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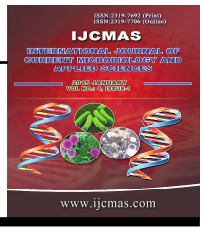

Original Research Article

Isolation and screening of bacteria from rhizospheric soils of rice fields in Northwestern Morocco for different plant growth promotion (PGP) activities: An *in vitro* study

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Biofertilizers, Phosphate Solubilization, PGPR, Rhizobacteria, Rice

A B S T R A C T

The development of a biological alternative to chemical fertilizers is of great importance for the improvement of agriculture as well as protection of the environment. Achieving this goal, 305 bacteria were isolated from the rhizosphere of rice fields in Northwestern Morocco, of which 136 rhizobacteria were tricalcium phosphate solubilizers. Based on diameter of solubilization halos, the 17 best phosphate solubilizers, Gram-negative, were selected. Six isolates were able to produce the indole acetic acid, while 3 bacteria were positive for hydrogen cyanide synthesis. Nine bacteria were chosen for more activities. Except the isolate P66, all bacteria were siderophores producers. No bacteria fixed nitrogen, while ACC deaminase was detected in three bacteria, E34, E68 and E85.The solubilization of other inorganic forms of insoluble phosphate was quantitatively evaluated and all isolates couldn't dissolve both forms $FePO_4$ and $AlPO_4$, while 7 bacteria were found to be $Ca_5HO_{13}P_3$ and $CaHPO_4$ solubilizers. Analysis of 16S rDNA partial sequencing demonstrated that these 9 bacteria belong to three genera: *Aeromonas*, *Pseudomonas* and *Enterobacter*. To evaluate the effectiveness of the most efficient rhizobacteria and confirm their role as biofertilizers, inoculation experiments on plants are required.

Introduction

The rhizosphere is a region of very intense microbial activity because of root exudates of plants. The rhizobacterial populations may have various effects on plants.

Rhizobacteria executing a phytobenefic effect and is included under the term Plant Growth Promoting Rhizobacteria (PGPR) that was coined for the first time by Joe

Kloepper in the early 1980s. The PGPR may use one or more mechanisms for plant growth improvement. These beneficial activities are either direct like phosphate solubilization, production of plant growth regulators and atmospheric nitrogen fixation or indirect presented by the biocontrol through the production of several antagonistic metabolites. Thus, inoculation of plants with these PGPR is accompanied by a significant increase in productivity that results from two main beneficial mechanisms: stimulation of plant growth and protection of plants against soil-borne diseases.

According to numerous studies, PGPR include different bacterial genera. Among the PGPR used in the inoculation of plants and have given significant results, isolates belonging to *Rhizobium* (Afzal and Bano, 2008), *Bacillus* (Orhan *et al.,* 2006), *Pseudomonas* (Naiman *et al.,* 2009) *Enterobacter* (Morales-García *et al.,* 2011), *Serratia* (Nico *et al.,* 2012) and *Pantoea* (Khalimi *et al.,* 2012).

The development of biofertilizer composed by these phytobenefic rhizobacteria could minimize or even replace the use of chemical fertilizers while assuring a sustainable agriculture and maintaining environmental quality. For this purpose, the present work is the first study that focuses on the isolation of bacteria from the rhizosphere of rice cultured in the Northwest of Morocco, and the selection of strains that are characterized *in vitro* by several positive activities for plants.

Materials and Methods

Isolation and selection of phosphate solubilizing bacteria (PSB)

Two grams of rhizospheric soil of three rice (*Oryza sativa*) varieties (Puntal, Elio and Guadiamar) were dissolved in 18 ml of sterile physiologic water. Then 100µl of dilutions were plated on TSA (Tryptic soy agar) medium.

To select PSB, the isolates were grown on Pikovskaya's (PVK) (Pikovskaya, 1948) agar plates containing 0.5% $Ca_3(PO_4)_2$ as P source. Only colonies surrounded by clear halos were chosen. To measure the solubilization halos, the inoculated PVK plates were incubated at 28 °C for 7 days. 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

Production of indole acetic acid (IAA)

To detect the IAA production, aliquots of 10µl bacterial cultures were deposited on a nitrocellulose membrane placed on tryptic soy agar (TSA) contained 0.05% tryptophan. After incubation at 28 ° C, the membrane was located on filter paper soaked with Salkowski reagent (2% $FeCl_3$ (0.5M), 35% perchloric acid) (Bric *et al.,* 1991). Development of pink halos around the bacterial colonies indicated IAA production.

Production of hydrogen cyanide (HCN)

To estimate HCN production, bacterial cultures were streaked on TSA amended with 4.4 gl^{-1} glycine. A filter paper 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.

Production of siderophores

The plates of TSA were spot inoculated with test bacteria and incubated at 28 ± 2°C for 3 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, 1 ml acetylene was injected in each tube and incubated again. As ethylene is proportional to the rate of N_2 fixed, formation of this gas was measured by the gas chromatography after every 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 contained 3 mM ACC as a 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 tubes.

Solubilization of other forms of inorganic P

Qualitative assay of solubilization of other sources of inorganic P was evaluated using modified PVK medium, substituting $Ca_3(PO_4)_2$byhydroxyapatite ($Ca_5HO_{13}P_3$), dicalcium phosphate ($CaHPO_4$), aluminum phosphate ($AlPO_4$) or ferric phosphate ($FePO_4$). The inoculated plates were incubated at 28°C for 7 days. Only isolates surrounded by clear halos were considered as P solubilizers.

PCR amplification of 16S rDNA and sequencing

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 from agarose gels with the 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 National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/blast/Blast.cgi).

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.

Result and Discussion

Isolation and selection of PSB with several plant-beneficial activities

Bacteria producing transparent halos on PVK solid medium are considered as phosphate solubilizing bacteria (PSB). So, a total of 305isolates obtained from the rhizosphere of three varieties of rice cultured in the Northwest of Morocco, of which 44.6%bacteriawerePSB. Based on the diameter of solubilizing halo (diameter $\geq$ 0.4 cm) 17 isolates, all Gram negative, were selected for further tests.

Six isolates were capable of producing IAA giving pinkish halos on nitrocellulose membranes, whereas just 3 bacteria were HCN positive. These 9 bacteria were screened for the siderophores test. Except the isolate P66, all the bacteria were able to produce siderophores (Table 1). For the nitrogen fixation assay, no bacteria were found nitrogenase positive. But three bacteria, E34, E68 and E85 were shown positive for the ACC deaminase activity (Table 1).

Quantitative assay of solubilization of inorganic forms of insoluble phosphate

Other mineral forms of insoluble phosphate were used to assess P solubilizing capacity of 9 selected bacteria. So, tricalcium phosphate was substituted by4inorganic sources of insoluble phosphate, $Ca_5HO_{13}P_3$, $CaHPO_4$ $FePO_4$ and $AlPO_4$. Based on the results obtained, the majority of the selected bacteria were able to solubilize both forms of calcium phosphate ($Ca_5HO_{13}P_3$ and $CaHPO_4$) (Table 2). Except G70 and E34, solubilizing halos were observed around 7isolates in plates containing $Ca_5HO_{13}P_3$ and $CaHPO_4$ as phosphate source. In contrast, no colonies exhibiting a clear halo were observed on agar plates supplemented with either $FePO_4$ or $AlPO_4$ (Table 2).

Identification of PSB isolates

Sequencing of PCR-amplified 16S rDNA genes and comparison of sequences obtained with available data in the GenBank using the BLAST allowed us to identify the 9 PSB isolates evaluated in this study (Table 3). Based on a sequence identity of 98% or more, the 9 isolates were closely related to species belonging to three bacterial genera *Pseudomonas*, *Aeromonas*, and *Enterobacter* (Table 3).

Selection of PSB with several plant growth promoting activities

Phosphorus (P) is one of the major elements in the mineral nutrition of plants for its importance to the growth and development (White and Brown, 2010). However, it is often a limiting mineral nutrient for many agricultural crops because it is abundant in many soils but in forms poorly assimilable (Khan *et al.*, 2009). The exploitation of rhizobacteria which are able to solubilize the inorganic P is a very promising approach to enhance the availability of soluble P in soils. This present work represents the first study realized in rice fields in Northwestern Morocco, in order to isolate rhizobacteria dissolving the inorganic phosphate and to select those exhibiting several plant-beneficial activities. According to the literature, the PSB are more abundant in the rhizosphere than non-rhizosphere soil (Chen *et al.*, 2006; Muleta *et al.*, 2013). So, it was found that almost 44.6% of rhizobacteria isolated from the rhizospheric soil were PSB characterized by production of transparent halos surrounding the colonies indicating the P release from $Ca_3(PO_4)_2$ and the highest halos diameters were measured by G18 and G70 that were identified as *Enterobacter* sp.

and *Aeromonas* sp. respectively. Regarding the molecular identification, selected PSB were found to belong to the genera *Pseudomonas*, *Enterobacter* and *Aeromonas*. Similarly, Prasanna *et al.* (2011) and Kumar *et al.* (2012) isolated from the rhizosphere of rice and green bean respectively PSB populations of which strains identified as *Pseudomonas* and *Enterobacter*. Moreover, Muleta *et al.* (2013) found that, among the PSB bacteria isolated from the rhizosphere of coffee grown in Ethiopia, strains that belong to *Pseudomonas* and *Aeromonas*.

Plant hormones are regulators that may influence plant growth and development, and IAA is one of the most physiologically active auxins. The majority of the selected bacteria were able to produce IAA from tryptophan added to the culture medium, which is in agreement with numerous studies that demonstrated that IAA is the common product of tryptophan metabolism for several rhizobacteria (Ahmad *et al.*, 2005; Joseph *et al.*, 2007). This phytohormone plays a very important role in the stimulation of root elongation and proliferation of root hairs and lateral roots (Patten and Glick, 2002; Shahab *et al.*, 2009). Biocontrol conferred by some PGPR is mainly due to a synergistic combination of different antagonistic metabolites (Haas and Defago, 2005; Jourdan *et al.*, 2008). Selected bacteria were assessed for HCN production. This volatile compound is one of effective antagonistic compounds particularly against fungi (Haas and Defago, 2005). Only 3 out of 9 isolates were HCN positive. These isolates were identified later as *Pseudomonas* sp. According to the literature, the most efficient bacterial antagonists in the soil belong to this genus, producing different antagonistic metabolites (Bakker *et al.*, 2007; Ramyasmruthi *et al.*, 2012; Trivedi *et al.*, 2008).

Another PGP feature that may play an important role in biocontrol is siderophores production. These molecules have high affinity to iron ion. Through this process, iron is made unavailable in the natural habitat of phytopathogens and more available for the beneficial bacteria and / or to the plant (Haas and Defago, 2005; Beneduzi *et al.*, 2008; Ramesh *et al.*, 2009). At the same time, the siderophores react as elicitors for plant inducing the systemic resistance (Höfte and Bakker, 2007). Except P66 strain, all selected PSB produced siderophores, which is in agreement with the results of Joseph *et al.* (2007) and Gull and Hafeez (2012) who isolated rhizobacteria producing siderophores.

The ability of rhizobacteria to fix atmospheric nitrogen has been of interest to agricultural microbiologists. These PGPR improve plant growth through the increase of nitrogen uptake (Shridhar, 2012). In current study, no bacteria of selected strains have shown nitrogenase positive, although many studies that have demonstrated the ability of certain rhizobacteria to fix atmospheric nitrogen (Kumar *et al.*, 2001; Beneduzi *et al.*, 2008; Gull and Hafeez, 2012).

Another direct phytostimulator activity searched in PGPR is ACC deaminase that reduces ethylene levels in plants during the stress (Glick *et al.*, 2007a). This enzyme was detected in 3 strains belonging to *Pseudomonas*, E34, E85 and E68. According to these results, Gravel *et al.* (2007) also reported the presence of this enzyme in *Pseudomonas putida* strain isolated from the rhizosphere of tomato. Nevertheless, this enzyme is present in strains from different rhizobacterial genera, and it hydrolyzes ACC, ethylene precursor that inhibits root elongation, thereby promoting root development (Penrose and Glick, 2003; Glick *et al.*, 2007b).

Solubilization of other inorganic sources of insoluble phosphate

The soluble P is rapidly immobilized in soils and becomes unavailable for plants, and this process of fixation is dependent on pH and soil type. Thus, in alkaline soils, P is precipitated by calcium, while in acid soils it is fixed by free oxides and hydroxides of aluminum and iron (Rodriguez and Fraga, 1999). Consequently, we decided to evaluate the ability of selected PSB to solubilize other mineral forms of insoluble phosphate (hydroxyapatite, dicalcium phosphate, aluminum phosphate and ferric phosphate). The results show that even though all isolates were able to grow on PVK supplemented with each of the aforementioned inorganic phosphates, solubilization halos surrounding colonies were observed only on plates containing Ca-phosphates, when none was able to produce clear halos on agar plates amended with $FePO_4$ or $AlPO_4$. These results are in agreement with those presented by Pérez *et al.* (2007) who reported that PSB strains isolated from an acid soil gave solubilization halos on NBRIP plates containing $Ca_3(PO_4)_2$ without showing any solubilization in the presence of $FePO_4$ and $AlPO_4$. Identical results were found also by Chang and Yang (2009). This phenomenon could be explained by the inadequacy of the protocols used for the evaluation of solubilization activity by PSB regarding the ferric and aluminum phosphates (Pérez *et al.*, 2007) or because of the toxicity of ions such as the Al^{3+} released during the phosphate solubilization (Illmer *et al.*, 1995) and their possible inhibitory effect on the solubilization activity PSB (Oliveira *et al.*, 2009).

Table.1 Origen of isolation, diameter of solubilization halos and evaluation of different plant growth promoting activities of selected PSB

Isolates	Rice variety	Halos (cm)	IAA	HCN	Siderophores	Nitrogen fixation	ACC deaminase
P29	Puntal	0.7 (±0.15)	+	-	+		-
P66	Puntal	0.4 (±0.1)	+	-	-	-	-
E34	Elio	0.4 (±0.06)	-	+	+	-	+
E68	Elio	0.6 (±0.06)	-	+	+	-	+
E85	Elio	0.6 (±0.1)	-	+	+	-	+
G70	Guadiamar	0.9 (±0.21)	+	-	+	-	-
G18	Guadiamar	1.1 (±0.12)	+	-	+	-	-
G46	Guadiamar	0.5 (±0.12)	+	-	+	-	-
G55	Guadiamar	0.4 (±0.1)	+	-	+	-	-

Table.2 Qualitative test of solubilization of inorganic phosphates by the PSB isolates

Isolates	Modified PVK			
	$CaHPO_4$	$Ca_5HO_{13}P_3$	$FePO_4$	$AlPO_4$
P29	+	+	-	-
P66	+	+	-	-
E34	-	-	-	-
E68	+	+	-	-
E85	+	+	-	-
G70	-	-	-	-
G18	+	+	-	-
G46	+	+	-	-
G55	+	+	-	-

Table.3 Identification of selected PSB by 16S rDNA partial sequencing

Isolates	Length of 16S rDNA gene sequenced	Most closely related organism	Identity (%)
P29	918	*Aeromonas media*	99%
P66	413	*Aeromonas allosaccharophila*	99%
E34	1021	*Pseudomonas brassicacearum*	99%
E68	649	*Pseudomonas corrugata*	99%
E85	538	*Pseudomonas brassicacearum*	99%
G70	1014	*Aeromonas hydrophila*	98%
G18	1020	*Enterobacter asburiae*	98%
G46	1008	*Enterobacter ludwigii*	98%
G55	1015	*Enterobacter ludwigii*	99%

Conclusively, this present work is a contribution to evaluate the PSB present in the rhizosphere of rice (*Oryza sativa*) cultured in North-western Morocco. The results make several isolates attractive as phosphate solubilizers and emphasize the importance of some PSB characterized by several phytobenefic activities *in vitro* such as bacteria and G18 E68, and the possibility of their exploitation for biotechnological applications such as the development of biofertilizers. However, further studies are required to assess their effect under pot culture as well as field conditions before they are recommended as bioinoculants for the plant-soil system.

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