Isolation and screening of inorganic phosphate solubilizing *Pseudomonas* strains from rice rhizosphere soil from Northwestern Morocco

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ABSTRACT

In order to make rice cultivation sustainable and less dependent on phosphate fertilizers, the use of phosphate solubilizing bacteria (PSB) exhibiting other beneficial traits is a promising alternative that can contribute to rice growth improvement. With the aim to develop biofertilizer, a total of 283 bacteria belonging to *Pseudomonas* were isolated from the rice rhizosphere of which 67.14% was capable of solubilizing tricalcium phosphate (TCP). Based on diameter of solubilization halos (diameter $\geq$ 0.4 cm), 73 strains were selected and evaluated for hydrogen cyanide production. Nine bacteria were chosen to be explored for more activities. All isolates were siderophores producers but none was able to fix nitrogen. Only two bacteria, PE76 and PE77, were positive for the 1-aminocyclopropane-1-carboxylate deaminase activity. Amounts of indole acetic acid produced by test bacteria ranged between 0.68 and 20.46 mg l^{-1}. They were also checked too for quantitative assay of TCP solubilization and thus, phosphorus concentrations were between 70.98 and 608.28 mg l^{-1}. Four bacteria were selected for rice inoculation substituting soluble P by TCP. Significant increases in plant length and dry matter were obtained with two strains PP22 and PG1 after 30 days under controlled conditions. This stimulation is possibly not the sole result of increasing phosphate availability in the medium. Because these two efficient strains produced IAA in high concentrations in comparison with bacteria PE77 and PE76, which may partly explain the observed increases. However, more evaluation of PP22 and PG1 under field conditions is needed before recommending them as biofertilizers.

Key words: biofertilizers, IAA, PGPR, PSB, *Pseudomonas*, Rice

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INTRODUCTION

Phosphorus (P) is a major element for plant nutrition. It is often a limiting mineral nutrient for many agricultural crops because it is abundant in many soils but in poorly assimilable forms (Harris et al., 2006). Plants utilize fewer amounts of applied phosphate fertilizers and the rest is rapidly converted to insoluble complexes (Khan et al., 2006). This leads to a permanent need for chemical inputs that generate economic and ecological complications (Harris et al., 2006**;** Khan et al., 2006). In calcareous soils like most Moroccan soils, the soluble phosphates are fixed by calcium forming calcium phosphates (CaP) which is poorly soluble. In last few decades, several studies have demonstrated the ability of different bacterial genera to dissolve insoluble mineral phosphate compounds, and strains from *Pseudomonas* are among the most powerful PSB (Ahmad et al., 2005; Gravel et al., 2007; Chen et al., 2008 ; Muleta et al., 2013; Rajkumar et al., 2008; Ramyasmruthi et al., 2012).

Besides providing P to the plants, numerous strains of *Pseudomonas* also have been reported to exhibit many other plant growth promoting traits as the ability to produce phytohormones such as indole acetic acid (Ahmad et al., 2005), 1-aminocyclopropane-1-carboxylate deaminase (Glick et al., 2007) and asymbiotic N_2 fixation (Gull and Hafeez, 2012). Furthermore many studies have demonstrated that the most efficient antagonists in the soil belong to this genus (Haas and Defago, 2005; Bakker et al., 2007; Rajkumar et al., 2008; Ramyasmruthi et al., 2012). These multiple intrinsic characteristics give a particular interest to this genus for its use in biofertilization and biocontrôle for the improvement of agriculture. Therefore, several studies in both laboratory and field trials have reported plant growth stimulation and yield increase by *Pseudomonas* strains either for rice (Lucas et al., 2009; Nico et al., 2012) or other crops such as tomato (Gravel et al., 2007), pepper (Rajkumar et al., 2008), sweet marjoram (Banchio et al., 2008), mung beans (Shahab et al., 2009), eggplant (Ramesh et al., 2009), and wheat (Gull and Hafeez, 2012). Hence, this category of bacteria is included under the term Plant Growth Promoting Rhizobacteria (PGPR) that was coined for the first time by Joe Kloepper in the late 1980s. The role of these beneficial bacteria in plant growth promotion has recently been reviewed (Hayat et al., 2010).

The development of biofertilizers with rhizobacteria, exhibiting different plant growth promoting (PGP) activities, can minimize or even replace the use of chemical fertilizers ensuring sustainable agriculture. To this end, the present work focused on the screening of PSB belonging to *Pseudomonas* isolated from the rhizosphere of rice grown in the Northwest of Morocco for their multiple PGP activities *in vitro* and estimation of their effect as inoculants on rice growth in pots.

MATERIALS AND METHODS

Isolation of *Pseudomonas* spp

All the isolates of *Pseudomonas* strains were isolated from the rhizospheric soil of three varieties of rice (Puntal, Elio and Guadiamar) grown in Northwest of Morocco. Two grams of rhizospheric soil were suspended in 18 ml of sterile physiologic water. After 1h of agitation (200 rpm, 28 °C), aliquots of 100 µl of each dilution (10^{-1} to 10^{-7}) were plated on King's B medium (King et al.,

1954) and incubated at 28 °C for 24-48 h. Colonies were isolated and purified on the same medium.

Selection of PSB

All isolates were first screened for their ability to dissolve inorganic phosphate on Pikovskaya's (PVK) (Pikovskaya, 1948) agar plates containing 0.5% TCP as P source. The inoculated plates were incubated at 28 °C for 7seven days. We measured, for each isolate, the total diameter (diameter of the halo + colony diameter) and the diameter of colony. The diameter of the halo of solubilization was calculated by subtracting the colony diameter from the total diameter. Only PSB that gave halos with diameters ≥ 4 cm were selected and conserved in 25% sterile glycerol at -20 °C.

***In vitro* screening of isolates for multiples (PGP) activities**

Hydrogen cyanide (HCN) production

To estimate HCN production, 100 µl of bacterial cultures was streaked on tryptic soy agar (TSA) amended with 4.4 gl^{-1} glycine. A Whatman filter paper no 1 soaked in 2% sodium carbonate in 0.5% picric acid solution was placed in the top of each plate (Bakker and Schippers, 1987). Plates were sealed with parafilm and incubated at 28 ± 2 °C. Development of orange to red colour indicated HCN production.

Siderophores production

The plates of TSA were spot inoculated with test bacteria and incubated at 28 ± 2 °C for three days. A layer of chrome azurol S medium (CAS) (Schwyn and Neilands, 1987) was poured on the surface of each plate. After 24 h in the dark, development of orange halo around the bacteria was considered as positive for siderophores production.

Nitrogenase activity

Tubes containing 3 ml of semisolid N-free Burk medium (Burk, 1930) were inoculated with 100 µl of bacterial culture, sealed and incubated at 28 ± 2 °C. After 24 h, air was replaced with 1 ml acetylene in each tube. As ethylene is proportional to the rate of N_2 fixed, formation of this gas was measured by the gas chromatography after each 72 h of incubation.

Aminocyclopropane-1-carboxylate (ACC) deaminase activity

Bacterial cultures grown in TSB and washed with sterile physiological water were used to inoculate tubes of M9 Minimal Medium containing per liter of distilled water: Na_2HPO_4. 7 H_2O, 12.8 g, KH_2PO_4, 3 g, NaCl, 0.5 g, NH_4Cl, 1 g, $MgSO_4$ (1 M), 2 ml, glucose (20%), 20 ml, $CaCl_2$ (1 M), 0.1 ml, and 3 mM ACC as sole nitrogen source. M9 medium without ACC served as a control. All inoculated tubes were incubated at 28 °C. The absorbance was recorded after 24 h and then after 48 h at 600 nm. Strains having ACC deaminase activity provided high values in the ACC tube.

Quantitative assay of indole acetic acid (IAA) production

Bacterial cultures grown in M9 Minimal Medium were used to inoculate other tubes containing the same M9 medium supplemented with tryptophan (500 µg ml^{-1}) and incubated at 28 °C for 24 h.

All media were centrifuged at 13,000 rpm for 3 min. The supernatant (1 ml) was mixed with 4 ml of Salkowski reagent and the development of a pinkish color indicated the production of IAA (Gordon and Weber, 1951). The optical density was taken at 535 nm after 20 min of incubation at room temperature. Concentrations of produced IAA were measured with the help of its standard curve.

Quantitative assay of P solubilization

The test isolates were inoculated in 50 ml PVK's broth (500 µl of 10^8 CFU ml^{-1}) and negative control consisted of uninoculated broth. All flasks were incubated at 28 ± 2 °C with shaking for seven days. The cultures were centrifuged at 13,000 rpm for 20 min and the P of supernatant was determined by the colorimetric method as described by Ames (Ames, 1966). The amount of soluble P was detected from the standard curve of KH_2PO_4. Dissolved P concentration was determined by subtracting the P concentration of control from the final concentration of soluble P obtained in the inoculated broths. The pH was determined using a pH meter.

Inoculation of rice

Oryza sativa (Puntal variety) was used to evaluate the performance of strains under culture chamber conditions. The seeds were surface sterilized by agitation in 95% ethanol for 1 min then in 1.2% sodium hypochlorite for 20 min, followed by five washings with sterile water. Seeds were then germinated in 1% agar water (w/v) plates for 48 to 72 h at 25°C. Each pot (12 cm diameter, 18 cm height) filled with vermiculite mixed with perlite in 4:1 ratio and 200 ml of nutrient solution (Rigaud and Puppo, 1975), received 230 µl of 10% TCP as the sole source of P, then autoclaved. Every pot was sowed by 5 germinated seeds and each seed was inoculated directly with 1 ml of bacterial culture (10^8 CFU ml^{-1}) grown in TSB. Uninoculated pots and uninoculated pots containing soluble P in the form PO_4H_2K were used as controls (negative (C-) and positive (C+) respectively). All pots were maintained at 26 ± 2 °C under a 16 h photoperiod and a light intensity of 400 µE $m^{-2}s^{-1}$. Three replications were maintained for each treatment. Plants were harvested after 30 days, washed and dehydrated at 80 °C for 24 h. The dry weight biomass and shoot size were measured.

Statistical analysis

The data are reported as means ± SD (standard deviation) for 3 replications or more. The results were subjected to analysis of variance (ANOVA) according to Fisher protected LSD test ($p < 0.05$) using the Statgraphics Plus version 4.0.

RESULTS

Isolation and selection of PSB

A total of 283 strains were obtained from the rhizosphere of three varieties of rice grown in the Northwest of Morocco, of which 67.14% were able to solubilize TCP. On the basis of halo's solubilization (diameter ≥ 0.4 cm), 73 strains were chosen for HCN test. Nine strains were selected: 6 isolates were HCN positive (PP7, PE15, PE76, PE77, PG1 and PG70) and 3 isolates with diameters ≥ 0.9 cm (PP16, PP22 and PP31) (Table 1).

Table1: Origin and biochemical tests of selected isolates

Isolates	Origin	Halos (cm)	HCN	Siderophores	Nitrogen fixation	ACC deaminase	AIA (mg/l)	P (mg/l)	pH
PP7	Puntal	1.1 (±0.12)	+	+	-	-	11.52^{d} (±0.55)	70.98^{a} (±4.09)	6.38^{f} (±0.04)
PP16	Puntal	1 (±0.12)	-	+	-	-	3.82^{b} (±0.42)	208.37^{d} (±7.61)	4.5^{b} (±0.05)
PP22	Puntal	0.9 (±0.21)	-	+	-	-	20.46^{f} (±1.84)	295.71^{f} (±6.63)	4.44^{b} (±0.08)
PP31	Puntal	1 (±0.15)	-	+	-	-	14.49^{e} (±0.61)	140.33^{c} (±2.73)	4.8^{c} (±0.08)
PE15	Elio	1.2 (±0.15)	+	+	-	-	13.99^{e} (±0.89)	91.1^{b} (±1.5)	5.88^{d} (±0.03)
PE76	Elio	0.8 (±0.15)	+	+	-	+	1.22^{a} (±0.39)	608.28^{h} (±2.96)	4.46^{b} (±0.02)
PE77	Elio	0.5 (±0.06)	+	+	-	+	0.68^{a} (±0.39)	339.39^{g} (±12.27)	4.81^{c} (±0.13)
PG1	Guadiamar	0.6 (±0.15)	+	+	-	-	10.66^{d} (±0.84)	235.69^{e} (±16.93)	4.30^{a} (±0.04)
PG70	Guadiamar	0.8 (±0.15)	+	+	-	-	5.97^{c} (±0.60)	87.67^{b} (±4.09)	6.23^{e} (±0.01)

Values are means of 3 replications or more. Means in the same column followed by the same letter are not significantly different at $P < 0.05$ (Fisher's least significant difference (LSD) test; ± values indicate tandard errors of the mea

***In vitro* PGP traits of test isolates**

Screening results of PGP activities of 9 selected bacteria are shown in Table 1. All isolates were able to produce siderophores, but none was able to fix atmospheric nitrogen. ACC deaminase was shown in only two bacteria, PE76 and PE77 (Table 1). IAA production was detected in all the nine isolates with different amounts. The highest concentration (20.46 mg l^{-1}) was produced by the isolate PP22 while the lowest values were presented by 0.68 and 1.22 mg l^{-1} obtained by PE77 and PE76 respectively (Table 1).

The quantitative test of phosphate solubilization was also checked for selected bacteria. The concentration of dissolved P was between 70.98 and 608.28 mg l^{-1} (Table 1). This TCP biosolubilisation was accompanied by a significant decrease in pH (6.38 to 4.30) compared to uninoculated control (pH 6.8 ± 2) (Table 1). Solubilized P values were significantly negatively correlated ($r = -0.66$, $p < 0.01$) with the final pH of the culture medium.

Effect of seed inoculation on rice growth

For plant inoculation test (*Oryza sativa*, Puntal variety), P was the limiting factor, as it is clearly shown by rice response to P fertilization (positive control, C+). After 30 days of growth, shoot length significantly increased by 90% and 98% in the presence of PP22 and PG1 respectively compared to the negative control (Table 2). Similarly, inoculation of rice with these both bacteria increased significantly dry matter by 140% and 160% for roots and by 65% and 68% for shoots respectively compared to non-inoculated control (C-) (Table 2). In comparison with C+, inoculated plants with isolates PP22 and PG1 showed non-significant difference for shoot dry matter, and we even had some important promotion for root dry matter.

Table 2. The shoot length, the dry matter of shoot and root of plants after 30 days of growth in pots

Isolates	Shoot length (cm)	Shoot dry weight (g/pot)	Root dry weight (g/pot)
PG1	24.77^{d} (± 0.88)	0.51^{c} (± 0.02)	0.36^{c} (± 0.04)
PP22	23.73^{d} (± 0.83)	0.52^{c} (± 0.02)	0.39^{c} (± 0.02)
PE76	10.51^{a} (± 0.15)	0.18^{a} (± 0.18)	0.11^{a} (± 0.02)
PE77	10.5^{a} (± 0.26)	0.19^{a} (± 0.01)	0.12^{a} (± 0.02)
C-	12.49^{b} (± 1.68)	0.31^{b} (± 0.05)	0.15^{a} (± 0.03)
C+	21.56^{c} (± 0.46)	0.53^{c} (± 0.01)	0.25^{b} (± 0.02)

The data presented are the mean of 3 replicates. Means in the same column followed by the same letter are not significantly different $P < 0.05$ (Fisher's least significant difference (LSD) test; ± values indicate standard errors of the means).

DISCUSSION

***In vitro* Plant growth promoting traits**

PGPR are free bacteria living in the rhizosphere of plants which exert a beneficial effect on plant growth. The mechanisms by which these PGPR contribute to plants growth improvement are either direct or indirect. Solubilization of inorganic phosphate precipitated in the soil and make it available to plants is one of the most important activities exhibiting by some PGPR termed PSB or Phosphobacteria (Chen et al., 2008; Muleta et al., 2013). The use of these PSB as inoculants represents a very promising approach to increase P bioavailability in soils (Chung et al., 2005; Ahmad et al., 2008; Oliveira et al., 2009). According to qualitative assay results of P solubilization, the rhizosphere of rice grown in Moroccan Northwest harbors PSB belonging to

the genus *Pseudomonas*, which are able to dissolve inorganic P. In this regard, several studies have shown that a high proportion of PSB is concentrated in the rhizosphere and *Pseudomonas* is among the genera that contain the most efficient PSB (Hayat et al., 2010; Muleta et al., 2013).

Many *Pseudomonas* strains have been reported as the most powerful antagonists by the production of several compounds that play a very important role in biological control (Haas and Defago, 2005). This bioantagonistic ability is mainly due to a synergistic combination of different metabolites such as siderophores, HCN, antibiotics and various hydrolytic exo-enzymes (Trivedi et al., 2008; Noori and Saud, 2012). In this study, HCN and siderophores production were exhibited by all isolated bacteria. These results are in accordance with Noori and Saud, (2012) who indicated that all strains identified as *Pseudomonas* spp. isolated from rice rhizosphere were all HCN and siderophores producers. Similar results were obtained by Gull and Hafeez, (2012) who reported the ability of *Pseudomonas* strains to produce HCN and different types of siderophores.

One of the important mechanisms that characterize PGPR is the nitrogen fixation to meet the need of the plant to this element. Although some *Pseudomonas* strains have been reported as free N_2 fixing bacteria (Gull and Hafeez, 2012), in our case, no bacteria have shown any positive nitrogenase. Deamination of ACC is another direct phytostimulator feature that may influence plant growth by reduction of ethylene levels (Glick et al., 2007). The enzyme catalyzing this reaction, ACC deaminase, hydrolyses ACC into ammonia and ketobutyrate, thereby promoting root elongation for inoculated plants (Glick et al., 2007). Among the nine selected bacteria, both PE76 and PE77 were capable to hydrolyze the ACC. According to these results, Gravel et al. (2007) also reported the production of this enzyme by *Pseudomonas putida* strain isolated from the rhizosphere of tomato. IAA is known as an important plant growth stimulating phytohormone produced by rhizobacteria to induce root elongation and proliferation of root hairs and lateral roots improving the mineral nutrition of the plant (Ahmad et al., 2005; Gravel et al., 2007; Shahab et al., 2009). As root tissues are especially sensitive to fluctuations in this phytohormone, its concentrations were evaluated. IAA production was a common trait in all selected bacteria which is in agreement with several studies that have reported the ability of *Pseudomonas* strains to produce this phytohormone (Joseph et al., 2007; Ahmad et al., 2008; Gull and Hafeez, 2012).

After confirming TCP solubilizing ability on solid medium, quantitative analysis of this activity was carried out. We found a negative correlation between pH values and P concentrations. Similar inverse relationship was also declared earlier by many studies that reported as well that the acidification of the medium is caused mainly by the production of organic acids by PSB (Chen et al., 2006; Pérez et al., 2007; Oliveira et al., 2009; Yu et al., 2011; Ng et al., 2012; Walpola and Yoon, 2013). Hence, the organic acids produced by PSB plays a significant role in the P solubilization. However, some studies have declared alkalinization during the rock phosphate solubilization and no production of organic acids was detected in the presence of actinomycetes isolated from Moroccan phosphate mines (Hamdali et al., 2008). This solubilization of rock phosphate has been attributed to some chelators, such as siderophores (Hamdali et al., 2008; 2012).

Effect of seed inoculation on rice growth

Rice is one of the most cultivated cereals in the world. In order to achieve a sustainable rice production, many authors have shown that inoculation of rice with PGPR could significantly increase different parameters such as plant height; root shoot biomass and grain yield and improve plant health (Beneduzi et al., 2008; Lucas et al., 2009; Ng et al., 2012). In the current study, inoculation with four selected bacteria has led to different results for the three parameters

studied in comparison with the non-inoculated control. The best results were obtained in the presence of both bacteria PP22 and PG1. While both bacteria PE76 and PE77 had no positive effect on rice growth even though they were the most powerful PSB in the liquid medium by giving the highest concentrations of solubilized P (608.28 and 335.69 mg l^{-1}), and they were the only ACC deaminase producers. Besides, the strain PE77 showed inhibition on shoot length compared to negative control. The two efficient rhizobacteria PP22 and PG1 were found to produce higher IAA than PE76 and PE77. It is assumed that PGPR producing plant hormones play an important role in growth and development of plants and IAA is one of the most physiologically active auxins (Ahmad et al., 2005). The production of this phytohormone by rhizobacteria is often associated with their ability to stimulate plant growth (Gravel et al., 2007; Shahab et al., 2009). Thus, the significant improvement obtained in the presence of PP22 and PG1 might be due to their ability to produce IAA at high concentrations (20.46 and 10.66 mg l^{-1} respectively) compared to PE76 and PE77 which produced low amounts of IAA.

The stimulating effect of phosphate-solubilizing *Pseudomonas* strains exhibiting other traits especially IAA production was confirmed by Shahab et al. (2009) who obtained a significant stimulation of the growth of mung beans treated with *Pseudomonas aeruginosa*, a phosphate solubilizer and IAA producer. Ramyasmruthi et al. (2012) as well, isolated a *Pseudomonas fluorescens* strain displaying varied growth promoting characters as phosphate solubilization, siderophores, HCN and IAA production and anti-fungal activity. They observed that bacterization of chilli seeds with this strain showed 100% germination index and almost 50% reduction in disease incidence by fungal phytopathogen *Collectotrichum gleosporidose* OGC1 suggesting both the biocontrôle and PGPR aspect of this strain (Ramyasmruthi et al., 2012).

Besides, the inoculation of rice with strains from other bacterial genera as *Bacillus* and *Serratia* characterized as IAA producers and phosphate solubilizers, showed significant increase in roots and shoot parts of plants (Beneduzi et al., 2008; Nico et al., 2012). Moreover, Ng et al (2012) assessed the role of the IAA-producing rhizobacteria in rice germination and seedling growth. They reported that the significant improvement of rice seed germination, radical length and vigor index obtained might be due to the production of IAA. Thus, to fully exploit the soluble P and other nutrients present in the soil, the plants need to have a well-developed root system to increase nutrients and water uptake from the soil. Consequently, phosphate dissolving rhizobacteria are of great importance for agriculture, but bacterial IAA increases root surface area and length, and thereby provides the plant greater access to nutrients and water (Ng et al., 2012).

In addition, different plant seedlings respond differently to variable auxin concentrations (Ahmad et al., 2005) and type of IAA-producing microorganisms (Shahab et al., 2009). Besides, one of two efficient bacteria, PP22, was isolated from the same variety of rice used for inoculation test (Puntal), showing the important effect of the origin of isolates on their efficiency. These results agree with those of Beneduzi et al. (2008) who had already demonstrated that strain SVPR30 was more efficient in providing benefits to the same variety of rice from which it was isolated. So, it is presumed that seed inoculation with IAA-producing PSB plays a critical role in plant growth promotion.

CONCLUSION

The present work highlights the importance of some PSB belonging to *Pseudomonas* present in the rhizosphere of rice grown in the Northwest of Morocco. The most effective isolates, PP22 and

PG1 could serve as biological inoculants for rice. Nevertheless, further experiments are needed to evaluate the beneficial effect of these two strains especially under field conditions in order to confirm their use as biofertilizers for rice.

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