Isolation and Characterization of Phosphate Solubilizing Bacteria from the Rhizosphere of Faba Bean (Vicia faba L.) in Meknes Region, Morocco

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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...
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 solubilize phosphorus include *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Microccocus*, *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\(_2\)HPO\(_4\); 0.024% KH\(_2\)PO\(_4\)) [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 tricalcium phosphate as insoluble inorganic phosphate source, and incubated at 27°C for 72-
2.3 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 g L\(^{-1}\)). 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].

2.4 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 rRNA 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 H2O 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 Ca3(PO4)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 Ca3(PO4)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 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                        | PT19 | P24 | BSP30 | Rh8  |
|----------------------------------------|------|-----|-------|------|
| Colony morphology<sup>a</sup>          | C, I, R, W, Cy | E, R, OW, Cy | E, R, OW, V | E, R, OW, V |
| Catalase                               | -    | +   | +     | -    |
| Oxidase                                | +    | -   | +     | +    |
| Motility                               | +    | +   | +     | +    |
| Urease                                 | +    | +   | +     | +    |
| Indole production                      | -    | +   | +     | +    |
| Methylenol                             | -    | -   | +     | +    |
| 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                       | -    | -   | -     | -    |

<sup>a</sup> Tested positive/utilized as substrate; <sup>-</sup> tested negative/non-utilized as substrate;<sup>**a**</sup> All PSB isolates were shown to be Gram-negative, reacted negative for H₂S production; <sup>b</sup> Colony morphology in YMA medium: C: circular; E/I: entire/irregular edge; R/Cr: raised/crateriform; 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<sup>ab(mm)</sup> |
|---------|-----------|-----------------------|--------------------------|-----------------------------------------|
|         | 4,8       | 5,8                   | 6,8                      | 8,8                                    |
|         | 0,5       | 1                     | 1,5                      | 2                                       |
|         | 10        | 20                    | 30                       | 40                                      |
|         | 50        |                        |                          | AMP, TE, S, K                          |
| PT19    | -         | +                     | +                        | +                                      |
| P24     | -         | +                     | -                        | +                                      |
| BSP30   | +         | +                     | ±                        | +                                      |
| Rh8     | -         | ±                     | -                        | +                                      |

<sup>a</sup> Growth, ±: weak growth, <: no growth; <sup>b</sup> AMP: Ampicillin (10 μg), TE: Tetracycline (30 μg), S: Streptomycin (25 μg), K: Kanamycin (30μg); <sup>**b**</sup>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 | Number of base pairs | Closest relative species        | Similarity (%) | Accession number | Accession number in Gen Bank (NCBI) of the strain |
|---------|----------------------|--------------------------------|----------------|------------------|-----------------------------------------------|
| 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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