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Full Length Research Paper

Molecular genosystematic and physiological characteristics of fluorescent pseudomonads isolated from the rice rhizosphere of Iranian paddy fields

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The great progress achieved in the use of molecular genosystematics permits the study to solve many problems of specificity in the relationship between plant and microbial population of the rhizosphere. In this study, the plant growth promoting properties (indolileacetic acid production, phosphate solubilization and siderophore production) and genetic diversity of isolated *Pseudomonas* strains were examined. Bacterial strains were isolated from the rice rhizosphere of paddy fields in three Northern Provinces (Mazandaran, Gollestan and Guillan) of Iran. Our studies revealed that pseudomonads have plant growth promoting properties. Isolated strains showed high ability of IAA production, phosphate solubilization and siderophore production, while genotyping analysis showed that pseudomonads isolated from the rhizosphere of rice are genetically diverse. Nevertheless, the strains were distributed into 11 genotypes, including five groups of fluorescent pseudomonads.

Key words: Fluorescent pseudomonads, rice rhizosphere, IAA production, phosphate solubilization, siderophore production, gene typing.

INTRODUCTION

Rice (*Oryza sativa* L.*)* is one of the most important sources of food and it provides cheap carbohydrate source for human consumption in many countries. In Iran, 650,000 ha of the agricultural fields are cultivated with rice. However, Mazandaran, Guillan and Golestan are major rice cultivating areas in Iran. Due to continuous crop cultivation, essential nutrients are depleted from the soil. Input of nutrients by chemical or biological means is essential for sustainable agriculture. All along its development, rice is subjected to the output of fertilizer, leaching of nitrogen, fixing of phosphorus and decrease of micronutrient elements available in calcic soils of Iran. So, the use of biofertilizers could constitute an efficient alternative to increase the yield and improve nutrients uptake by the plant.

Rhizosphere bacteria have an important role that positively affects plant growth and yield via different mechanisms (Misko and Germida, 2002; Garbeva et al., 2004a, b; Aloni et al., 2005; Ljung et al., 2005). Aerobic non-sporeforming bacteria, described as pseudomonades, are widely distributed in soil and other natural substrates (Anzai et al., 2000; Yamamoto et al., 2000). *Pseudomonas* spp. are important plant growth promoting rhizobacteria (PGPR) used as biofertilizers and are able to enhance crop yield by direct and indirect mechanisms (Walsh et al., 2001). Several researchers have shown that fluorescent pseudomonads are abundant in the rhizosphere of different crops (Khayyati and Anvari, 2001; Misko and Germida, 2002; Xie et al. , 2003; Kumar and Sugitha, 2004; Ramos Solano et al., 2006). Effectively, they produce a variety of biologically active substances among which growth-promoting compounds represent a keen interest (Asghar et al., 2002; Rodríguez, 2006). It has been shown that many strains of pseudomonads are able to solubilize phosphorous in soil and increase its availability to plants (Sundara et al., 2002). Some strains of pseudomonas produce chelating agents, called siderophores with high affinity for Fe absorption. Microbial siderophores can enhance plant

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growth through increasing Fe solubility in the plant rhizosphere (Kloepper et al., 1980). Such products are also able to alleviate the unfavorable effects of pathogens on plant growth.

Genetic diversity of pseudomonads in the rhizosphere of rice can influence their efficiency to enhance growth of inoculated plants. PCR-RFLP based on 16S rRNA has been used by several authors for rapid taxonomic and strain-typing purposes (Misko and Germida, 2002). However, Thakuria et al. (2004) isolated three groups of rhizobacteria from rice rhizosphere. RAPD analysis of the PGPR isolates indicated that they belonged to four distinct genotypes. The aim of this study is to evaluate plant growth promoting characteristics of pseudomonas isolated from the rhizosphere of rice. The genetic diversity of the isolates was also studied.

MATERIALS AND METHODS

One hundred and eleven strains of fluorescent pseudomonads were used in this study. The strains were isolated from the rhizosphere of rice cultivated in the Northern parts of Iran and identified in our previous study using biochemical and physiological tests (Ramezanpour et al., 2008). For all isolates, we determined three PGPR properties: IAA and siderophore production, as well as the ability for phosphate solubilization. Determination of these properties was carried out in three replicates for each sample.

IAA production was determined by the colorimetric method as described by Benziri et al. (1998). Bacterial isolates were inoculated into the TSB medium supplemented with tryptophan (50 mg/ml) and incubated at 28°C for 72 h. The cultures were centrifuged at 7160 rpm for 5 min, and IAA was determined by spectrophotometer at 530 nm using Salkowski reagent (mixture of 2 ml of 0.5 M FeCl and 98 ml of 35% HClO) as the coloring agent. Consequently, the level of IAA production was estimated by a standard IAA curve.

The Sperber (Sperber, 1958) medium with insoluble tricalcium phosphate $Ca_3 (PO_4)_2$, was used to determine the potential of bacterial strains in solubilizing mineral phosphates. First, the bacteria were grown on a TSB medium for 48 h and then 50 µL of the bacterial suspension was inoculated in 25 ml of the Sperber medium. The samples were then shaken for 120 h on a shaker at 125 rpm (at 128°C). Bacterial suspension was centrifuged at 10,000 g for 15 min, and 1 ml of the supernatant was mixed with 3 ml of the distilled water and 1 ml of the indicator ammonium molybdate-vanadate. After 20 min of incubation, light absorption by the samples at 470 nm was determined by spectrophotometer. P solubility was determined using the standard curve of KH2PO4 (Jeon et al., 2003).

Siderophore production by the bacterial isolates was performed following the chrome azurol S (CAS) method of Alexander and Zuberer (1991). The CAS agar medium was prepared and distributed in Petri dishes. Changing the medium's color (blue to orange) indicated siderophore-producing bacterial isolates. After three days, the diameter of the colonies was measured. The results were expressed as a ratio between the diameter of halos and the diameter of colonies for all bacterial isolates, while the data were subjected to analysis of variance using MSTATC computer software (Bricker, 1991) and Duncan's Multiple Range Test (Duncan, 1955).

Fifty isolates were randomly selected among superior strains along with three reference strains, *Pseudomonas fluorescens* ATCC 49642, *P. putida* ATCC 12633 and *Pseudomonas aeruginosa* GRP3 were subjected to 16S rDNA-PCR-RFLP analysis. DNA was extracted and purified by a modified phenol chloroform isoamyl alcohol (PCI) method (Marmur, 1961).

The 16S-rDNA region was amplified using the primers fD1: 5´-AGAGTTTGATCCTGGCTCAG and rD1: 5´-AAGGAGGTGATCCAGCC (W eisburg et al., 1991). Amplifications were carried out in a thermo-cycler (Perkin Elmer PCR system 2400) with the following program: initial lysis of bacterial cells and denaturation for 2.5 min at 95°C, 32 cycles of denaturation (35 s at 94°C), annealing (1 min at 51°C), extension (2 min at 72°C) and final extension (10 min at 72°C). Amplification of DNA was confirmed by electrophoresis in 1.3% (w/v) agarose gels. Gels were stained in an aqueous solution of 1 mg/l ethidium bromide and photographed using UV light.

PCR products were digested with the restriction endonucleases *HaeIII* and *Msp*I. The digestion products were separated by electrophoresis on 3.0% agarose gels for 2 h using a current of 100 V. Gels were stained with ethidium bromide and photographed under UV light. The genotypic diversity data were converted into a binary matrix that was analyzed using the simple matching similarity coefficient. The Dice similarity coefficient (Dice, 1945) matrix was subjected to UPGMA analysis and a phenogram was created using average linkage procedure. The calculation of the simple matching similarity coefficients and the construction of the UPGMA dendrograms were conducted using the NTSYS-pc (Rohlf, 1993), while the Multivariate Statistical Package (NTSYSpc2.0, V.2.02e.) software was used in the analyses.

RESULTS AND DISCUSSION

Plant growth promoting properties of strains

Bacterial isolates were different in their ability to produce IAA. All 111 isolates were able to produce IAA ranging from 17.7 to 95.9 mg L^{-1} with an average of 39.8 mg L^{-1}. The highest amount of IAA (95.9 mgL^{-1}) was produced by the *P. putida* strain MZ15 isolated from Mazandaran. The results showed that the average production of IAA by the species was very close to each other and the amounts of 42.3, 42.8 and 40.7 mgL^{-1} were obtained from *P. aeruginosa*, *P. putida* and *P. fluorescens*, respectively. Eighteen strains produced IAA more than average and considered them as superior strains. IAA production is widespread among soil rhizobacteria (Sarwar and Kremer, 1995; Patten and Glick, 1996), even though differences among isolates of *Pseudomonas* spp. in their ability to produce IAA were previously reported (Glickmann et al., 1998; Ahmad et al., 2005).

The ability of the strains to solubilize phosphate was also different and varied greatly within the range of 139.3 to 272.7 mg L^{-1} with an average of 205.6 mg L^{-1}. The maximum phosphate solubilization rate (272.7 mg L^{-1}) was obtained from strain MZ4. Thirty-one strains solubilized phosphorus more than average and were considered as superior strains. Studies of Jeon et al. (2003) showed that three strains of *P. fluorescens* including MCO7, M45 and B16, grown in a PKV medium in a 5-days period solubilized P at 458.3, 447.6 and 427.7 mg/L, respectively. The ability of *Pseudomonas* sp. To produce organic acid and hence, solubilize immobile P sources, such as tricalcium phosphate, in the soil is very advantageous as it can enhance the availability of P in

Table 1. Plant growth promoting properties of the superior strains of fluorescent pseudomonads.

Strains	IAA production (mg L^{-1})	P-solubilization (mg L^{-1})	Siderophore production (halo/colony diameter)
MZ 1	-	226.5^{hi}	-
MZ 3	-	237.4^{f}	-
MZ 4	-	272.2^{b}	-
MZ 9	-	209.4^{mno}	-
MZ 11	-	220.7^{j}	-
MZ 13	-	-	3.47^{ghijk}
MZ 15	*95.9^{a}	-	-
MZ 16	-	207.2^{o}	-
MZ 18	-	210.5^{imn}	-
MZ 20	63.2^{defg}	-	3.53^{ghi}
MZ 21	57.5^{g}	-	3.76^{fgh}
MZ 22	61.9^{efg}	-	3.11^{ijk}
MZ 24	60.3^{fg}	212.2^{klm}	-
MZ 26	84.5^{a}	-	-
MZ 27	-	223.9^{i}	-
MZ 33	-	–	4.82^{abc}
MZ 34	-	213.4^{kl}	-
MZ 42	-	-	3.45^{ghijkl}
MZ 43	57.9^{g}	207.4^{o}	-
MZ 44	-	-	4.68^{abc}
MZ 45	69.8^{bc}	-	-
MZ 47	-	233.4^{g}	-
MZ 48	-	219.5^{j}	-
MZ 49	-	220.3^{j}	3.29^{hijk}
MZ 50	-	217.9^{j}	-
MZ 51	-	-	3.14^{ijk}
GO 2	68.4^{bcd}	240.6^{e}	-
GO 3	-	-	3.35^{hijk}
GO 5	-	-	3.02^{jk}
GO 7	-	214.1^{k}	-
GO 8	-	237.5^{f}	-
GO 11	-	272.0^{a}	-
GO 12	-	213.6^{k}	-
GO 13	-	214.3^{k}	-
GO 14	65.3^{cdef}	-	-
GO 15	-	255.1^{c}	-
GO 16	64.6^{cdef}	-	-
GO 17	61.9^{efg}	244.8^{d}	-
GO 19	-	-	4.90^{ab}
GO 21	67.0^{bcde}	-	4.17^{def}
GO 22	-	231.1^{g}	3.53^{ghi}
GO 23	72.3^{b}	-	4.06^{ef}
GU 1	-	-	3.29^{hijk}
GU 3		208.2^{no}	-
GU 5	-	213.3^{kl}	4.54^{bcd}
GU 6	-	214.1^{k}	3.07^{ijk}
GU 7	-	-	3.18^{ijk}
GU 8	68.3^{bcd}	-	3.49^{ghij}
GU 10	-	220.6^{j}	3.46^{ijk}
GU 12	-	228.2^{h}	-

Table 1. Cont'd.

GU 13	–	–	3.03 ^{jk}
GU 15	–	208.1 ^{no}	–
GU 20	–	212.0 ^{klm}	–
GU 22	–	–	4.00 ^{ef}
GU 23	–	–	3.93 ^{fg}
GU 24	51.1 ^{n}	–	–
GU 25	–	–	3.88 ^{fg}
GU 26	63.1 ^{defg}	–	4.39 ^{cde}
GU 28	–	–	5.11 ^{a}
GU 34	62.2 ^{efg}	–	–
GU 36	–	–	3.22 ^{ijk}

*****Values followed by different letters were significantly different ($P < 0.05$).

the plant and hence increase plant growth (Dey et al., 2004).

Production of the orange halo indicates that the strains are able to produce siderophores. The ratio of halo/ colony diameter was different in various strains ranging from 0.71 to 5.1 with an average of 2.9, while the highest ratio of halo to colony diameter was seen in strain GU28. Twenty-eight strains produced siderophores more than average and were considered as superior strains. Meyer (2000) reported that different *pseudomonas* strains have the ability to produce high amounts of siderophore, whereas Rasuli et al. (2006) found that 201 strains of *P. fluorescens,* isolated from wheat rhizosphere, were able to produce siderophore. The Fe-chelating property of siderophore can greatly enhance Fe absorption by plant and plant growth, especially in calcareous soils where Fe deficiency is very common. Consequently, plant growth promoting properties of the strains are shown in Table 1.

Genetic diversity of the strains

PCR of 16S rDNA from all 50 strains (except one strain) produced a single band around 1200 bp as estimated by summing the sizes of the restriction fragments after digestion with restriction enzymes. In general, enzyme digestions of the 16S rDNA produced restriction patterns including 3 to 6 bands with molecular sizes ranging from 130 to 950 bp for enzymes HaeIII and MspI. Differences between genotypic RFLP profiles of isolated strains were observed for enzymes HaeIII and MspI. In general, enzyme digestions of the 16S rDNA products resulted in six distinct restriction patterns: with two to four restricted fragments per pattern detected for HaeIII restriction enzyme and three to five restricted fragments per pattern detected for MspI restriction enzyme. For the type of strains, three to four restricted fragments per pattern were detected for MspI and HaeIII restriction enzymes.

Eleven 16S rDNA types were obtained after combining the data for two enzymes (Table 2). A dendrogram was constructed based on the UPGMA algorithm by analyzing the similarity between different RFLP patterns (Figure 1). The clustering data showed that all strains could be clustered into five groups at a similarity level of 83%. Thirty five strains were genetically related to *P. fluorescens* ATCC 49642, among which twenty one strains showed a similar gene type of *P. fluorescens* ATCC 49642 and fourteen isolates were genetically close to it. *P. putida* ATCC 12633*P* was a cluster and eight strains were genetically related and close to *P. putida* ATCC 12633*P* in different similarity levels. Four strains were genetically related to *P. aeruginosa* GRP3, among which two strains showed a similar gene type of *P. fluorescens* ATCC 49642 and two isolates were genetically close to it.

In the present study, we used PCR-RFLP analysis of 16S rDNA region to evaluate the genetic diversity of the isolated bacterial strains. Fingerprinting of 16S rDNA has been used by several researchers to discri - minate genetic differences between bacterial species (Solano et al., 2006; Fischer et al., 2007; Sun et al., 2008). In conclusion, the results obtained from this study showed that the majority of the selected isolates belong to *P. fluorescens*. Determination of biodiversity and analysis of the genotypic rDNA by means of PCR - RFLP can be employed as a good marker for identifying pseudomonades due to its easiness and rapidity. Furthermore, this method was used to confirm the results of the biochemical tests obtained in our previous study (Ramezanpour et al., 2008).

Conclusion

It is shown that the fluorescent pseudomonads used in this study have high activity for IAA biosynthesis, phosphate solubilization and siderophore production, and also, they have growth promoting action for the development of rice and its crop yield. Conclusively, the strains'

Table 2. 16S rDNA types of selected strains.

Strain	Origin	16S rDNA type
MZ 3	Mazandaran	I
MZ 7	Mazandaran	I
MZ 8	Mazandaran	VIII
MZ 9	Mazandaran	I
MZ 10	Mazandaran	I
MZ 11	Mazandaran	I
MZ 13	Mazandaran	I
MZ 14	Mazandaran	I
MZ 15	Mazandaran	I
MZ 16	Mazandaran	I
MZ 18	Mazandaran	I
MZ 20	Mazandaran	I
MZ 21	Mazandaran	I
MZ 22	Mazandaran	I
MZ 24	Mazandaran	I
MZ 26	Mazandaran	I
MZ 29	Mazandaran	VI
MZ 33	Mazandaran	I
MZ 36	Mazandaran	I
MZ 37	Mazandaran	I
MZ 42	Mazandaran	III
MZ 44	Mazandaran	III
MZ 45	Mazandaran	IV
MZ 47	Mazandaran	VIII
MZ 49	Mazandaran	III
GO 1	Golestan	I
GO 2	Golestan	II
GO 5	Golestan	X
GO 7	Golestan	X
GO 11	Golestan	VII
GO 12	Golestan	III
GO 15	Golestan	II
GO 20	Golestan	III
GO 22	Golestan	II
GO 23	Golestan	III
GU 1	Guilan	I
GU 4	Guilan	IV
GU 5	Guilan	IX
GU 7	Guilan	III
GU 8	Guilan	I
GU 10	Guilan	IV
GU 22	Guilan	IV
GU 23	Guilan	IX
GU 24	Guilan	III
GU 25	Guilan	XI
GU 28	Guilan	VI
GU 34	Guilan	III
GU 35	Guilan	III
GU 36	Guilan	VI
GU 37	Guilan	III
P. fluorescens ATCC 49642	bSWRI Culture collection	I

Table 2. Contd.

P. putida ATCC 12633	SWRI Culture collection	V
P. aeruginosa GRP 3	SWRI Culture collection	VIII

[a]The 16S rDNA type represents a combination of restriction patterns obtained by using enzymes. [b]Soil and Water Research Institute, Tehran, Iran.

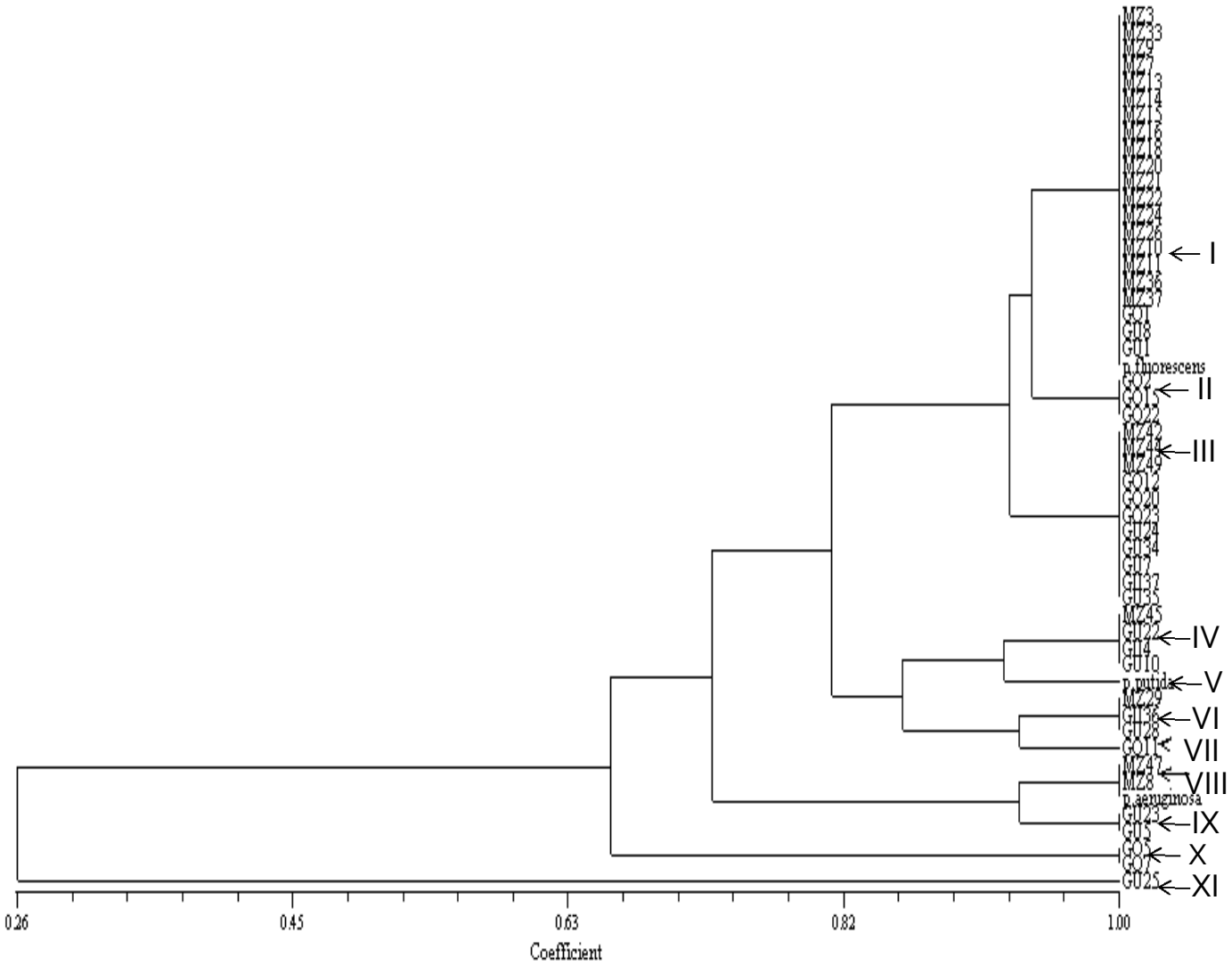

Figure 1. UPGMA dendrogram of fluorescent pseudomonad, based on 16S rDNA profiles obtained by using restriction enzymes *Msp*I and *Hae*III, and NTSYSpc software (distance calculation with the Dice index) in *P. fluorescens* (ATCC 49642) and *P. putida* (ATCC 12633).

strains' RFLP of 16S rDNA confirmed that these bacteria are genetically diverse and mainly belong to *P. fluorescens*, *P. putida* and *P. aeruginosa*.

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