

Original Research Article

Phenotypic and genotypic characteristics of phosphate solubilizing rhizobacteria isolated from fababeanrhizosphere 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 and 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 **identified as** *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 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 *Photorhabdus*[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 former 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 (*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 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 faba bean (*Vicia faba* 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% Na₂HPO₄; 0,024% KH₂PO₄) [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 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 (HgCl₂) (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. 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 solubilize insoluble phosphate was described by the solubilization index (SI) = the ratio of the total diameter (colony + halo zone) to the colony diameter[17].

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 ($5 \times 10^8 \text{ CFU ml}^{-1}$). 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
97 stresses. Growth in Lysogeny broth (LB) agar at different temperatures (from 10 to 50 °C),
98 pH (from 4.8 to 8.8) and salinities (from 0.5 to 2 $\text{g L}^{-1} \text{NaCl}$) 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.

109 2.6. Molecular identification based on 16S rRNA gene sequence

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

124 2.7. Statistical analysis

125 Simple correlation was run to determine correlation coefficients (r) by the method of
126 Ordinary Least Squares (OLS). The result means were depicted diagrammatically using
127 Microsoft Excel version 2010.

128

129 3. RESULTS AND DISCUSSION

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

140 A total of seventy Eight bacteria strains (encoded FN and FR) were isolated on NBRIP
 141 plates from the rhizosphere of faba bean collected from Meknes region in Morocco.
 142 Most of PSB isolated strains in this study lost their ability to form halozone on agar
 143 medium on repeated subculturing. However, only four strains maintained phosphate
 144 solubilization activity as reflected by formation of a translucent to clear around their
 145 colonies in the NBRIP agar after 72-120 h of growth at 27 °C. This result is in
 146 accordance with Illmer and Schinner [21] and Rodriguez and Fraga [3]. Solubilization
 147 index based on colony diameter and halozone for each PSB isolate is presented in Table
 148 1. Results showed that among PSB, FN30 was the most efficient phosphate solubilizer
 149 on NBRIP plates with SI = 4.65. Generally, halozone increased with increase in colony
 150 diameter. These results are in accordance with that of Baig et al. [22] and Yang et al.
 151 [23]. The P-solubilizing activity of these isolates varied from 62.45 to 119.95 µgm⁻¹ in
 152 NBRIP broth at 27 °C in seven days (Table 1). However, in this assay, the maximum P
 153 solubilization achieved with FN30 (119.95 mg/l) was followed by FN10 (84.44 mg/l). The
 154 results of this analysis were found in accordance with the previously reported studies
 155 [4, 24, 13, 25]. Drop in pH by PSB ranged from 4.1 to 3.5 at the end of incubation
 156 period (Table 1).

157 **Table 1. Tricalcium phosphate solubilisation by bacterial isolates in agar and**
 158 **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 (<i>Pseudomonas</i> sp.)	2,84	3,18	3,72	41,20	58,40	62,45	3,8	3,6	4,1
FR24 (<i>Rahnella</i> sp.)	2,84	3,28	3,73	48,98	56,89	68,33	3,9	3,7	3,8
FN10 (<i>Rhizobium</i> sp.)	3,17	3,23	3,51	29,12	47,87	84,44	4,5	4,1	3,6
FN30 (<i>Rhizobium</i> sp.)	2,88	3,96	4,65	64,94	80,96	119,95	4,8	4,6	3,5

159 *Initial pH = 6.8

160 The release of soluble phosphate in the broth was associated with a concomitant
 161 decrease in pH. Simple regression analysis suggest that there is a significant negative
 162 correlation ($r = -0.71$) between the soluble phosphate released and pH (Fig. 1). This
 163 observation is consistent with previous reports demonstrating that phosphate
 164 solubilization was paralleled with decrease in pH [26, 4, 27]. On the contrary Yang et al.
 165 (2012) [23] reported no significant negative correlation between the final culture pH and
 166 tri-calcium phosphate solubilization activity ($r = -0.15$).

167

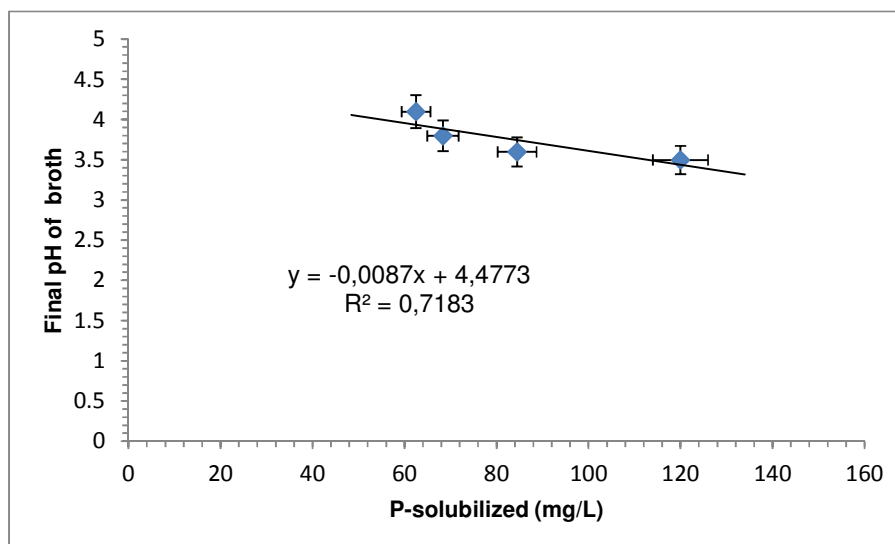


Fig. 1. Correlation analysis between final culture pH and tricalcium phosphate solubilizing ability of phosphate solubilizing bacteria.

Those isolates presumably identified as PSB further characterized by a series of biochemical **reactions**. So, they were Gram- negative, with positive for catalase activity (except FR18 and FN30). All strains have been negative for oxidase activity, 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 °C, but some of them were also able to grow at 50 °C (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 Chaiarn and Lumyong[29]. Regarding the intrinsic resistance, all PSB isolated strains in this study showed a **good** resistance to different antibiotics (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	-	-	-	-

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

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

^cColony 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 ^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	-	+	±	±	+	+	+	±	+	+	+	+	±	+	+	±	-

^a+: Growth, ±: weak growth, -: no growth

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

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), *Rahnella aquatilis* (FR24), *Rhizobium leguminosarum* (FN10) and *Rhizobium pusense* (FN30). The similarity among the sequences studied through GenBank access was between 97 and 99 % (Table 4).

224

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

Isolate	Length of 16S rDNA sequenced (bp)	GenBank accession no.	Closest relative species	Similarity (%)
FR18	1239	NR102835	<i>Pseudomonas fluorescens</i> Pf0-1	97
FR24	1545	NR074921	<i>Rahnella aquatilis</i> HX2	99
FN10	1475	NR036938	<i>Rhizobium leguminosarum</i> 3Hoq18	98
FN30	1379	NR116874	<i>Rhizobium pusense</i> NRCPB10	99

226

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 $\text{Ca}_3(\text{PO}_4)_2$ 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 of P. Also, all of the bacterial strains isolated could reduce the plant stress against extreme temperature, salinity, 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.

248

REFERENCES

- 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.
- Antoun H. Beneficial microorganisms for the sustainable use of phosphates in agriculture. *Procedia Eng.* 2012;46:62–67.
- Rodríguez H, Fraga R. Phosphate solubilizing bacteria and their role in plant growth promotion. *Biotechnol Adv.* 1999;17:319–339.
- 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.

259

- 260 5. Ribeiro CM and Cardoso EJBN. Isolation, selection and characterization of root-
261 associated growth promoting bacteria in Brazil pine (*Araucaria angustifolia*).
262 Microbiol Res. 2012;167:69–78.
- 263 6. Ullah I, Khan AR, Park GS, Lim JH, Waqas M, Lee IJ, and Shin JH. Analysis of
264 Phytohormones and Phosphate Solubilization in *Photorhabdus* spp. Food
265 SciBiotechnol. 2013;22:25-31.
- 266 7. Nautiyal CS. An efficient microbiological growth medium for screening phosphate-
267 solubilizing microorganisms. FEMS MicrobiolLett. 1999;170:265–270.
- 268 8. Gopalakrishnan S, Humayun P, Kiran BK, Kannan IKG, Vidya MS, Deepthi K,
269 Rupela O. Evaluation of bacteria isolated from rice rhizosphere for biological control
270 of charcoal rot of sorghum caused by *Macrophominaphaseolina* (Tassi) Goid. World
271 J MicrobiolBiotechnol.2011;27:1313–1321.
- 272 9. Bakhshandeh E., Rahimian H., Pirdashti H., Nematzadeh G.A.Phosphate
273 solubilization potential and modeling of stress tolerance of rhizobacteria from rice
274 paddy soil in northern Iran. World J MicrobiolBiotechnol. 2014;doi: 10.1007/s11274-
275 014-1669-1.
- 276 10. Ahemad M, Kibret M. Mechanisms and applications of plant growth promoting
277 rhizobacteria: current perspective. J King Saud Univ Sci.2014;26:1–20.
- 278 11. Nadeem SM, Zahir ZA, Naveed M, Arshad M, Shahzad S. Variation in growth and
279 ion uptake of maize due to inoculation with plant growth promoting rhizobacteria
280 under salt stress. Soil Environ.2006;25:78–84.
- 281 12. Lavakush JY, Verma JP. Isolation and characterization of effective plant growth
282 promoting rhizobacteria from rice rhizosphere of Indian soil. Asian J Biol
283 Sci.2012;5:294–303.
- 284 13. Leandro FA, Gleika LODS, Silvia N, Adelica AX, Marcia RC, AcleideMSC, Marlon
285 CTP and Débora FGSP. Analysis of the Abilities of Endophytic Bacteria Associated
286 with Banana Tree Roots to Promote Plant Growth. J Microbiol. 2014;52(1):27–34.
- 287 14. Araújo WL, Lima AOS, Azevedo JL, Marcon J, Sobral JK, Lakava PL, editors.
288 Manual de isolamento de microrganismosendofíticos. Departamento de Genética –
289 Escola Superior de Agricultura “Luiz de Queiroz” – Piracicaba ESALQ – USP ;
290 2002. Portuguese.
- 291 15. Vincent JM. The cultivation, isolation and maintenance of rhizobia. In :Vincent JM,
292 editors.A Manual for the PracticalStudy of Root-Nodule. Oxford: Blackwell Scientific
293 Publications; 1970.
- 294 16. Gyaneshwar P, Parekh LJ, Archana G, Poole PS, Collins MD, Hutson RA, Kumar
295 GN. Involvement of a phosphate starvation inducible glucose dehydrogenase in soil
296 phosphate solubilization by *Enterobacterasburiae*. FEMS MicrobiolLett.
297 1999;171:223–229.
- 298 17. Edi–Premono M, Moawad MA, Vleck PLG. Effect of phosphate solubilizing
299 *Pseudomonasputida* on the growth of maize and its survival in the rhizosphere.
300 Indonasian J Crop Sci. 1996;11:13–23.
- 301 18. Murphy J, Riley JP. A modified single solution method for the determination of
302 phosphate in natural waters. Anal Chim Acta. 1962;27:31–36.
- 303 19. Holt JG, Krieg NR, Sneath PHA, Stanley JT, William ST, editors.Bergey’sManual
304 of Determinative Bacteriology. 9th ed.Lippincott Williams and Willikins: Baltimore
305 Maryland USA; 1994.
- 306 20. Weisburg WG, Barns SM, Pelletier DA, Lane DJ. 16S ribosomal amplification for
307 phylogenetic study. J Bacteriol. 1991;173:697-703.
- 308 21. Illmer P, Schinner F.Solubilization of inorganic calcium phosphates-
309 solubilizationmechanisms. Soil Biol. Biochem.1995;27:257–263.
- 310 22. Baig KS, Arshad M, Zahir ZA, Cheema MA. Comparative efficacy of qualitative and
311 quantitative methods for rock phosphate Solubilization with phosphate solubilizing
312 rhizobacteria. Soil Environ. 2010;29:82–86.
- 313 23. Yang PX, Ma L, Chen MH, Xi JQ, He F, Duan C Q, Mo MH, Fang DH, Duan YQ,
314 Yang FX. Phosphate solubilizing ability and phylogenetic diversity of bacteria from
315 P-rich soils around Dianchi Lake drainage area of China. Pedosphere.2012;22:707–
316 716.
- 317 24. Chatli AS, Beri V, Sidhu BS. Isolation and characterisation of phosphate solubilising
318 microorganisms from the cold desert habitat of *Salix alba* Linn. in trans Himalayan
319 region of Himachal Pradesh. Indian JMicrobiol. 2008;48:267–273.

- 320 25. Elizabeth A, Tania GC, Fausto P, Mónica N, HernánMR. Phosphate-solubilizing
321 microorganisms associated with the rhizosphere of oil palm
322 (*Elaeisguineensis*Jacq.) in Colombia. Appl Soil Ecol. 2014;80:26–33.
- 323 26. Hwangbo H, Park RD, Kim YW, Rim YS, Park KH, Kim TH, Suh JS, Kim KY. 2-
324 Ketogluconicacid production and phosphate solubilization by *Enterobacterinter*
325 medium. CurrMicrobiol. 2003;47:87–92.
- 326 27. Yu X, Liu X, Zhu TH, Liu GH, Mao C. Isolation and charac-terization of phosphate-
327 solubilizing bacteria from walnut and their effect on growth and phosphorus
328 mobilization. BiolFert Soils.2011;47:437–446.
- 329 28. Thakuria D, Talukdar NC, Goswami C, Hazarika S, Boro RC, Khan MR.
330 Characterization and screening of bacteria from rhizosphere of rice grown in acidic
331 soils of Assam. Curr Sci. 2004;86:978–985.
- 332 29. Chaiarn M,Lumyong S. Phosphate solubilization potential and stress tolerance of
333 rhizobacteria from rice soil in Northern Thailand. World J MicrobiolBiotechnol.
334 2009;25:305–314.
- 335 30. Araujo FF, Guaberto LM, Silva IF. Bioprospection of plant growth promoter
336 rhizobacteria in *Brachiariabrizantha*. Rev Bras Zootec. 2012;41:521–527.
- 337 31. Luo S, Xu T, Chen L, Chen J, Rao C, Xiao X, Wan Y, Zeng G, Long F, Liu C, Liu Y.
338 Endophyte assisted promotion of biomass production and metal-uptake of energy
339 crop sweet sorghum by plant-growth-promotion endophyte *Bacillus* sp. SLS18.
340 ApplMicrobiolBiotechnol. 2012;93:1745–1753.
- 341 32. Atemkeng MF, Remans R, Michiels J, Tagne A, NgonkeuELM. Inoculationwith
342 *Rhizobium etli* enhances organic acid exudation in common bean (*Phaseolus*
343 *vulgaris* L.) subjected to phosphorus deficiency. Afr J Agric Res. 2011;6: 2235–
344 2242.
- 345 33. Azziz G, Bajsa N, Haghjou T, Taulé T, Valverde A, Igual JM, Arias A. Abundance,
346 diversity and prospecting of culturable phosphate solubilizing bacteria on soils
347 under crop-pasture rotations in a no-tillage regime in Uruguay. Appl Soil Ecol.
348 2012;61:320–326.

349