Isolation and Identification of Potential Phosphate Solubilizing Bacteria from the Rhizosphere of *Lupinus hirsutus* L. in the north of Morocco

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Abstract

The use of biological approaches instead of chemicals to improve agricultural production has captured the interest of agronomists for a long time. With the aim to select beneficial bacteria exhibiting several plant growth promoting (PGP) traits**,** 44 bacteria were isolated from the rhizosphere of the legume *Lupinus hirsutus* L., and tested for solubilization of tricalcium phosphate (Ca_3 $(PO_4)_2$). Of 35 phosphate solubilizing rhizobacteria, 14 isolates were selected for their solubilization diameters (0.6-1cm). Four bacteria were able to produce indole acetic acid (IAA), while none was positive for hydrocyanic acid (HCN). Other PGP traits (siderophores production, Atmospheric nitrogen fixation and ACC deaminase) were searched for these 4 bacteria. Except the isolate RL5 strain which showed ACC deaminase activity, all strains were unable to produce siderophores or to fixe nitrogen. Phosphorus solubilizing ability of these 4 strains was tested in liquid medium with 0.5% $Ca_3(PO_4)_2$, and the values of soluble P were ranged between 81.94 and 298.66 mg / l after 7 days of incubation, obtained respectively by RL77 and RL10. This bacterial solubilization of P was accompanied by a fall in pH to pH 4.83 in presence of RL10. These isolates were shown to belong to the genus *Enterobacter*. The rice inoculation test (Puntal variety) was performed in pots substituting soluble P by Ca_3 $(PO_4)_2$. Significant increases were obtained for shoot length and dry weight of plants in presence of RL5, RL10 and RL16, as compared to the control. However, RL77 showed a significant decrease of the different parameters.

Keywords: rhizosphere; *Lupinus hirsutus*; legume; PGP traits; phosphate solubilization; *Enterobacter*; rice.

Introduction

Phosphorus (P) is one of the essential macronutrients for plant growth and reproduction. However, it is a limiting factor in many soils, because an important part of this element is insoluble (Del Campillo *et al*., 1999). Mineral P solubilization is a common phenotype in several rhizobacteria, hence the term "phosphate solubilizing bacteria" PSB (Pérez *et al*., 2007). The application of these bacteria in the soil can increase plant productivity by improving P nutrition (Hameeda *et al*., 2008). These PSB also can stimulate plant growth by other mechanisms such as the production of phytohormones, nitrogen fixation, inhibition of phytopathogenic microorganisms,

production of siderophores and ACC deaminase (Bhattacharyya & Jha, 2012).

Consequently, the application of PSB as inoculants represents the most promising solution to mobilize large reserves of insoluble P in the soil. The long-term goal of our work is to explore the PSB naturally colonizing plants rhizosphere to develop biofertilizers for rice and assure a sustainable agriculture in the future for this cereal. With this objective in mind, the present study was conducted to isolate, select and characterize PSB with other PGP activities, from rhizosphere of the legume *Lupinus hirsutus* and test their effect as inoculum on rice.

Materials & Methods

Isolation and selection of PSB

Two grams of rhizospheric soil of the legume *Lupinus hirsutus* collected from Tanakoub in the province of Chefchaouen (Latitude 35 ° 5 '27.6 "N and longitude 5 ° 25' 19.2" W; Altitude 670m) were dissolved in 18ml of sterile physiologic water, and then serially diluted up to 10^{-7}. Then after 100µl of each dilution were plated on tryptic soy agar (TSA) containing casein peptone15g, soya peptone 5g, sodium chloride 5g, agar 15g, pH 7.3 in 1 L distilled water. After incubation for 3 days at 28 ° C, bacteria were purified on the same medium. To select the PSB, the isolates were streaked on PVK agar (Pikovskaya, 1948) with 0.5% $Ca_3(PO_4)_2$, incubated at 28 ° C. Only colonies surrounded by clear halos were selected.

Production of indole acetic acid (IAA)

To detect the IAA production, 10µl of bacterial culture were deposited on a nitrocellulose membrane placed on TSA contained 0.05% tryptophan. After incubation at 28 ° C, the membrane is located on filter paper soaked with Salkowski reagent (2% FeCl3 (0.5M), 35% perchloric acid) (Bric *et al*., 1991). Development of pink halo around the bacterial colony indicated IAA production.

Producing hydrogen cyanide (HCN)

To estimate HCN production, 100µl of bacterial culture were streaked on TSA supplemented with 4.4g/l glycine. Filter paper discs (9cm diameter) soaked in 2% sodium carbonate in 0.5% picric acid solution were placed in the lid of each Petri dish (Bakker & Schippers, 1987). The plates were sealed with parafilm and incubated at 28 ° C. Change in color from yellow to orange or brown indicated the synthesis of HCN production.

Production of siderophores

The bacteria were spot inoculated on TSA and the plates were incubated for 3 days at 28 ° C. A layer of chrome azurol S medium (CAS) (Schwyn & Neilands, 1987) was poured on the surface of these plates. After 24 h in the dark, change in color of CAS medium from blue to orange indicated the production of siderophores.

Nitrogen fixation

Tubes containing 3ml semisolid N-free Burk medium (Burk, 1930) were inoculated with 100µl of bacterial culture, sealed and incubated at 28 ° C. After 24 h, 1 ml acetylene was injected in each tube and incubated again. After each 72 h, ethylene formation was measured by the gas chromatography as this gas is proportional to the rate of N_2 fixed.

ACC deaminase activity

Bacterial cultures grown in Tryptic Soy Broth (TSB) and washed

with sterile physiological water were used to inoculate tubes of M9 medium contained 3 mM ACC as the sole source of nitrogen and M9 without ACC, and then incubated at 28 ° C. The absorbance was recorded after 24h and 48h at 600 nm. Strain having ACC deaminase activity provided a high value in the ACC tube.

Activity estimate solubilisatrice of Ca3 (PO4) 2 and pH

The PSB were inoculated in 50ml PVK broth. Controls consisted of the uninoculated culture medium. The cultures were incubated at 28 ° C with shaking for 7 days. The media was centrifuged at 13,000 rpm for 20 min and the P of supernatant was determined by the colorimetric method (Ames, 1966). Dissolved P concentration was determined by subtracting the concentration of soluble P of control from the concentration of soluble P obtained in the inoculated media. The pH was determined using a pH meter.

Identification of PSB by 16S rDNA sequence

Total genomic DNA was extracted with the Quantum prep Aquapure Genomic DNA kit (Bio-Rad). Amplification of 16S rDNA using universal bacterial primers fD1 (5'-AGAGTTTGATCCTGGCTCAG-3') and rD1 (5'-AAGGAGGTGATCCAGCCGCA-3') was carried out in a 20µl final volume containing 0.2 mM of each primer, 0.2 mM dNTPs, 1X PCR buffer and 0.1 U of Taq polymerase. The reaction mixture was incubated in a thermocycler under the following conditions: an initial denaturation for 5 min at 95 °C, followed by 35 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 45s and extension at 72 °C for 2 min. PCR products were purified by PCR Clean-up Gel Extraction kit (Macherey-Nagel, Germany) and sequenced. The nucleotide sequences obtained were compared using the BlastN program on the page of NCBI (www.ncbi.nlm.nih.gov / blast / Blast.cgi).

Inoculation assays of rice

Rice (*Oryza sativa*, Puntal variety) was used to evaluate the performance of strains under culture chamber conditions. The seeds were surface sterilized by soaking in 95% ethanol for 1 min then in 1.2% sodium hypochlorite for 20 min, and rinsed 5 times in sterile distilled water and placed on plates of agar/water 1% (w / v) to germinate. Each pot (12×18cm) filled with vermiculite/perlite (4:1) and 200 ml of nutrient solution (Rigaud & Puppo, 1975), received 230µl of 10% $Ca_3 (PO_4)_2$ as the sole source of P, then autoclaved. Every pot was sowed by 5 seeds and each seed was inoculated directly with 1 ml of bacterial culture. Uninoculated pot was negative control. Uninoculated pot that contained soluble P in the form PO_4H_2K was considered as positive control. The pots were placed in the growth chamber under controlled conditions: 16h day at 26°C and 8h night at 18 °C, and a light intensity of 400µE m^{-2} s^{-1}. Three repetitions were made for each bacterial isolate. After 30 days of growth, plants were harvested, washed with tap water and dried in at 80 °C for 24 h. The dry weight of plants and shoot size were noted.

Statistical analysis

The data are reported as means ± SD (standard deviation) for three replicates. The results were compared by analysis of variance (ANOVA) according to Fisher protected LSD test ($p < 0.05$) using the Statgraphics Plus version 4.0.

Results and Discussion

Isolation of PSB

Many studies have demonstrated the ability of bacteria isolated from the rhizosphere of different plants to dissolve insoluble P (Chung *et al.*, 2005; Pérez *et al.*, 2007, Chen *et al.*, 2008; Keneni *et al.*, 2010; Prasanna *et al.*, 2011, Kumar *et al.*, 2012). In the present work, 79.5% out of the 44 isolates were able to solubilize $Ca_3(PO_4)_2$. Based on the diameter of the solubilizing halos on PVK medium ranged between 0.6cm and 1cm, 14 bacteria were screened for further analysis. According to the Gram stain, these isolates were all Gram negative.

PGP activities, direct and indirect

PSB can improve plant growth thanks to other activities. The first PGP characters searched in this study were the production of IAA and HCN. Among these selected 14 PSB, IAA production was shown in 4 bacteria (Table 1). This phytohormone is one of the auxins the most physiologically active and it is the product common of tryptophan metabolism by several microorganisms (Gravel *et al.*, 2007). No isolate was able to produce HCN, the gas that plays an indirect role in biocontrol. For other promoting activities evaluated for these four isolates, no bacteria produced siderophores or fixed nitrogen. As for the ACC deaminase, RL5 strain was the only one that showed this activity (Table 1). This enzyme indirectly stimulates root growth by hydrolyzing the ACC, the precursor of ethylene that inhibits root elongation (Gravel *et al.*, 2007).

Solubilization of P in the liquid medium

The four selected bacteria showed different abilities to dissolve soluble P from $Ca_3(PO_4)_2$ (Table 2). The maximum P solubilization was recorded by RL10 (298.66 mg/l) compared to other isolates RL5, RL16 and RL77 (272, 264.31 and 81.94 mg/l respectively).

Table 1. Direct and indirect PGP traits.

PGP traits	RL5	RL10	RL16	RL77
IAA	+	+	+	+
HCN	-	-	-	-
Siderophores	-	-	-	-
Dinitrogen fixation	-	-	-	-
ACC deaminase	+	-	-	-

This solubilization was accompanied by a significant drop in pH (especially in presence of RL10, RL15 and RL16) as compared to control (pH 7.0) (Table 2). A significant negative correlation ($r = -0.91$; $p < 0.01$) was observed between P concentration and pH, which has been founded also by a previous study (Keneni *et al.*, 2010). According to several studies, this acidification is caused by the production of organic acids by bacteria, and the negative relationship between pH and P indicates the significant role of these organic acids in mineral P solubilization (Chen *et al.*, 2006; Pérez *et al.*, 2007). Nevertheless, Hamdali *et al.* (2008) have reported alkalinization during the solubilization of natural P by Actinomycetes isolated from Moroccan phosphate mines and absence of organic acids in the growth medium. This explains the involvement of other mechanisms in the process of inorganic P solubilization as the production of siderophores (Hamdali *et al.*, 2008; 2012).

	RL5	RL10	RL16	RL77
P*(mg/l)	272±3.48a	298.66±2.88b	264.31±13.75a	81.94±10.25c
pH*	5±0.01a	4.83±0.01a	4.87±0.02a	5.75±0.44b

Table 2. Solubilization of P and pH of the culture medium.

* Data in the same line followed by the same letter are not significantly different according to Fisher protected LSD test ($p < 0.05$). Correlation between P concentration and the pH was $r = -0.91$ ($p < 0.01$).

Molecular identification

Based on 16S rDNA sequences, the four strains showed maximum similarity (97% or more) with the genus *Enterobacter* (Table 3).

Previous studies have reported the ability of certain strains *Enterobacter* spp. to solubilize inorganic P (Chung *et al*., 2005), and to stimulate plant growth (Chabot *et al*., 1993; Shoebitz *et al*., 2009). And recently, an *Enterobacter* sp. strain with multiple PGP activities was reported as bacterial phytostimulator for maize and rice (Frank & Julius 2012).

Table 3. Identification of PSB isolates by 16S rDNA sequencing.

Isolates	Length of 16S rDNA gene sequenced	Most closely bacteria Species (strain)	% Identity
RL5	1014	*Enterobacter aerogenes* (JCM1235)	97%
RL10	999	*Enterobacter ludwigii* (EN-119=DSMZ 16688=CIP 108491)	98%
RL16	935	*Enterobacter amnigenus* (JCM1237)	98%
RL77	1071	*Enterobacter cancerogenus* (LMG 2693)	99%

Effect of selected strains on rice growth

The inoculation results showed a significant stimulating effect of strains on rice growth ($p < 0.05$) with respect to negative control (C-) (Figure 1). Indeed, after 30 days of growth, a significant stimulation of shoot length was registered in the presence of RL5, RL10 and RL16, reaching 53% for RL10 and the dry weight was also increased especially in the presence of RL5 strain getting to 71% for shoots and 51% for roots (Figure 1). However, RL77 was the only strain that showed a significant reduction of dry weight of rice. This could be explained by a decrease in its P solubilization ability with respect to the other strains (Table 2). The stimulation of the growth of rice inoculated by RL5, RL10 and RL66 reported in this work could be mainly caused by the solubilization of P and also the ACC deaminase activity. Similar results have been reported about the use of PSB as bioinoculum and their promoting effect on rice germination and its productivity (Meunchang *et al*., 2006, Ng *et al*., 2012). Moreover, it has been shown that the inoculation by PSB stimulates the growth of other cereals such as maize (Hameeda *et al*., 2008; Frank & Julius, 2012) and wheat (Afzal & Asghari, 2008).

Conclusion

The present study demonstrated the importance of some free PSB with various PGP activities living in the rhizosphere of the natural legume. RL5, RL10 and RL16 strains could be applied as biofertilizers based on positive effect showed on the growth of rice under chamber conditions. However, tests in the field are required to complete this

work, in order to evaluate the effect of biotic and abiotic factors on these strains.

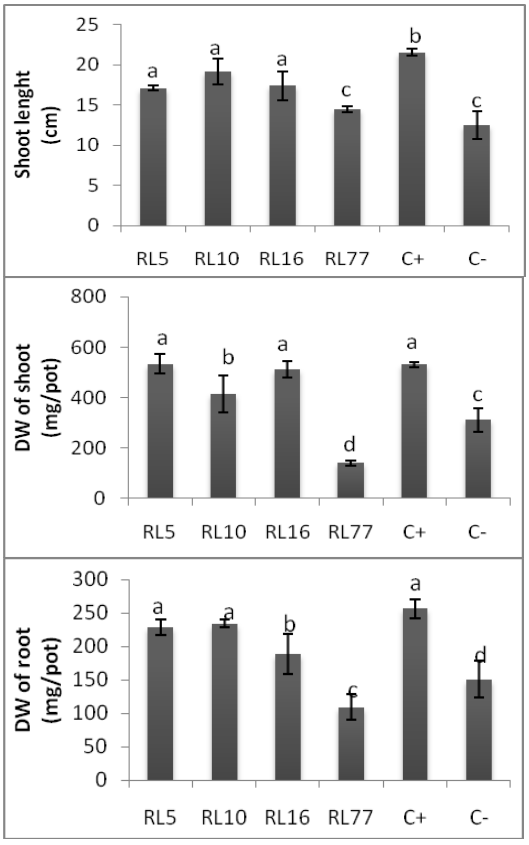

Figure 1. Shoot length and dry weight (DW) of shoots and of roots of inoculated plants after 30 days of growth in pot (mg/pot). The letters on the bars of the same parameter indicate significant differences according to Fisher protected LSD test ($p < 0.05$).

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