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The study of inorganic insoluble phosphate solubilization and other plant growth promoting characteristics of indigenous *Pseudomonas fluorescens* bacteria of Kordan and Gonbad regions

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Key words: Phosphate solubilizing bacteria, *Pseudomonas fluorescens*, siderophore; auxin, Acc-deaminase.

Abstract

This study investigated inorganic phosphate solubilization ability of *Pseudomonas fluorescens* bacteria isolated from Gonbad and Kordan regions as well as some of the plant growth promoting characteristics of the superior phosphate solubilizing isolates. In this study, 58 strains of *Pseudomonas fluorescens* bacteria were isolated from the soil and evaluated in term of phosphate solubilization ability. Among these 58 isolates, 10 strains showed the most inorganic phosphate solubilization ability. The results showed that there is a 5% level of significant difference from the point of inorganic phosphate solubilization ability. Plant growth promoting characteristics including ACC-deaminase, Auxin and siderophore production ability was studied as well and the results showed that among these ten superior strains, isolate 12, 26 and 42 in spite of having the most ability to solubilize inorganic phosphate, had more significant superiority than other strains in term of studied plant growth promoting characteristics. Totally among these 58 bacterial isolates, 84% were able to solubilize inorganic phosphate and 16% were unable. Furthermore, 70%, 100% and 80 % of the superior phosphate solubilizing bacteria respectively showed the ability to produce siderophore, Auxin and ACC-deaminase enzyme. According to the results, there is no correlation between the ability of strains to solubilize the phosphates, produce IAA, siderophore and ACC-deaminase enzyme. Altogether the bacteria isolated from Kordan soil were more able than Gonbad soil.

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Introduction

After nitrogen, phosphorus is the most important element limiting plant growth. Although abundant in soils, phosphorus (P) is one of the essential macronutrients required for plant growth and development, and is by far the least available factor for plants (Poonguzhali *et al.*, 2007). The most important role of this element in the plants is its role in the process of energy product and transfer. The amount of available phosphorus for the plant in soil is controlled by several factors such as soil pH, calcium ion concentration, soil organic matter content, clay type and content, soil moisture, soil texture, root exudates and its density (Barber, 1995). Most agricultural soils in Iran have large reserves of phosphorus but due to the special characteristics of these soils such as being calcareous and alkaline and also having large amounts of accumulated phosphorus which is the consequence of chemical phosphate fertilizers application this considerable amount of phosphorus is not practically available for the plants. This accumulated phosphorus in soil has lots of ill-effects and causes irreparable damage to the environment and soil. So a number of scientists and intellectuals throughout the world started working on a solution like using biological agents like microorganisms for increasing the yield without causing any damage associated with chemical fertilizers. For the first time in 1903, the dissolution of calcium phosphate, bone meal and rock phosphate by bacteria on solid medium was reported. Then in 1908, tricalcium phosphate solubilization by isolated bacteria from soil was reported and these bacteria were generally called Phosphate Solubilizing Bacteria (PSB). Apart from insoluble phosphates solubilization, it has been reported that PSBs can produce growth-promoting substances that can contribute to the plant growth and its development (Selvakumar *et al.*, 2009). PSBs can convert the insoluble phosphates into soluble form by acidification, chelation, exchange reaction and production of gluconic acid (Chen *et al.*, 2006). *Fluorescence pseudomonas* bacteria are the best plant growth promoting rhizobacteria. They increase the growth and yield of the crops by the use various methods, including production of phytohormones. Inorganic acids like Hydrochloric can solubilize phosphate. However compared with organic acids they have less efficiency in the same pH. Using PSB inoculants have attracted a lot of attention in recent years (Pandey *et al.*, 2006). PSB inoculants result the improvement of growth, yield and phosphorus uptake in several plants (Hameeda and Rupela, 2006).

In most of Iranian soils, although there is large content of some nutritional elements like phosphorus, soluble and available content of these elements is lower than essential amount for providing proper plant growth. A common method to deal with the current shortage of phosphorus is using chemical fertilizers but in addition to the high cost and low efficiency, they can cause environmental contamination. So in recent years it has been necessitated finding biological solutions for this problem. Using soil microorganisms which have the potential of solubilizing insoluble phosphates and changing them into soluble forms is one of the most effective ways to increase the uptake of phosphorus in alkaline soils (Attoe et al., 1966). There are several reports concerning the ability of bacteria genus *Pseudomonas* to solubilize insoluble phosphate. Bacteria Genus *Pseudomonas* has demonstrated a considerable potential to improve the efficiency of phosphate absorption and due to its spread and the diversity of species and being resistant to environmental stresses, it is considered as a biological fertilizer (Kim *et al.*, 1989). Therefore the present investigation was designated for the isolation and identification of bacteria which had high potential of inorganic insoluble phosphates solubilization and also studying other indices of plant growth promotion (such as Indole Acetic Acid phytohormone production, siderophore and ACC deaminase enzyme) in two different soils and ultimately selecting superior strains to be used in future studies as inoculants.

Materials and methods

Soil sample collection

The soils used for bacterial isolation were collected from Gonbad region N37°19′ 11′′, E55°10′ 19′′placed

at an altitude of 45 m above mean sea level in the northwest of Iran and Kordan in Alborz province N39°73′ 23′′, E48°57′ 23′′ located at an altitude of 1360 m above mean sea level.

The top 2 cm soil was taken out and soil samples were collected at a depth of 0-30 cm from randomly selected sites in each area. The samples were then transferred to a sterile polythene bags and transported immediately to the laboratory. For microbiological isolation and analysis they were preserved at 4˚C.

Isolation of Pseudomonas fluorescens bacteria

To obtain standard soil suspension 10 gram of soil samples was shaken with 90 ml of sterile physiological saline solution for 10-20 minutes. So 10-fold serial dilutions of the samples were prepared and soil samples were serially diluted, spread plated on S1 agar medium to isolate different strains of *Pseudomonas fluorescens* .One ml of soil suspension from aliquot dilutions (10^{-2} to 10^{-6}) was aseptically added to sterile petriplates containing sterile medium and incubated at 28±2 ˚C for 24-48 h and after incubation, well separated individual colonies with yellow green and blue white pigments were marked and detected by viewing under UV light. The individual colonies were picked up and purified on individual plates with sterile loop and then transferred to fresh slants and these obtained pure cultures were stored in refrigerator at 4 ˚C for further use.

Phosphate solubilization test

Pseudomonas fluorescens bacterial isolates were applied for phosphate solubilization by inoculating them on the Sperber's agar medium (Sperber, 1958) containing inorganic phosphate [Ca_3 $(PO_4)_2$]. The halo and colony diameters were measured after 2, 4, 8 and 10 days of incubation of the plates. The ability of the bacteria to solubilize insoluble phosphate was described by the ratio of halo to colony diameter. After screening superior isolates, they were characterized on the basis of plant growth promoting activities. These isolates were tested for their ability to produce Indole acetic acid (IAA), siderophore and ACC-deaminase.

Evaluation of PGPR characteristics of isolates

Production of Indole Acetic Acid by *Pseudomonas fluorescens* bacteria was assayed as described by Patten and Glick (1996). Bacterial isolates were propagated in LB minimal medium with L-Tryptophane and after incubation; Salkowski's reagent was applied to observe the development of a pink color indicating indole. The absorbance of supernatant mixture with reagent was measured at 535 nm. The quantity of indoles was determined by comparison with a standard curve using an IAA standard graph.

Semi quantitative test of siderophore production ability

As far as Chrome Azurol Sulphonate (CAS) agar effectively differentiates bacteria that are capable of excreting siderophore, we used Schwyn and Neilands method (1987) for evaluating isolates. The change in color of the blue dye Chrome Azurol Sulphonate (CAS) assay solution to orange indicates the presence of siderophore (Iron chelating compounds) production by isolated *Pseudomonas fluorescens* Bacteria. Visual observation of the bacterial growth and measurement of the halo area diameter around the colonies employed for the evaluation (Alexander and Zuberer, 1991).

Semi quantitative test of ACC-deaminase enzyme production

This test was carried out based on Penrose and Glick method (2001) using three media of ACC+RMM, RMM+Ammonium Chloride as positive control and RMM as negative control. The visual observation and measurement of colonies on 3, 6 and 9th day was employed to evaluate the *Pseudomonas fluorescens* bacteria.

Biochemical test of isolates

Superior bacterial isolates were studied for their morphological and biochemical characteristics according to the standard techniques.

Results and discussion

Phosphate solubilization test

Semi quantitative test of inorganic phosphate solubilization for assessing the ability of 58 isolates to solubilize inorganic insoluble phosphate was carried out by the observation of a clear halo around the colony and measuring the halo diameter to colony diameter ratio. Among these 58 isolates, 32 isolates were related to the soil of Kordan region which was coded from 1 to 32 and 26 isolates were related to the soil of Gonbad region and coded from 33 to 58. Among the bacteria *Pseudomonas fluorescens*, 49 strains had the ability of solublizing inorganic insoluble phosphates which were recognized by producing clear halo around the colony. Comparing to the other strains, the efficiency of each strain was different based on observation of clear halo diameter around the colonies. Among these 49 strains, 10 strains were selected for further studies because of having the highest clear halo diameter to colony diameter and supposed as superior and the most efficient strains. It must be mentioned that 7 strains were related to Kordan soil and 3 strains were related to Gonbad soil. The results of halo to colony diameter mean in the test of inorganic insoluble phosphates solubilization by selected isolates after 3 and 8 days of inoculation is illustrated in **Error! Reference source not found.**.

Table 1. The ability of inorganic insoluble phosphate solubilization by *Pseudomonas fluorescens* strains on different days after inoculation.

Isolates code	3 days	8 days
4	1.83^{cdef}	2.54^{a}
10	2.16^{bcd}	2.33^{a}
12	2.25^{bc}	2.45^{a}
21	1.95^{cde}	1.43^{b}
26	2.91^{a}	2.78^{a}
31	1.39^{f}	1.56^{b}
32	1.57^{ef}	1.58^{b}
42	2.58^{ab}	2.7^{a}
54	1.64^{def}	1.47^{b}
57	1.7^{def}	1.62^{b}

The results showed that there is a significant difference at the 5% level between isolates in the term of insoluble inorganic phosphates solubilization. As it can be observed in **Error! Reference source not found.** there is a significant difference among all isolates in the term of halo to colony diameter of inorganic phosphate solubilization. However by the time, after eight day this significant difference decreased among isolates that strains 4, 10, 12, 26 and 42 showed more halo to colony diameter mean than other strains but there was no significant difference among these isolates. Although the bacteria isolated from Kordan soil were more able than Gonbad soil.

Table 2. Comparison of halo to colony diameter ratio of inorganic insoluble phosphate solubilizing superior strains in CAS agar medium measured at different intervals.

Isolates code	3 days	5 days	7days
4	2.01^{b}	1.72^{b}	1.53^{b}
10	0^{g}	0^{d}	0^{c}
12	1.17^{f}	1.28^{c}	1.23^{b}
21	1.33^{ef}	1.25^{c}	1.16^{b}
26	1.6^{cd}	1.55^{b}	1.50^{b}
31	0^{g}	0^{d}	0^{c}
32	1.41^{de}	1.23^{c}	1.22^{b}
42	2.25^{a}	2.11^{a}	2.21^{a}
54	1.64^{c}	1.35^{c}	1.27^{b}
57	0^{g}	0^{d}	0^{c}

There are several reports on the ability of different bacteria strains to solubilize insoluble phosphates such as tricalcium phosphate, dicalcium phosphate, hydroxyl apatite and rock phosphates. Sperber (1958) reported that approximately 26-39% of the total populations of bacteria are able to solubilize phosphate. Plant Growth Promoting Bacteria cause the solubilization of insoluble inorganic phosphates by producing organic acids especially gluconic acid and it improves plant phosphorus nutrition and increases its growth (Vassilev et al., 2002). Kenei *et al* (2010) showed that bacteria isolated from different regions show various potential of insoluble phosphate solubilization.

Semiquantitative test of siderophore production ability

Based on the test and **Error! Reference source not found.**, the isolates 42 with halo to colony

diameter ratio of 2.2, isolates 4 and 26 with halo to colony diameter ratio of 1.5 and isolates 12, 21, 32 and 54 with halo to colony diameter ratio of 1.2, after 7 days of inoculation on CAS-agar medium had the ability of siderophore production.

Furthermore, strains 10, 31 and 57 were not able of siderophore production. The results indicate significant difference at 5% level among the isolates in term of siderophore production.

Table 3. comparison of ACC-deaminase enzyme production by superior isolates of solubilizing inorganic insoluble phosphates.

Isolates code	Average colony diameter 10 days after inoculation			P:PC	P:NC	ACC-deaminase enzyme production capability	Colony size on ACC medium
	NC	PC	ACC				
12	3.1^{ghi}	2.9^{i}	5.1^{b}	64.79^{a}	76.19^{a}	Very high	Diameter increase compared with PC
57	2.15^{jk}	2.4^{j}	3.45^{ef}	60.49^{a}	43.91^{b}		
42	3.1^{ghi}	4.1^{cd}	5.25^{b}	69.27^{a}	27.97^{c}		
54	3.25^{efgh}	3.55^{e}	3.9^{d}	20.07^{bc}	9.92^{d}		
26	3.1^{ghi}	4^{d}	4^{d}	29.16^{b}	0^{de}	High	Equal colony diameter to PC
10	3.35^{efg}	4.35^{c}	3.9^{d}	16.51^{c}	-10.31^{e}	Moderate	Diameter decrease compared with PC <% 30
4	4^{d}	5.9^{a}	4.4^{c}	10^{cd}	-25.43^{f}		
32	1.75^{l}	5.1^{b}	1.9^{kl}	8.49^{cd}	-62.69^{g}	Very low	Diameter decrease compared with PC ≥% 60
21	3.9^{d}	4.1^{cd}	3.9^{d}	0^{d}	-4.88^{e}	Unable	Equal colony diameter to NC
31	3^{hi}	3.15^{fghi}	3^{hi}	0^{d}	-4.73^{e}		

PC: positive control, **NC:** negative control, **P: PC**= increase or decrease percentage in colony diameter compared with the positive control, **P: NC**= increase or decrease percentage in colony diameter compared with the negative control

Plant growth promoting bacteria increase plant growth and the bioavailability and solubility of micronutrients like Fe, Mn, Zn and Cu by ionophores production and also increase phosphorus adsorption. One of the most important ionophores is siderophore which links specifically with Ferric-Fe and in addition to providing and increasing plant essential Fe uptake, competitively controls plant diseases (Hu and Boyer, 1996).

Quantitative test of IAA production:

Among 10 superior isolates of insoluble inorganic phosphates, strain 10 significantly had lower ability to produce IAA than other 9 strains. Auxin production was more in isolates 4, 12, 21, 57 than strain 10 but there was not a significant difference among these 4 strains (**Error! Reference source not found.**).

Strains 32, 26 and 54 produced 20.48, 21.7 and 24.35 mg/l IAA respectively and were located in the third and fourth place after strains 4, 12, 21 and 57. Eventually the most ability of IAA production is related to strains 31 and 42 with IAA production of 27.4 and 26.9 mg/l which statistically had remarkable and significant ability of IAA production than 8 other strains. IAA as a hormone doesn't haveany special activity in bacterial cell (Glick, 2002) but this hormone increases the plant growth and metabolites

so it improves the plant growth condition (Glick and Patten, 1996). Khakipour *et al* (2008) reported that 79% of *Pseudomonas fluorescens* isolates are able to produce IAA in the presence of L-Tryptophane.

Srideve and Mallaiah (2007) reported that more production of IAA in an isolate is probably due to the better use of the ambient compounds by that isolate. On assessing indigenous bacteria of Iranian soils, Soltani *et al* (2007) showed that all isolates are able to produce IAA.

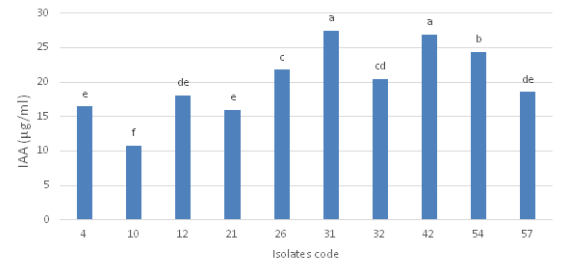

Fig. 1. Comparison of IAA production in superior isolates of solubilizing inorganic insoluble phosphate.

Qualitative test of ACC-deaminase enzyme production

Among superior strains of insoluble inorganic phosphate solubilization, isolates 12, 57, 42 and 54 had the highest capacity and isolates 21 and 31 were unable to produce ACC deaminase enzyme. According to the ACC-deaminase production ability, isolates can be classified into six groups of very high, high, medium, low, very low, and unable. Isolate 26 was able to produce satisfactory level of ACC-deaminase and isolates 4 and 10 produced an average amount of ACC-deaminase enzyme and isolate 32 produced the lowest amount of ACC-deaminase (**Error! Reference source not found.**). These isolates can convert ACC to Ammonia and α- keto butyric acid by the secretion of ACC-deaminase enzyme which is an important enzyme to prevent stress ethylene production in plant so at phosphorus deficiency, they can decrease the stress ethylene level.

On the study of ACC-deaminase enzyme production capacity of different *Pseudomonas fluorescens* isolates, Jalili *et al* (2009) reported that several isolates are able to produce this enzyme. On their study on two strains of *Pseudomonas fluorescens*, Shaharoona, *et al* (2008) reported that both isolates had the capacity for ACC-deaminase enzyme production.

Biochemical tests and bacteria identification

All the superior isolates were Gram negative, straight, non-spore formers, motile, non-gas producing, in oxidative fermentative medium with glucose: open tube acid produced, tube sealed acid not produced, oxidase positive, catalase positive, indole negative, methyle red negative and voges proskauer negative. These characteristics render all the isolate as belonging to genus *Pseudomonas*.

These findings proved that the bacteria isolated from Kordan soil were more able than Gonbad soil and these isolates inspite of having the ability of inorganic phosphate solubilization and increasing the solubility of phosphorus, they are able to increase plant growth by producing Indole Acetic Acid and ACC-deaminase and guarantee the plant survival in phosphorus deficiency. According to the results, there is no correlation between the ability of strains to solubilize the phosphates, produce IAA, siderophore and ACC-deaminase enzyme. So it is suggested that the effects of superior strains which solubilize inorganic insoluble phosphate and have the ability of plant growth promoting like isolates 26 and 42 would be applied to increase the efficiency of phosphoric chemical fertilizers in future greenhouse and field studies.

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