***Original Research Article***

# Isolation and characterization of phosphate solubilizing rhizobacteria isolated from faba bean (*Viciafaba*L.)rhizosphere in Meknes region, Morocco.


## 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 solubilizationas 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&EnvironmentMicrobiologyUnit)Faculty of Sciences, Moulay Ismail University, Meknes, Morocco; betweenFebruaryand August 2014.
**Methodology:**Four of the most effective strains 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 fluorescens*(FR18), *Rahnellaaquatilis* (FR24), *Rhizobium leguminosarum* (FN10), and *Rhizobium pusense*(FN30). P solubilization index of these isolates ranged from 3.51-4.65 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; fababean*


## 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 soilthrough

transforminginsoluble phosphate to available, soluble phosphate [2]. Genera with the ability to solublize phosphorus include *Pseudomonas*, *Bacillus*, *Rhizobium*, *Burkholderia*, *Achromobacter*, *Agrobacterium*, *Microccocus*, *Flavobacterium*, *Erwinia*[3], *Serratia*, *Ralstonia*, and *Pantoea*[4], *Ewingella*, *Enterobacter*[5], and *Photorhabdus*[6]. ~~Phosphorus is required to all living being in the form of phosphate ion [7]. Microorganisms have the ability to solublize these compounds.~~ Although several mechanisms may be involved, the main one is through the production of organic acids [7]. It is assumed that these organic acids solublize insoluble forms of phosphate to usable forms which increases 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, 13]**.**The application of bacterial inoculants as biofertilizers result in improved plant growth and productivity [1,10]. Faba bean (*Viciafaba L.*) is a legume which forms an important part of the diet of many people worldwide including Morocco. It is grown in all agriculture soils in Meknes region.The aims of this study was to isolate and characterize bacterial strains with phosphate solubilization activity from the rhizosphere of Faba bean in Meknes region. Also,the effect of temperature (T), NaCl concentration, pH and the antibiotics on the growth of these isolates was investigated.

## 2. Materials and Methods

### 2.1. Isolation of Phosphate Solubilizing Bacteria

From the rhizosphere of fababean (*Viciafaba L.*), 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% $KH_2PO_4$) [14]. The flasks were incubated on rotary shaker (180 rpm) at 30°C. After one h of incubation,phosph ate solubilizing bacteria were isolated from soil samples by serial dilution using spread plating on NBRIP medium [14]supplemented with tri-calcium phosphate as insoluble inorganic phosphate source, and incubated at 27°C for 72-120 h. For isolating b acteria from nodules, two undamaged, healthy root nodules of similar size and root location were sampled from the lateral roots of unsterilized faba bean plants 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[15]**.** The macerate were plated onto Yeast Mannitol Agar (YMA) and restreaked on to fresh NBRIP agar medium. Colonies showing clear zone of P-solubilization were counted as PSB [16]. 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 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 solu bilize insoluble phosphate was described by the solubilization index (SI) = the ratio of the total diameter (colony + halo zone) to the colony diameter[17].

## 2.3. Quantification of inorganic 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 ($5 \times 10^8$ CFU $ml^{-1}$). Autoclaved uninoculated NBRIP medium served as control. The flasks were incubated on rotary shaker (180 rpm) at 30℃. After incubation for seven days, daily the growth medium was centrifuged at 10,000 rpm for 20 min. Supernatant was decanted and autoclaved at 121℃ fo r 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)~~ [18]. 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. 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. ~~(1994)~~[19].

## 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 inLysogeny broth(LB)agar at different temperatures (from 10 to 50℃), pH (from 4.8 to 8.8) and salinities (from 0.5 to 2 $gL^{-1}$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 onto 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℃.

## 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 BacterialGenomic DNA kit" from SIGMA, Aldrich according to the protocol providedand PCR amplification of 16S rDNA gene was carried out by using the primers: FD1(5′ AGAGTTTGATCCTGGCTCAG 3′) and rp2 (5’ ACGGCTACCTTGTTACGACTT 3’) [20]. 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℃ for 1 min, 96℃ for 10s, 50℃ for 5s, and 60℃ for 4 min, followed by a 4℃ 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.

## 2.7. 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.

## 3. RESULTS AND DISCUSSION

Faba bean (*Viciafaba L.*) 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 solubilizationbacteriaisolated 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.

A total of seventy eight bacteria strains (encoded FN and FR) were isolated on NBRIP plates from the rhizosphere of faba bean collected from Meknes region in Morocco. Most of PSB isolated strains in this study lost their ability to form halozone on agar medium on repeated subculturing.However,only four strainsmaintainedphosphate solubilization activity as reflected by formation of a translucent to clear around their colonies in the NBRIP agar after 72-120 h of growth at 27 ℃. This result is in accordance withIllmer and Schinner[21] and Rodriguez and Fraga[3].Solubilization index based on colony diameter and holozone for each PSB isolate is presented in Table 1.Results showed that among PSB, FN30 was the most efficient phosphate solubilizer on NBRIP plates with SI = 4.65. Generally, halozone increased with increase in colony diameter(Fig. 1).These results are in accordance with that of Baig et al. [22]and Yang et al. [23].The P-solubilised in Broth mediumof these isolates varied from 62.45 to 119.95 $\mu gml^{-1}$ in NBRIP broth at 27℃ in seven days (Table 1). Ho wever, in this assay, the maximum P solubilization achieved with FN30 (119.95 mg/l) was followed by FN10 (84.44mg/l). The results of this analysis were found in accordance with the previously reported studies [4,24,13,25].Drop in pH by PSB ranged from 4.1 to 3.5 at the end of incubation period(Table 1).

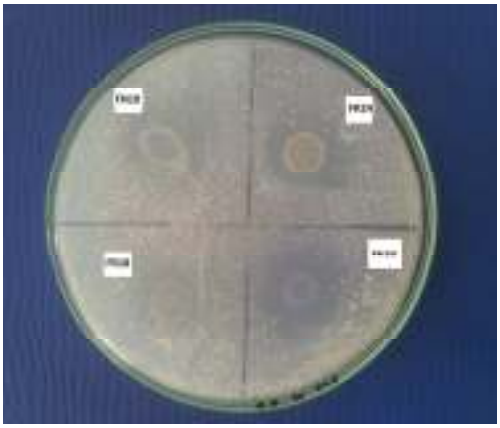

**Fig. 1. Phosphate solubilization halo produced by bacterial isolates in NBRIP agar plates**

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

| Bacteria | Agar (solubilization index (SI)) | | | Broth(P-solubilised (µg /ml)) | | | pH in broth medium* | | |
|---|---|---|---|---|---|---|---|---|---|
| | 10 days | 20 days | 30 days | 3rd day | 5th day | 7th day | 3rd day | 5th day | 7th day |
| FR18 (*Pseudomonas sp.*) | 2,84 | 3,18 | 3,72 | 41,20 | 58,40 | 62,45 | 3,8 | 3,6 | 4,1 |
| FR24 (*Rahnellasp.*) | 2,84 | 3,28 | 3,73 | 48,98 | 56,89 | 68,33 | 3,9 | 3,7 | 3,8 |
| FN10 (*Rhizobium sp.*) | 3,17 | 3,23 | 3,51 | 29,12 | 47,87 | 84,44 | 4,5 | 4,1 | 3,6 |
| FN30 (*Rhizobium sp.*) | 2,88 | 3,96 | 4,65 | 64,94 | 80,96 | 119,95 | 4,8 | 4,6 | 3,5 |

**Initial pH = 6.8*

The release of soluble phosphate in the broth was associated with a concomitant decrease in pH. Simple regression analysis suggest that there is a significant negative correlation (r =-0.71) between the soluble phosphate released and pH (Fig. 2)**.** This observation is consistent with previous reports demonstrating that phosphate solubilizationwas paralleled with decrease in pH [26,4, 27]. On the contrary Yang et al.~~(2012)~~[23] reported no significant negative correlation between the final culture pH and tri-calcium phosphate solubilization activity (r = -0.15).

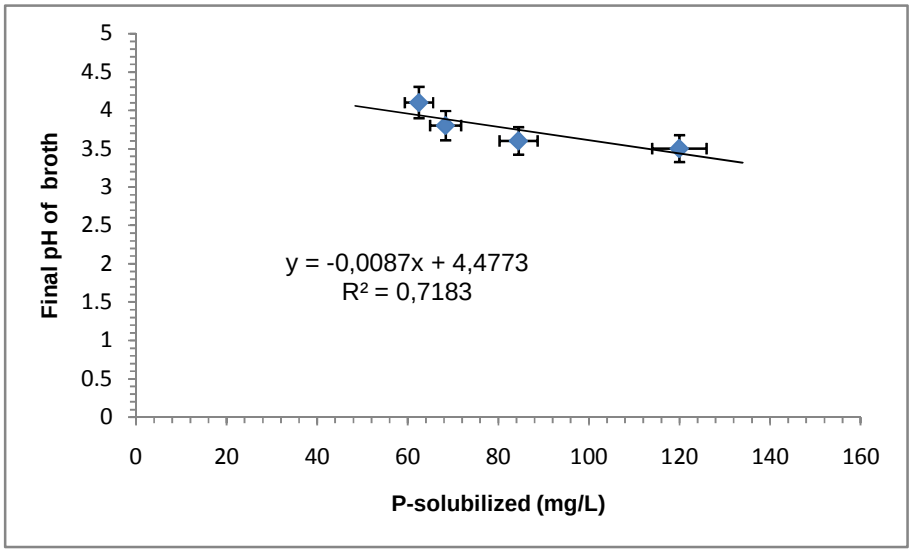


**Fig. 2.Correlation analysis between final culture pH and tricalcium phosphate solubilizing ability of phosphate solubilizing bacteria.**

Those isolates presumably identified as PSB further characterized by a series of biochemical tests. So, they were Gram- negative, with positive for catalase activity (except FR18 and FN30).All strains have been negative for oxidaseactivity, $H_2S$ production, gelatin, starch (except FR24 and FN30) and lipid hydrolysis. Details on the physiological and biochemical characterizations of all bacterial strains are given in Table 2.

The ability to adapt to temperature stress may be important in the survival of microorganisms during drought. All the isolates in this study could tolerate temperature ranging between 10 and 40℃, but some of them were also able to grow at 50℃ (FR24 and FN30) (Table 3).The results of this test are in concordance with previous studies [28, 29].

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[29].Regarding the intrinsic resistance, all PSB isolated strains in this study showed a ~~good~~resistance to different antibioticsthrough a qualitative assessment of bacterial growth around the discs (Table 3).

Otherwise, several species of phosphobacteria isolated from rhizospheric soil are known to support plant growth by synthesizing auxins, mainly indole-3-acetic acid, solubilizing insoluble phosphates, producing siderophores or fixing atmospheric nitrogen [30, 31].

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

| CHARACTERISTICS | PSB isolates[a] | | | |
|---|---|---|---|---|
| | **FR18** | **FR24** | **FN10** | **FN30** |
| Colonymorphology[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 | + | + | + | + |
| Gelatinhydrolysis | - | - | - | - |
| Starchhydrolysis | - | + | - | + |
| Lipidhydrolysis | - | - | - | - |

*+, 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/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 | | | | Growthsalinity (g/l) | | | | Growthtemperature (℃) | | | | | Antibioticresistance[a] | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 4,8 | 5,8 | 6,8 | 8,8 | 0,5 | 1 | 1,5 | 2 | 10 | 20 | 30 | 40 | 50 | AMP | TE | S | K |
| FR18 | - | + | + | + | ± | + | + | ± | + | + | + | ± | - | + | + | + | + |
| FR24 | - | + | + | - | + | + | + | + | ± | + | + | ± | ± | + | - | + | ± |
| FN10 | + | + | ± | - | ± | + | + | ± | ± | + | + | ± | - | - | ± | + | + |
| FN30 | - | + | ± | ± | + | + | + | ± | + | + | + | + | ± | + | + | ± | - |

*+: Growth, ±: weak growth, -: no growth*

*[a]AMP: Ampicillin (10 ug), TE: Tetracycline (30µg), S: Streptomycin (25µg), K: kanamycin (30µg)*

The 16S rRNA sequences from the four bacterial isolates placed the isolates to the family *Enterobacteriaceae*, *Pseudomonadaceae* and *Rhizobiaceae* from various soils. Evaluated were aligned against sequences available from GenBank data as *Pseudomonas fluorescens* (FR18), *Rahnellaaquatilis*(FR24), *Rhizobium leguminosarum*(FN10) and *Rhizobium pusense* (FN30). The similarity among the sequences studied through GenBank access was between 97 and 99 % (Table 4).

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

| Isolate | Length of 16S rDNAgenesequenced (bp) | GenBank accession no. | Closest relative species | Similarity (% ) |
|---|---|---|---|---|
| FR18 | 1239 | NR102835 | *Pseudomonas fluorescens Pf0-1* | 97 |
| FR24 | 1545 | NR074921 | *Rahnellaaquatilis HX2* | 99 |
| FN10 | 1475 | NR036938 | *Rhizobium leguminosarum 3Hoq18* | 98 |
| FN30 | 1379 | NR116874 | *Rhizobium pusense NRCPB10* | 99 |

All of the genera identified in this study have been associated with plant rhizosphere and have been studied as promising phosphate-solubilizing microorganisms [3,32, 33]. The isolated strains in our study, can effectively solubilize $Ca_3(PO_4)_2$ and release soluble phosphate in NBRIP medium.

The studied phosphate solubilizing bacteria tolerate high concentrations of NaCl (2 g $L^{-1}$), were tolerant the extreme temperature (50 ℃) and pH (from 4.8 to 8.8). The antibiotic resistance of the isolated strains showed a ~~high level of~~resistance to streptomycin, kanamycin, ampicillin and tetracycline. However, ~~It~~ further investigation on the bacterial strains is needed to exploit them as biofertilizers for faba bean to enhance its growth in Meknes area.

## 4. CONCLUSION

In conclusion,~~after~~ we isolated four bacterial strains from faba bean rhizosphere soil and root nodules in Meknes region, wepurified, 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 amount of P. Also, all of the bacterial strains isolated tolerate high concentrations of NaCl, extreme temperature, pH and antibiotics. These may be a versatile phosphate-solubilizer to make an efficient bioinoculant to facilitate solubilization of complexed P in soils, to minimize fertilizer application,all this could reduce environmental pollution and promote sustainable agriculture for faba bean field soils.

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