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Genetic Diversity and Efficiency of Indole Acetic Acid Production by the Isolates of Fluorescent Pseudomonads from Rhizosphere of Rice (*Oryza sativa* L.)

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Abstract: A total of 111 strains of fluorescent pseudomonads were isolated from rhizosphere of rice (*Oryza sativa* L., in specific) from different provinces in northern Iran and characterized by standard morphological and biochemical methods. Those isolates were tested to identify the production of indole acetic acid (IAA). The IAA production, presence of tryptophan (50 mg L^{-1}) in the strain was recorded within the range of 17.7 - 95.9 μg mL^{-1}. Twelve strains were randomly selected for further studies. Genetic diversity of selected strains was evaluated by restriction fragment length polymorphism (RFLP) analysis. The isolates were distributed into four distinct of 16S rDNA genetic types, clustering into three major groups. Based on clustering data, five different strains were selected to study their effects on growth, yield and nutrient uptake of rice. It was found that fluorescent pseudomonads isolated from rhizosphere of rice were significantly increased growth, yield and nutrient uptake of plants comparison to the control treatment.

Key words: Rice · *Fluorescent Pseudomonads* · IAA · RFLP · Nutrient uptake

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are group of soil bacteria that actively colonize plant roots and increase plant growth and yield [1,2]. PGPR belong to a range of genera, including *Pseudomonas*, *Azotobacter*, *Azospirillum*, *Bacillus etc* [3,4]. The mechanisms by which PGPR can promote plant growth are not fully understood, but are thought to include: symbiotic nitrogen fixation [5], the ability to produce phytohormones [6], solubilization of phosphates [7] and production of ACC deaminase [8]. Significant increases in growth and yield of agronomical important crops in response to inoculation with PGPR have been demonstrated by many researchers [9,3,10]. In last few decades a large array of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Enterobacter*, *Alcaligens*, *Arthobacter*, *Burkholderia*, *Bacillus* and *Serratia* have reported to enhance plant growth [11,12,1].

Occurrence of species of Pseudomonas in the rhizosphere of different crop plants [12-15] has been previously reported. Strains of *Pseudomonas putida* and *Pseudomonas fluorescens* were particularly effective in increasing root and shoot elongation in canola, lettuce and tomato and yield of potato, radish, sugar beet, tomato, lettuce, apple, citrus, bean, ornamental plants and wheat [16]. Several reports indicating that pseudomonades are dominant in the rhizosphere of rice and their inoculation can increase growth and yield production in different parts of the world [11,17,18]. An evaluation of rice rhizosphere from Cultivars Espinal, Tolima, Colombia, showed to included 69 bacteria; among them, 51% were *Pseudomonas* sp., mainly of species *P. putida, P. aeruginosa, P. fluorescens* and *P. citchori* [42].

Genetic diversity of pseudomonads in the rhizosphere of rice can influence their efficiency to enhance growth of inoculated plants. Ramehskumar *et al.* 2005 reported that all fluorescent pseudomonads isolated from rhizosphere of rice were able to produce IAA. They analyzed genetic diversity of the isolates using RAPD and found that the isolates were genetically diverse.

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The aim of this study was to isolate and characterize *Pseudomonads* from rhizosphere of rice. We studied genetic diversity of the isolates and finally the effect of the selected isolates on growth of rice was also evaluated.

MATERIALS AND METHODS

Samples of rhizospheric soil were collected from fifty rice fields of different locations within three provinces of northern Iran including Mazandaran, Guillan and Golestan. For each sample, one kg of rhizospheric soil (root + adhering soil) was taken and transferred to the laboratory in an ice-box and stored at 4°C for further analysis.

Ten grams of each sample was added to a 250 ml erlenmeyer flask containing 90 ml sterile distilled water and shaken (120rpm) for 30 minutes. Serial dilutions were made and 0.1 ml aliquots (10^3-10^7) were spread on plates containing King's B (KB) medium. Plates were incubated at 28°C for 48 hours and colonies with fluorescent radiation under UV exposure (260nm) were selected and purified on the King's B agar medium [19].

Bacterial isolates were identified according to Bergey's Manual of Systematic Bacteriology [20]. The following general physiological and biochemical tests including gram staining, determination of catalase and oxidase activities, gelatin liquefaction, growth at 41°C, arginine hydrolysis, utilizing citrate as a sole carbon and energy source, acid formation in carbohydrate (trehalose, mesoinositol and glucose) broths were used for identification of pseudomonas species [21].

IAA production was determined by colorimetric as described by Benziri, *et al.* [22]. Bacterial isolates were inoculated into the TSB medium supplemented with tryptophan (50 mg/ml) and incubated at 28°C for 72 hours. Cultures were centrifuged at 7160 rpm for 5 min. IAA was determined by spectrophotometer at 530 nm using Salkowski reagent (mixture of two ml of 0.5 M $FeCl_3$ and 98 ml of 35% $HClO_4$) as coloring agent. The level of IAA production was estimated by a standard IAA curve. Twelve isolates with highest rates of IAA production along with three reference strains, *Pseudomonas fluorescens* ATCC 49642, *P. putida* ATCC 12633 and *P. aeruginosa* GRP3 were subjected to 16S rDNA-PCR-RFLP analysis. Total DNA was extracted according to the method of Ausubel, *et al.* [23]. The concentration of DNA was determined by comparison with known concentrations of λ DNA in 1.5% agarose gel electrophoresis. Primers fD1 (5′-AGAGTTTGATCCTGGCTCAG3-′) and rD1 (5′-AAGGAGGTGATCCAGCC-3′) [24] were used to amplify 16S rDNA region. PCR amplification was done in a Perkin Elmer 2400 PCR system with the following temperature profile: an initial denaturation at 95°C for 2 min and 30 s; 34 cycles of denaturation (35 sec at 94°C), annealing (1 min at 51°C), extension (2 min at 72°C); and final extension at 72°C for 10 min. The size of the amplified DNA was examined by electrophoresis in 1.3% agarose gel.

The restriction enzymes, *Hae*III (Promega) and *Msp*I (Fermentas), were used to digest the amplified DNA. Five microlitre of the PCR products were digested with 4.5 units of enzyme at 37°C overnight. The restricted DNA fragments were analysed by horizontal gel electrophoresis (100 V for 2 h) in TAE buffer on 3% agarose gel containing ethidium bromide and photographed under UV illumination. The analysis of the restriction patterns were carried out as described by Faria da Mota *et al.* [25]. The results of PCR fingerprinting were collected into matrixes indicating the presence and absence of specific bands in each PCR analysis. In each case, a simple matrix was obtained by comparing pair of strains using the simple matching coefficient and a dendrogram was constructed sing the UPGMA. For these analyses, the NTSYS software package (version 2.02) was used. Isolates sharing the same RFLP pattern were defined as rDNA type.

A pot experiment was carried out to investigate the effects of IAA-producing *pseudomonas* strains on growth and yield of three cultivars of rice. Five strains were selected from RFLP patterns (one strain from each gene type) and were used in the experiment. Seeds of rice (cultivars: Tarom, Neda and Khazar) were obtained from Rice Research Centre (RRC) Amol, Iran. The soil for pot experiment was loamy sand in texture characterized by pH, 7.9; N, 0.05%; O.C., 0.56%; P_{ava}, 6.6 mg kg^{-1}; K_{ava}, 135 mg kg^{-1}; Fe, 4.6 mg kg^{-1}; Zn, 2.1 mg kg^{-1}; Mn, 11.6 mg kg^{-1}; and Cu, 9.5 mg kg^{-1}. Pots were filled with sieved soil and 4.5 g urea, 1.5 g Triple Super Phosphate (TSP) and 4.5 g Potassium Sulphate were applied to each pot. The soil was moistened with water and ten seeds were sown in each pot and inoculated with 1 ml of bacterial cultures to provide approximately 10^8 cells $seed^{-1}$. One non-inoculated control was also included. The experiment was carried out in a complete randomized design (CRD) with four replications. After germination, plants were thinned to three per pot. The data collected included fresh shoot weight, fresh root weight, root length and nutrient uptake in the shoots at flowering stage and plant height, yield and yield

components at maturity. The data recovered from pot experiment were subjected to analysis of variance using MSTATC software and means were compared by Duncan's multiple range test.

RESULTS

All soil samples had indigenous populations of fluorescent pseudomonads and a total of 111 isolates were obtained. Results showed that the isolates consisted of three species including *P. fluorescens*, *P. putida* and *P. aeruginosa*. However, the species composition in the provinces was different and *P. fluorescens* was dominant in Golestan and Guilan provinces, whereas in Mazandaran, *P. putida* was the most abundant species (Table 1).

Bacterial isolates were different in their ability to produce IAA. All 111 isolates were able to produce IAA ranging from 17.7- 95.9 mg L^{-1} in the presence of L-TRP. The highest amount of IAA (95.9 mg L^{-1}) was produced by the *P. putida* strain MZ15 isolated from Mazandaran, while the minimum (17.7 mg L^{-1}) was produced by *P. fluorescens* MZ39 as isolate from the same province. Results showed that the average production of IAA by species was very close to each other and the amounts of 42.3, 42.8 and 40.7 mg L^{-1} were obtained from *P. aeruginosa*, *P. putida* and *P. fluorescens*, respectively (data no shown).

PCR of 16S rDNA from all strains produced single band around 1200 bp as estimated by summing the sizes of the restriction fragments after digestion with restriction enzymes. After digestion by restriction enzymes, six 16S-RFLP patterns were identified among 15 studied strains (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 3 groups at similarity level of 90%. Eleven strains were genetically related to *P. fluorescens* ATCC 49642 therefore seven strains showed similar genetic type to *P. fluorescens* ATCC 49642. Strain MZ 45 was separated from other strains and genetically characterized close to *P. putida* ATCC 12633. There was not a clear relationship between grouping pattern of the strains and their geographical origin of isolation however, 75% of the strains isolated from Mazandaran were clustered into two one gene types.

It was found that there were no significant different effects induced by inoculation of IAA-production strains on growth, yield and nutrient uptake of rice. Plant cultivars also showed different responses due to inoculation with bacterial strains.

Inoculation significantly ($p<0.05$) increased plant height and weight, root weight and length, number of panicles and number of spikelets per panicle, length of panicle, number of tillers, weight of 1000 grains and

Table 1: Bacteria isolated and identified in this study

Province	Number of isolates	*P. fluorescens*	*P. putida*	*P. aeruginosa*
Mazandaran	51	15	19	17
Golestan	23	14	6	3
Guilan	37	21	12	4

Table 2: IAA production and 16S rDNA types of selected strains

Strains	Place of isolation	IAA production (mg L^{-1})	16S rDNA type[a]	Source
MZ15	Mazandaran	95.9	I	This study
MZ20	Mazandaran	62.4	I	This study
MZ21	Mazandaran	58.1	I	This study
MZ22	Mazandaran	61.2	I	This study
MZ24	Mazandaran	56.9	I	This study
MZ26	Mazandaran	86.1	I	This study
MZ45	Mazandaran	68.9	V	This study
GO2	Golestan	67.2	II	This study
GO23	Golestan	71.7	III	This study
GU8	Guilan	66.7	I	This study
GU24	Guilan	56.9	III	This study
GU34	Guilan	61.7	III	This study
P. fluorescens ATCC 49642	-	-	I	SWRI[b] culture collection
P. putida ATCC 12633	-	-	VI	SWRI culture collection
P. aeruginosa GRP 3	-	-	IV	SWRI culture collection

[a]The 16S rDNA type represents a combination of restriction patterns obtained by using enzymes

[b]Soil and Water Research Institute, Tehran, Iran

Table 3: Plant height (PH), root length (RL), root fresh weight (RW), shoot fresh weight (SFW), number of tillers (NT), number of panicles (NP), panicle length (PL), spikelets per panicle (SP), weight of 1000 grains and grain yield in the pot experiment

Cultivar	Strains	PH (cm)	RL (cm)	RW (g)	SFW (g)	NT	NP	PL (cm)	SP	W1000 (g)	GY (g pot^{-1})
Tarom	MZ15	115.8a	35.25bc	24.00ab	114.9ab	14.75efgh	19.75defg	26.18bcd	12.00g	29.50fg	28.52b
	MZ26	111.8ab	32.50bcdef	21.33bc	115.8ab	13.50gh	18.75efg	26.05bcde	11.00g	29.00fg	28.75b
	GO23	109.5ab	33.50bcd	17.55de	119.0ab	15.50cdefg	19.50defg	24.66cde	12.00g	30.00efg	28.92b
	GU24	110.8ab	27.50fg	15.75e	118.9ab	14.00fgh	18.25fg	24.14de	11.25g	30.50defg	28.02bc
	MZ45	111.3ab	30.50cdef	19.33cd	116.50ab	12.50hi	19.75defg	25.52cde	11.75g	29.25fg	26.15bc
	Control	106.0bc	19.25h	14.40ef	78.84d	10.75i	13.25h	23.40e	11g	28.50g	24.75cd
Neda	MZ15	72.00d	41.50a	26.48a	122.4ab	21.75a	27.00a	17.50f	35.00a	34.00ab	34.80a
	MZ26	74.25d	36.50b	25.02a	15.5ab	20.00ab	26.50a	18.25f	32.50ab	34.50a	34.33a
	GO23	72.75d	36.00b	26.17a	118.7ab	17.50bcde	24.50ab	17.00fg	33.00ab	33.50ab	34.63a
	GU24	71.50d	31.25bcdef	19.63cd	117.1ab	16.50cdef	21.75bcde	17.50f	29.00c	33.25ab	33.90a
	MZ45	72.25d	34.00bcd	24.20ab	113.6b	15.25defgh	22.75bcd	18.75f	31.75b	32.50abcd	32.60a
	Control	61.00e	29.25defg	15.75e	100.8c	14.25fgh	20.75cdefg	14.75g	24.25e	32.25bcd	26.17bc
Khazar	MZ15	110.3ab	35.25bc	19.85cd	121.9ab	18.00bcd	23.75abc	30.56a	25.00de	32.00bcde	28.69b
	MZ26	110.5ab	33.75bcd	19.95cd	126.1a	18.25bc	24.50ab	30.75a	27.50cd	33.50ab	28.33b
	GO23	113.0ab	32.50bcdef	19.99cd	119.1ab	16.75cdef	22.25bcd	28.80ab	25.50de	33.00abc	27.52bc
	GU24	108.3ab	27.75efg	17.51de	120.7ab	16.50cdef	21.50bcdef	28.80ab	26.00de	34.50a	27.75bc
	MZ45	107.8b	33.00bcde	20.20cd	117.6ab	15.75cdefg	22.75bcd	28.61ab	26.75cde	31.00cdef	28.07bc
	Control	100.8c	24.75g	12.17f	98.14c	13.25ghi	18.00g	27.02bc	19.00f	30.00efg	22.30d

Table 4: Effect of different strains on Plant height (PH), root length (RL), root fresh weight (RW), shoot fresh weight (SFW), number of tillers (NT), number of panicles (NP), panicle length (PL), spikelets per panicle (SP), weight of 1000 grains and grain yield in the pot experiment

Strains	PH (cm)	RL (cm)	RW (g)	SFW (g)	NT	NP	PL (cm)	SP	W1000 (g)	GY (g pot^{-1})
MZ15	99.33a	37.33a	23.44a	119.8a	18.17a	23.50a	24.75a	24.00a	31.83ab	30.67a
MZ26	98.83a	34.25b	22.10ab	119.2a	17.25ab	23.25a	25.01a	23.67ab	32.33a	30.47a
GO23	98.42a	34.00b	21.24b	118.9a	16.58bc	22.08ab	23.49a	23.50ab	32.17a	30.36a
GU24	96.83a	28.83c	17.63c	118.9a	15.67cd	20.50b	23.48a	22.08b	32.75a	29.89a
MZ45	97.08a	32.50b	21.18b	115.9a	14.50d	21.75ab	24.30a	23.42ab	30.92bc	28.94a
Control	89.25b	24.42d	14.11d	92.60b	12.75e	17.33c	21.73b	18.08c	30.25c	24.41b

Table 5: Effect of rice cultivars on Plant height (PH), root length (RL), root fresh weight (RW), shoot fresh weight (SDW), number of tillers (NT), number of panicles (NP), panicle length (PL), spikelets per panicle (SP), weight of 1000 grains (W1000) and grain yield (GY) in the pot experiment

Cultivar	PH (cm)	RL (cm)	RW (g)	SFW (g)	NT	NP	PL (cm)	SP	W1000 (g)	GY (g pot^{-1})
Tarom	110.8a	29.75b	18.73b	110.7b	13.50c	18.21c	24.99b	11.50c	29.46c	27.52b
Neda	70.63b	34.75a	22.88a	114.7a	17.54a	23.88a	17.29c	30.92a	33.33a	32.74a
Khazar	108.4a	31.17b	18.25b	117.3a	16.42b	22.13b	29.09a	24.96b	32.33b	27.11b

Table 6: Effect of inoculation on nutrient uptake of leaves and grains of different cultivaes of rice

		Leaves			Grains		
Cultivars	Strains	N (mg kg^{-1})	P (mg kg^{-1})	K (mg kg^{-1})	N (mg kg^{-1})	P (mg kg^{-1})	K (mg kg^{-1})
Tarom	MZ15	175.6c	62.90b	646.5cde	467.90bc	187.00a	106.50abc
	MZ26	203.6bc	77.24ab	630.1de	500.20ab	193.10a	114.20ab
	GO23	253.3abc	79.10ab	668.8bcde	511.10ab	174.60a	110.30abc
	GU24	205.4bc	65.46b	616.7de	493.50ab	38.91cde	102.00abc
	MZ45	199.8bc	84.59ab	638.8de	460.50bc	24.68e	94.84abc
	Control	191.1bc	60.55b	532.4e	441.00bc	26.41e	88.66bc
Neda	MZ15	545.7a	185.5a	1385.0abc	525.60ab	45.13bcde	127.80a
	MZ26	577.3a	186.4a	1563.0a	478.60b	34.29de	112.30ab
	GO23	433.0abc	151.0ab	1246.0abcde	524.2ab	38.76cde	110.00abc
	GU24	323.5abc	123.5ab	1006.0abcde	572.80a	39.01cde	101.60abc
	MZ45	410.9abc	127.0ab	999.9abcde	490.70ab	35.61cde	101.90abc
	Control	372.8abc	126.6ab	1035.0abcde	382.20c	30.30de	76.77c
Khazar	MZ15	517.6ab	160.7ab	1319.0abcd	114.60d	56.41bc	128.30a
	MZ26	495.0abc	159.0ab	1379.0abc	118.20d	47.82bcd	113.50ab
	GO23	486.1abc	146.7ab	1140.0abcde	114.10d	50.57bcd	110.80abc
	GU24	518.5ab	164.0ab	1385.0abc	115.70d	50.10bcd	113.80ab
	MZ45	544.9a	170.6ab	1224.0abcde	120.60d	65.14b	111.80ab
	Control	524.7ab	147.3ab	1401.0ab	94.63d	44.82bcde	86.85bc

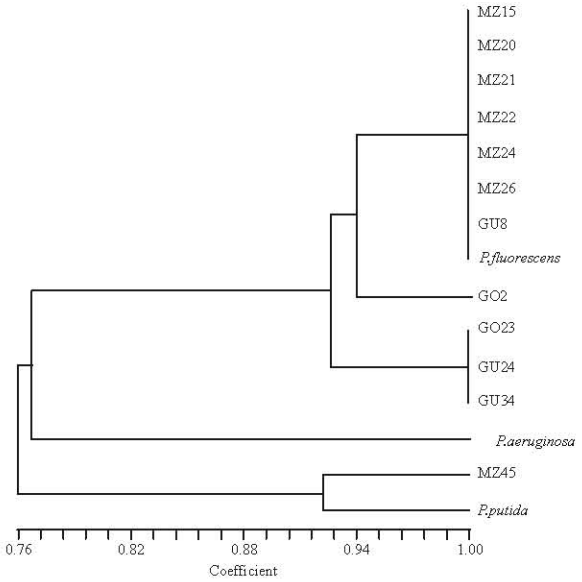

Fig. 1: Dendrogram based on the UPGMA cluster analysis of RFLP patterns of 16S rDNA profiles showing the genetic relationships among the bacteria. TSF: *P. fluorescens* (ATCC 49642), TSP: *P. putida* (ATCC 1 2633) and TSA: *P. aeruginosa* (GRP3)

grain yield compared to un-inoculated control. The highest grain yield was obtained from strain MZ15 when it was inoculated to cultivar Neda, which showed 33% increase over control plants (Table 3). This strain showed the superior performance to enhance growth and yield of rice plants among all tested strains (Table 4). Plant cultivars showed different responses to inoculation with strains. Cultivar Neda showed the greatest potential for growth and yield production where the majority of measured factors were increased due to inoculation compared to the cultivars Tarom and Khazar (Table 5). The results showed a clear and significant increase in total nutrient content of rice leaves due to inoculation with bacteria (Table 6). Inoculation with strain MZ26 increased content of N (55%), P (47%) and K (51%) compared to un-inoculated control. Nutrient uptake of grains was also affected by inoculation with strains. Strains GU24, MZ26 and MZ15 significantly increased N, P and K content of the grains compared to control treatment (Table 6).

DISCUSSION

In this study, the occurrence and IAA-production potential of fluorescent pseudomonads isolated from rice fields was evaluated. Genetic diversity and efficiency of the selected strains on rice was also studied. Biochemical identification methods were used to characterize the isolates. These methods have been used by many researchers to identify soil bacterial isolates [26,27,28]. The study showed that *Pseudomonas* sp. was dominated in the rhizosphere of rice. The isolates of this study were distributed into three species including *P. fluorescens*, *P. putida*, *P. aeruginosa*. This is consistent with some previous reports, which have isolated and identified rhizospheric bacteria from rice [29,30,31]. In addition, Tripathi, *et al.* [32] was also found that *P. aeruginosa* was the most dominant member of the bacterial community inside rice roots.

IAA production is widespread among soil rhizobacteria [33,34]. Isolates of this study were able to produce different amounts of IAA ranging from 17.7- 95.9 mg L^{-1}. Differences among isolates of *Pseudomonas* sp. in their ability to produce IAA were previously observed by other workers [35,36]. Strain MZ15 produced 86.6 mg L^{-1} IAA and showed the best performance for IAA production.

Twelve selected strains with highest IAA production were used to study their genetic diversity. The PCR-RFLP analysis of 16S rDNA region was used to evaluate genetic diversity of the bacteria. Fingerprinting of 16S rDNA has been used by several researchers to discriminate genetic differences between bacterial species [37,27,10]. In this study, bacteria exhibited relatively low genetic diversity either in the length of uncut 16S or in the patterns revealed after digestion with restriction enzymes. This is consistent with some previous reports, which have used 16S RFLP to study the genetic diversity of *Pseudomonas* sp. Isolated from rhizosphere of crop plants [27]. The 16S rDNA-RFLP analysis revealed that tested isolates contained of 4 genotypes and be clustered into 3 groups at the similarity of 93%. Group 1 contained 8 isolates and *P. fluorescens* ATCC 49462. This is demonstrating relatively high similarity between *Pseudomonas* sp. isolates from rice and a *P. fluorescens* reference strain.

In this study, the clustering of isolates based on 16S-RFLP profiles did not reflect their geographic origins. Similar results have been reported for other soil bacterial species [38].

Several researchers reported the positive effect of pseudomonads on growth characteristics of different crop plants [1, 39, 29, 40]. Isolates used in pot experiment showed positive effects on growth and yield of rice. However, they were different in their potential to enhance plant growth. In a study of Kloepper *et al.* [11], inoculated

different strains of Pseudomonas to the rice and an increase in the yield production ranging from 3-160% were obtained. The isolates of this study were also able to enhance nutrient content in the leaves and grains of rice plants. Similar results have been reported previously [41].

In conclusion some efficient strains of *Pseudomonas* were identified that effectively promote growth and yield of rice in greenhouse condition.

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