+
Lipid hydrolysis	-	-	-	-

+, Tested positive/utilized as substrate; -, tested negative/non-utilized as substrate; [a]All PSB isolates were shown to be Gram-negative, reacted negative for H_2S production; [b]Colony morphology in YMA medium: C: circular; E/I: entire/irregular edge; R/Cr: raised/crateri form; Y/W/OW: yellow/white/off-white; V/Cy: viscous/creamy

Table 3. Phenotypic characteristics of the strains under environmental stresses

Isolate	Growth pH				Growth salinity (g/l)				Growth temperature (°C)					Antibiotic-resistance[ab(mm)]			
	4,8	5,8	6,8	8,8	0,5	1	1,5	2	10	20	30	40	50	AMP	TE	S	K
PT19	-	+	+	+	±	+	+	±	+	+	+	±	-	R	R	R	R
P24	-	+	+	-	+	+	+	+	±	+	+	±	±	R	19	R	R
BSP30	+	+	±	-	±	+	+	±	±	+	+	±	-	25	R	R	R
Rh8	-	+	±	±	+	+	+	±	+	+	+	+	±	R	R	R	17

+: Growth, ±: weak growth, -: no growth; [a]AMP: Ampicillin (10 ug), TE: Tetracycline (30µg), S: Streptomycin (25µg), K: kanamycin (30µg); [b]Inhibition distances(ID) (mm): (ID = 0.00 = resistant (R)),intermediate or sensitive (ID ≥ 01.00)

Table 4. Identification of PSB isolates by 16S rDNA sequencing

Isolate	Identification based in the most similar sequence found in GenBank				Accession number in Gen Bank (NCBI) of the strain
	Number of base pairs	Closest relative species	Similarity (%)	Accession number	
PT19	1526	*Pseudomonas cedrina*	99%	NR024912	KF465842
P24	1231	*Rahnella aquatilis HX2*	99%	NR074921	KF465836
BSP30	1141	*Rhizobium nepotum*	99%	NR117203	KF432826
Rh8	1191	*Rhizobium tibeticum*	99%	NR116254	KF360080

4. CONCLUSION

In conclusion, we isolated four bacterial strains from faba bean rhizosphere soil and root nodules in Meknes region, we purified, characterized and identified them by 16S rRNA gene sequencing. These bacterial strains were identified as belonging to the genera *Pseudomonas*, *Rahnella* and *Rhizobium*. From this study, we have isolated efficient phosphate solubilizing bacteria which released high amounts of P. Also, all of the bacterial strains isolated tolerated high concentrations of NaCl, grew at a wide range of temperature, pH and showed an intrinsic resistance against different antibiotics tested. The results of our isolates highlight their importance as phosphate solubilizers. And in order to recommend this strains as biofertilizers, further studies are required particularly their effect on the plant growth promoting under the greenhouse conditions as well as the field ones.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
http://www.sciencedomain.org/review-history.php?iid=831&id=8&aid=7911