

Original Research Article
**Isolation and characterization of phosphate
solubilizing rhizobacteria isolated from the
rhizosphere of faba bean (*Vicia faba* 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 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*, *Micrococcus*, *Flavobacterium*, *Erwinia* [3], *Serratia*, *Ralstonia*, and *Pantoea* [4], *Ewingella*, *Enterobacter* [5], and *Photobacterium* [6]. Phosphorus is required to all living being in the form of phosphate ion [7]. Microorganisms have the ability to solubilize 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 solubilize 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 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 including Morocco. It is grown in all agriculture soils in Meknes region. The aims of this study were 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 faba bean (*Vicia faba* L.), *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) [14]. 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 [14] 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 [15]. The macerate was plated onto Yeast Mannitol Agar (YMA) and restreaked on to into 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 g/L). 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 10, 20 and 30 days 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 [17].

77

78 2.3. Quantification of inorganic phosphate solubilization

79 The quantitative bioassay was carried out using Erlenmeyer flasks (250ml) containing
80 50 ml of NBRIP broth medium supplemented with $(\text{Ca}_3(\text{PO}_4)_2)$ and inoculated by 200 μL
81 of bacteria (5×10^8 CFU ml^{-1}). Autoclaved uninoculated NBRIP medium served as
82 control. The flasks were incubated on rotary shaker (180 rpm) at 30°C. After incubation
83 for seven days, daily the growth medium was centrifuged at 10,000 rpm for 20 min.
84 Supernatant was decanted and autoclaved at 121°C for 20 min. Autoclaved samples
85 were then filtered through Whatman paper no. 42 followed by 0.2 μm millipore
86 membrane and were used for the determination of the pH and the soluble P released
87 into the solution. P was measured with molybdenum blue method as described by
88 Murphy and Riley (1962) [18]. The pH of the supernatant was measured in each case
89 by pH meter (Metrohm 620 pH meter, ~~swiss made~~). All the data were an average of
90 three replicates.

91 2.4. Morphological and biochemical identification of strains

92 Identification based on morphological, physiological and biochemical characteristics of
93 selected bacterial isolates were carried as described by Holt et al. (1994)[19].

94 2.5. Bacterial extrinsic and intrinsic stress resistance

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

110 2.6. Molecular identification based on 16S rRNA gene sequence

111 Genetic characterization based on 16S rRNA gene sequence was also done. Briefly,
112 DNA extraction from bacterial strains on liquid culture using the kit "Gen Elute Bacteria/
113 Genomic DNA kit" from SIGMA, Aldrich according to the protocol provided and PCR
114 amplification of 16S rDNA gene was carried out by using the primers: FD1(5'
115 AGAGTTTGATCCTGGCTCAG 3') and rp2 (5' ACGGCTACCTTGTTACGACTT 3') [20].
116 Sequencing was performed on the 515 bp to 907 bp region of the 16S rRNA gene using
117 the 3130XL Dye Terminator Cycle Sequencing (DTCS) Quick Start kit (Applied
118 Biosystems) according to manufacturer instructions. The optimal thermocycling
119 conditions for the cycle sequencing reaction were as follows: 25 cycles of 96°C for 1
120 min, 96°C for 10s, 50°C for 5s, and 60°C for 4 min, followed by a 4°C infinite hold. The
121 Sephadex G50 superfine (Sigma Aldrich) was used to remove unincorporated dye
122 terminators from the cycle sequencing reaction, according to manufacturer's instructions
123 with an additional 300 μL wash of the column with distilled H_2O and centrifugation at
124 1500 $\times$ g for 3 min prior applying the sample to the column. The sequences obtained
125 were compared with sequences in the GenBank database from the National Centre for
126 Biotechnology Information (NCBI) using blastn program (<http://blast.ncbi.nlm.nih.gov/>) to
127 obtain most similar accessions and their similarities.

128 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 (*Vicia faba* L.) *V. faba* 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.

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 Halo-zone on agar medium on repeated subculturing. However, only four strains maintained phosphate solubilization activity. However, four strains retained their P-solubilizing ability. However, four strains retained their P-solubilizing ability as reflected by formation of a translucent to clear around their colonies in the NBRIP agar after 72-120 h of growth at 27 °C. This result is in accordance with Illmer and Schinner [21] and Rodriguez and Fraga [3]. Solubilization index based on colony diameter and halozone-Halo-zone for each PSB isolate is presented in Table 1. Results showed that among PSB, Rh8 was the most efficient phosphate solubilizer on NBRIP plates with SI = 2.88. Generally, Halo-zone 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 medium of these isolates varied from 62.45 to 119.95 $\mu\text{g ml}^{-1}$ in NBRIP broth at 27°C in seven days (Table 1). However, in this assay, the maximum P solubilization achieved with Rh8 (119.95 mg/l) was followed by BSP30 (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).

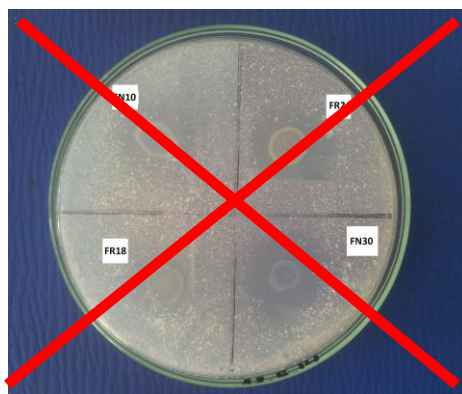


Fig. 1. Phosphate solubilization halo produced by bacterial isolates in NBRIP agar plates

Commentaire [A1]: Delete(duplicated)

174

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

Bacteria Isolate name	Agar (solubilization index (SI))			Broth (P-solubilised (µg /ml))			pH in broth media	
	3days	5days	7days	3rd day	5th day	7th day	3rd day	5th day
PT19 (<i>Pseudomonas cedrina</i>)	1,23	1,18	1,73	41,20	58,40	62,45	3,8	3,6
P24 (<i>Rahnella aquatilis</i> HX2)	1,33	1,28	1,85	48,98	56,89	68,33	3,9	3,7
BSP30 (<i>Rhizobium nepotum</i>)	1,12	1,25	1,57	29,12	47,87	84,44	4,5	4,1
Rh8 (<i>Rhizobium tibeticum</i>)	1,34	2,11	2,88	64,94	80,96	119,95	4,8	4,6

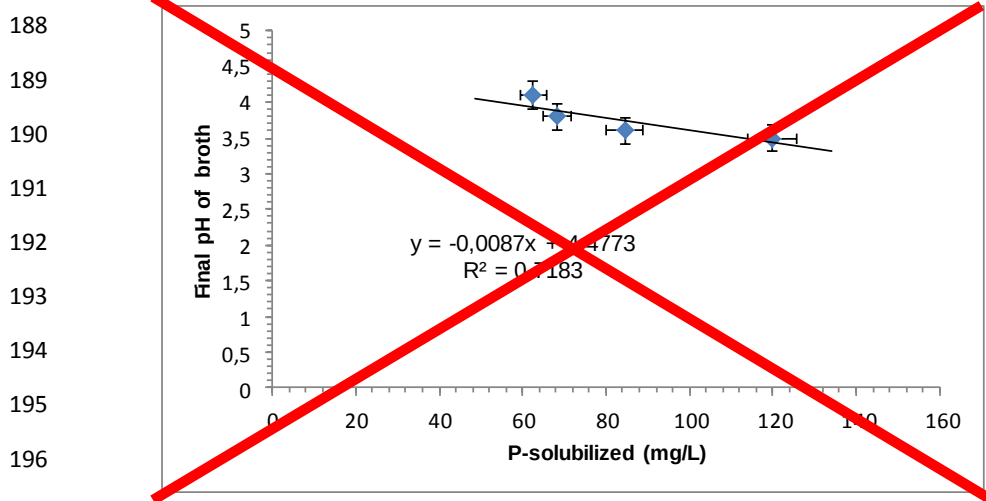
177 *Initial pH = 6.8

178 The release of soluble phosphate in the broth was associated with a concomitant
179 decrease in pH. Simple regression analysis suggest that there is a significant negative
180 correlation ($r = -0.71$) between the soluble phosphate released and pH (Fig. 2). This
181 observation is consistent with previous reports demonstrating that phosphate
182 solubilization was paralleled with decrease in pH [26, 4, 27]. On the contrary Yang et al.
183 (2012) [23] reported no significant negative correlation between the final culture pH and
184 tri-calcium phosphate solubilization activity ($r = -0.15$).

185

186

187



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

200 Those isolates presumably identified as PSB further were further characterized by a
201 series of biochemical tests. So, they were Gram- negative, with positive for catalase
202 activity (except PT19 and Rh8). All strains have been negative for oxidase activity, H₂S
203 production, gelatin, starch (except P24 and Rh8) and lipid hydrolysis. Details on the
204 physiological and biochemical characterizations of all bacterial strains are given in Table
205 2.

206 Phosphate solubilizing microorganisms were isolated from rhizosphere soils of different
207 crops. The establishment and performance of these microbes are affected severely
208 under stress such as high salt, pH and temperature prevalent in degraded ecosystems

Commentaire [A2]: Isolate identification

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209 such as alkaline soils with a tendency to fix phosphorus [26]. The data of this study
210 showed that the isolates studied are globally tolerant of alkalinity, acidity, salinity,
211 temperature and antibiotics. The ability to adapt to temperature stress may be important
212 in the survival of microorganisms during drought. All the isolates in this study could
213 tolerate temperature ranging between 10 and 40°C, but some of them were also able to
214 grow at 50°C (P24 and Rh8) (Table 3). The results of this test are in concordance with
215 previous studies [28,27,29,28]. The conditions in which the bacteria live greatly influence
216 their metabolism and especially their nutrition. All of our strains are mesophilic showed
217 a good temperature tolerance (between 20 and 40 ° C) with an optimum growth at
218 30°C, some strains are thermophilic were able to grow at 50°C like P24 and Rh8.

Commentaire [A8]: I do not see the sense of this statement...

Commentaire [A9]: Redundant. Please delete.

219 The bacteria tested in this study tolerated 2 g L⁻¹ salt concentration (Table 3). These
220 results are in accordance with those obtained by Chaiham and Lumyong [29,27]. All of
221 the isolated strains in the present study are halo bacteria. Therefore, the studied strains
222 showed a variable tolerance to different limiting factors, such as, temperature, pH, and
223 salinity. Also, what will increase the opportunity of strains to survive under limiting
224 factors and came into positive interaction with the plant and to promote growth.
225 Regarding the intrinsic resistance, all PSB isolated strains in this study showed a good
226 resistance to different antibiotics through a qualitative assessment of bacterial growth
227 around the discs. Regarding the intrinsic resistance, the investigated strains were
228 classified based on their inhibition distances (ID) in (mm) as demonstrating resistant (ID
229 = 00.00) and intermediate or sensitive (ID ≥ 01.00). The strain PT19 it's resistant at all
230 of the antibiotics studied. The other strains P24, BSP30 and Rh8 did not resistant for
231 one from all antibiotics tested (table 3). All of the strains that were efficient in phosphate
232 solubilization exhibited an initial resistance to four antibiotics studied (Table 3).

Commentaire [A10]: This entire paragraph needs revision. Discussion must be improved !

233 They were able to grow at an initial pH of 8.8 and 4.8 (Table 3). The isolates which can
234 survive on a wide pH range are candidates for further strain improvement to highly
235 acidic or alkaline conditions. Extremes of pH can be a major factor limiting
236 microorganisms in soil [3]. These results indicated that PSB isolated in our study which
237 can be applied to the acidic or alkaline soil. In this study, all the isolates grew at higher
238 NaCl concentration (2 g L⁻¹). This could be due to the synthesis of protective factors and
239 adaptation of current environmental conditions [29]. These results are in accordance
240 with those obtained by Chaiham and Lumyong [28]. Salt tolerance is important in the
241 growth and survival of microorganisms in V. faba soil. Looking into the perspectives of
242 crop production losses due to the severity of abiotic stresses, especially salinity,
243 tolerance to stress provided by microbial inoculants becomes more important. Beneficial
244 effect of PGPR under salinity has been related to hydraulic conductance, osmolyte
245 accumulation, sequestering toxic Na⁺ ions, maintaining higher stomatal conductance
246 and photosynthetic activities [30]. It was suggested that the anti-stress effect of
247 rhizobacteria on the plants is an important mechanism of their interaction and mutual
248 resistance to unfavorable conditions [31]. Microorganisms surviving at extreme
249 environmental conditions have been found suitable for use in different agricultural
250 practices [32]. Antagonism between microorganisms is a parameter capable of
251 influencing population dynamics in the soil. The investigated strains were classified
252 based on their inhibition distances (ID). Generally, PSB isolated strains in this study
253 showed an intrinsic resistance against different antibiotics tested (Table 3). However,
254 PT19 was the best intrinsic resistance strain showed a resistance for all antibiotics
255 tested. Also, obtaining a specific set of resistance genes is likely to give some
256 advantages to a population of PSB as a way to establish suitable inoculants for the
257 plant growth.

259 Otherwise, several species of phosphobacteria isolated from rhizospheric soil are
260 known to support plant growth by synthesizing auxins, mainly indole-3-acetic acid,

261 solubilizing insoluble phosphates, producing siderophores or fixing atmospheric nitrogen
262 [3033, 3134].

263 **Table 2. Morphological, physiological and biochemical characteristics of**
264 **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	-	-	-	-

265 +, Tested positive/utilized as substrate; -, tested negative/non-utilized as substrate.

266 ^aAll PSB isolates were shown to be Gram-negative, reacted negative for H₂S production.

267 ^bColony morphology in YMA medium: C: circular; E/I: entire/irregular edge; R/Cr: raised/crateriform; Y/W/OW:
268 yellow/white/off-white; V/Cy: viscous/creamy.

269

270 **Table 3. Phenotypic characteristics of the strains under environmental stresses.**

isolate	Growth pH				Growthsalinity (g/l)				Growthtemperature (°C)					Antibioticresistance ^{a,b}			
	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

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

272 ^aAMP: Ampicillin (10 ug), TE: Tetracycline (30µg), S: Streptomycin (25µg), K: kanamycin (30µg)

273 ^bInhibition distances(ID) (mm): (ID = 0.00 = resistant (R)),intermediate or sensitive (ID ≥ 01.00)

274

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 cedrina* (PT19), *Rahnella aquatilis* HX2 (P24), *Rhizobium nepotum* (BSP30) and *Rhizobium tibeticum* (Rh8). The similarity among the sequences studied through GenBank access was between 97 and 99 %. 16S rRNA gene sequences data of all isolates were deposited in the GenBank nucleotide sequence database under the accessions numbers (Table 4).

Commentaire [A11]: delete

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

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

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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, 35, 33, 36]. The isolated strains in our study, can effectively solubilize $\text{Ca}_3(\text{PO}_4)_2$ 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 °C) 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, 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 amount amounts of P. Also, all of the bacterial strains isolated tolerate tolerated high concentrations of NaCl, extreme temperature grew grew at a wide range of temperature, pH and antibiotics, 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. 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.

Commentaire [A13]: tolerated

Commentaire [A14]: grew

Commentaire [A15]: Please, revisespelling, grammar and punctuation.

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REFERENCES

1. Gupta M, Kiran S, Gulati A, Singh B, Tewari R. Isolation and identification of phosphate solubilizing bacteria able to enhance the growth and aloin-A biosynthesis of *Aloe barbadensis* Miller. Microbiol Res. 2012;167:358–363.
2. Antoun H. Beneficial microorganisms for the sustainable use of phosphates in agriculture. Procedia Eng. 2012;46:62–67.
3. Rodríguez H, Fraga R. Phosphate solubilizing bacteria and their role in plant growth promotion. Biotechnol Adv. 1999;17:319–339.
4. Pérez E, Sulbarán M, Ball MM, Yarzabal LA. Isolation and characterization of mineral phosphate-solubilizing bacteria naturally colonizing a limonitic crust in the south-eastern Venezuelan region. Soil Biol Biochem. 2007;39:2905–2914.
5. Ribeiro CM and Cardoso EJBN. Isolation, selection and characterization of root-associated growth promoting bacteria in Brazil pine (*Araucaria angustifolia*). Microbiol Res. 2012;167:69–78.
6. Ullah I, Khan AR, Park GS, Lim JH, Waqas M, Lee IJ, and Shin JH. Analysis of phytohormones and phosphate solubilization in *Photorhabdus* spp. Food Sci Biotechnol. 2013;22:25–31.
7. Nautiyal CS. An efficient microbiological growth medium for screening phosphate-solubilizing microorganisms. FEMS Microbiol Lett. 1999;170:265–270.
8. Gopalakrishnan S, Humayun P, Kiran BK, Kannan IGK, Vidya MS, Deepthi K, Rupela O. Evaluation of bacteria isolated from rice rhizosphere for biological control of charcoal rot of sorghum caused by *Macrophomina phaseolina* (Tassi) Goid. World J Microbiol Biotechnol. 2011;27:1313–1321.
9. Bakhshandeh E., Rahimian H., Pirdashti H., Nematzadeh G.A. Phosphate solubilization potential and modeling of stress tolerance of rhizobacteria from rice paddy soil in northern Iran. World J Microbiol Biotechnol. 2014;doi: 10.1007/s11274-014-1669-1.
10. Ahemad M, Kibret M. Mechanisms and applications of plant growth promoting rhizobacteria: current perspective. J King Saud Univ Sci. 2014;26:1–20.
11. Nadeem SM, Zahir ZA, Naveed M, Arshad M, Shahzad S. Variation in growth and ion uptake of maize due to inoculation with plant growth promoting rhizobacteria under salt stress. Soil Environ. 2006;25:78–84.
12. Lavakush JY, Verma JP. Isolation and characterization of effective plant growth promoting rhizobacteria from rice rhizosphere of Indian soil. Asian J Biol Sci. 2012;5:294–303.
13. Andrade LF, de Souza GLOD, Nietsche S, Xavier AA, Costa MR, Cardoso AMS, Pereira MCT, Pereira DFGS. Analysis of the abilities of endophytic bacteria associated with banana tree roots to promote plant growth. J Microbiol. 2014;52(1):27–34.
14. Araújo WL, Lima AOS, Azevedo JL, Marcon J, Sobral JK, Lakava PL, editors. Manual de isolamento de microrganismos endofíticos. Departamento de Genética – Escola Superior de Agricultura “Luiz de Queiroz” – Piracicaba ESALQ – USP ; 2002. Portuguese.
15. Vincent JM. The cultivation, isolation and maintenance of rhizobia. In : Vincent JM, editors. A Manual for the Practical Study of Root-Nodule. Oxford: Blackwell Scientific Publications; 1970.
16. Gyaneshwar P, Parekh LJ, Archana G, Poole PS, Collins MD, Hutson RA, Kumar GN. Involvement of a phosphate starvation inducible glucose dehydrogenase in soil phosphate solubilization by *Enterobacter asburiae*. FEMS Microbiol Lett. 1999;171:223–229.
17. Edi-Premono M, Moawad MA, Vleck PLG. Effect of phosphate solubilizing *Pseudomonas putida* on the growth of maize and its survival in the rhizosphere. Indonesian J Crop Sci. 1996;11:13–23.
18. Murphy J, Riley JP. A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta. 1962;27:31–36.
19. Holt JG, Krieg NR, Sneath PHA, Stanley JT, William ST, editors. Bergey's Manual of Determinative Bacteriology. 9th ed. Lippincott Williams and Wilkins: Baltimore Maryland USA; 1994.
20. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal amplification for phylogenetic study. J Bacteriol. 1991;173:697–703.

21. Illmer P, Schinner F. Solubilization of inorganic calcium phosphates-solubilization mechanisms. *Soil Biol. Biochem.* 1995;27:257–263.
22. Baig KS, Arshad M, Zahir ZA, Cheema MA. Comparative efficacy of qualitative and quantitative methods for rock phosphate solubilization with phosphate solubilizing rhizobacteria. *Soil Environ.* 2010;29:82–86.
23. Yang PX, Ma L, Chen MH, Xi JQ, He F, Duan C Q, Mo MH, Fang DH, Duan YQ, Yang FX. Phosphate solubilizing ability and phylogenetic diversity of bacteria from P-rich soils around Dianchi Lake drainage area of China. *Pedosphere.* 2012;22:707–716.
24. Chatli AS, Beri V, Sidhu BS. Isolation and characterisation of phosphate solubilising microorganisms from the cold desert habitat of *Salix alba* Linn. in trans Himalayan region of Himachal Pradesh. *Indian J Microbiol.* 2008;48:267–273.
25. Acevedo E, Galindo-Castañeda T, Prada F, Navia M, Romero HM. Phosphate-solubilizing microorganisms associated with the rhizosphere of oil palm (*Elaeis guineensis* Jacq.) in Colombia. *Appl Soil Ecol.* 2014;80:26–33.
26. Hwangbo H, Park RD, Kim YW, Rim YS, Park KH, Kim TH, Suh JS, Kim KY. 2-Ketogluconic acid production and phosphate solubilization by *Enterobacter intermedium*. *Curr Microbiol.* 2003;47:87–92.
27. Yu X, Liu X, Zhu TH, Liu GH, Mao C. Isolation and characterization of phosphate solubilizing bacteria from walnut and their effect on growth and phosphorus mobilization. *Biol Fert Soils.* 2011;47:437–446.
28. Johri JK, Surange S, Nautiyal CS. Occurrence of Salt, pH, and Temperature-tolerant, Phosphate-solubilizing Bacteria in Alkaline Soils. *Curr. Microbiol.* 1999;39:89–93.
29. Thakuria D, Talukdar NC, Goswami C, Hazarika S, Boro RC, Khan MR. Characterization and screening of bacteria from rhizosphere of rice grown in acidic soils of Assam. *Curr Sci.* 2004;86:978–985.
30. Chaihar M, Lumyong S. Phosphate solubilization potential and stress tolerance of rhizobacteria from rice soil in Northern Thailand. *World J Microbiol Biotechnol.* 2009;25:305–314.
31. Finkel SE, Kolter R. Evolution of microbial diversity during prolonged starvation. *Proc Natl Acad Sci USA.* 1999;96:4023–4027.
32. Dodd IC, Perez-Alfocea F. Microbial alleviation of crop salinity. *J Exptl Bot.* 2012;63:3415–3428.
33. Belimov AA, Postavskaya SM, Khamova OF, Kozehmvakov AP, Kunakova AM, Gruzdeva EV. Effectiveness of barley inoculation with root diazotrophic bacteria and their survival depending on soil-temperature and moisture. *Microbiol.* 1994;63:506–510.
34. Egamberdieva D, Kucharova Z. Selection for root colonizing bacteria stimulating wheat growth in saline soils. *Biol Fert Soil.* 2009;45:563–571.
35. Araujo FF, Guaberto LM, Silva IF. Bioprospection of plant growth promoter rhizobacteria in *Brachiaria brizantha*. *Rev Bras Zootec.* 2012;41:521–527.
36. Luo S, Xu T, Chen L, Chen J, Rao C, Xiao X, Wan Y, Zeng G, Long F, Liu C, Liu Y. Endophyte assisted promotion of biomass production and metal-uptake of energy crop sweet sorghum by plant-growth-promotion endophyte *Bacillus* sp. SLS18. *Appl Microbiol Biotechnol.* 2012;93:1745–1753.
37. Atemkeng MF, Remans R, Michiels J, Tagne A, Ngonkeu ELM. Inoculation with *Rhizobium etli* enhances organic acid exudation in common bean (*Phaseolus vulgaris* L.) subjected to phosphorus deficiency. *Afr J Agric Res.* 2011;6: 2235–2242.
38. Azziz G, Bajsa N, Haghjou T, Taulé T, Valverde A, Igual JM, Arias A. Abundance, diversity and prospecting of culturable phosphate solubilizing bacteria on soils under crop-pasture rotations in a no-tillage regime in Uruguay. *Appl Soil Ecol.* 2012;61:320–326.