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Identification of Phosphate Solubilizing *Pseudomonas sp.* of Rice Rhizosphere Based on 16s rDNA Genotyping

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Abstract: Specific strains of fluorescent pseudomonas inhabit the environment surrounding plant roots and some even the root interior. Introducing such bacterial strains to plant roots may lead to increased plant growth. That is usually due to direct and indirect mechanisms action, such as phytohormons production, phosphate solubilization, sidrophore production and other actions. *Pseudomonas* species were isolated from the rhizospheres of rice (*Oryza sativa*) cultivars (Tarom, Neda and khazar) in 50 different paddy fields of three provinces in north Iran (Golestan, Guilan and Mazandaran). The isolates were identified as fluorescent species of *Pseudomonas* on the basis of their morphological properties and the ability to fluoresce under UV light. The isolates were characterized by biochemical and physiological traits too. Based on Sperberg method, the rate of phosphate adsorption of the samples was determined by Spectrophotometer at wavelength of 470nm. Thirty one isolates with high ability of phosphate solubilization (with rates more than average 205.64 mg/l) were selected and their phosphate solubilization rate was determined in three replications. Based on the obtained results, it was concluded that among the strains there were significant differences at 0.05% level (Duncan test). The maximum phosphate solubilization achieved by the selected strains was 263.2 mg/l for MZ4 strain. The minimum phosphate solubilization obtained by the desired strains was 207.2 mg/l for MZ16 strain. The genotypic diversity of sixteen isolates (with different rates of phosphate solubilization) together with three type strains was determined on basis of amplified 16s rDNA restriction analysis (RFLP). This analysis showed that the majority of *pseudomonas* isolates obtained from rice rhizosphere which showed great ability for phosphate solubilization were *Pseudomonas fluorescens* > *P. putida* > *P. aeruginosa.*

Key word: Rice rhizosphere · *Fluorescent pseudomonas* · Phosphate solubilization · RFLP

INTRODUCTION

The use of 16S rRNA gene as a phylogenetic marker has been criticized, as it assumes one molecule reflects the evolution of an organism and the rate of evolution of the 16S rRNA gene is not sufficient to permit resolution of intrageneric relationships [1]. Despite this, there remains widespread confidence in 16S rRNA based phylogenetic inferences [2, 3]. Malik and his co-workers isolated various bacteria from rice roots and its rhizosphere, which were found to belong to the genera of *Azospirillum*, *Azotobacter*, *Flavobacterium*, *Pseudomonas*, *Xanthomonas* and *Zooglea* [4]. Hirkala showed that diazotrophs predominate among the complete bacterial microbiota of rice rhizosphere; he has isolated the diazotrophs belonging to the genera of *Bacillus*, *Azospirillum*, *Pseudomonas* and *Flavobacterium* [5]. In general, *Azospirillum* was the dominant diazotroph in every ecosystem. Khayyati and Anvari [6] confirmed that all diazotrophic isolates of the rice rhizosphere soil were associative nitrogen fixers and belonged to *Enterobacteriaceae*, organisms in particular are *Pseudomonas* and *Bacillus* genera. The isolates of the genus *Pseudomonas* were dominant, while those from *Serratia* and *Enterobacter* were present in small numbers. Xie and his group [7] have found that bacteria isolated from the paddy field and identified rice farms that the isolates were belonging to the genus such as *Bacillus*, *Burkholderia*, *Agrobacterium*, *Pseudomonas*, *Derxia*, *Alcaligenes*, *Aeromonas*, *Citrobacter* and *Corynebacteria* group. Kumar and Kumari Sugitha [8] have reported that the proportion of total diazotrophs to

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total heterotrophs was in the range of 12.39 to 20.65% in the rhizosphere of various rice cultivars. The distribution pattern of these diazotrophs was in order of *Pseudomonas* > *Azospirillum* > *Azotobacter* > *Beijerinckia* > *Derxia* > *Klebsiella* > *Enterobacter* [8].

PCR-RFLP based on 16s rRNA has been used by several researchers [9-12], since this method is useful and rapid for taxonomic and strain-typing purposes. Thakuria and his coworkers [13] have isolated three groups of rhizobacteria from rice rhizosphere, *viz*., phosphate solubilizing, fluorescent bacteria and *Azospirilla* group. RAPD analysis of the *Azospirillum* isolates indicated that they belonged to four distinct genotypes [13].

The aim of the present research paper was to study and analyze the genotype diversity among fluorescent *Pseudomonas* spp. with the purpose of phosphate solubilization potential which was isolated from rhizosphere of rice fields in north Iran.

MATERIALS AND METHODS

Collection of 111 strains isolated from rhizosphere of rice fields in north Iran were identified previously as *Pseudomonas* species according to methods discussed in Bergey's Manual of Systematic Bacteriology [14, 15].

For definition of phosphate solubilization rate, phosphate adsorption of samples was determined. Briefly, bacteria were grown in 20 ml trypticase soy broth (TSB) in 50ml Erlenmeyer flasks in an orbital incubator shaker (125 rpm) at 27°C for 72 h. Then 1 ml of TSB suspension was transferred into a 50ml Erlenmeyer flask containing 20 ml Sperber medium and grown at the same condition. No bacteria were introduced as inoculums into the control flasks. Sample size of 10ml aliquots were aseptically taken from each flask and centrifuged at 15000 g for 5 min. One ml of culture supernatant was transferred to the tube, where 1ml of ammonium vanadate and molibdate were added. These samples were held for 20 minutes at laboratory temperature, then the rate of phosphate adsorption of samples was determined by spectrophotometer at the wavelength of 470 nm [16]. Thirty one isolates with the maximum ability for phosphate solubilization (with rates more than average 205.64 mg/l) were selected and their phosphate solubilization was determined by three replications.

Sixteen of these isolates were randomly selected with high, medium and low quantities with respect to ability of phosphate solubilization using together three types of strains for the genotype diversity analysis based on RFLP marker. Total bacterial DNA was prepared by using a CTAB extract method [17, 18]. Briefly, cultures were incubated in trypticase soy broth (TSB) (Difco) medium and cells were lysed by SDS and the resulting lysate was treated with proteinase K. DNA was purified with a solution of CTAB/NaCl (10% cetyltrimethylammonium bromide in 0.7 M NaCl) followed by chloroform/ isoamylalcohol (24: 1) and phenol/ chloroform/ isoamylalcohol (25: 24: 1) extractions. DNA was recovered by isopropanol precipitation and washed in 70% ethanol. The pellet was suspended in 50 µl of TE buffer (10 mM Tris pH 8.0 1 mM EDTANa_2) with addition of 1 µl of DNA, RNAse free and stored at 4°C.

The 16S rDNA region was amplified using the primers fD1 (5´AGAGTTTGATCCTGGCTCAG3´) and rD1 (5´AAGGAGGTGATCCAGCC3´) [19]. Amplification was conducted in a final volume of 50 µl with 1 µl of bacterial suspension (optical density (OD600) of 0.5), 5 µL of buffer, 0.2 tM (0.4 µl) of each primer, 1.0 mM (1.5 µl)$MgCl_2$, 200 tM (0.4 µl) dNTPs and 2.5 U (0.4 µl) of RED*Taq*™ DNA polymerase. 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 min and 30s at 95°C, 32 cycles of denaturation (35s at 94°C), annealing (1 min at 51°C), extension (2 min at 72°C), final extension (10 min at 72°C). Amplification of DNA was confirmed by electrophoresis in 1.3% (w/v) agarose gel. 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. A 5 µl of PCR products were digested with 2 U (3 µl) of the respective restriction enzyme for overnight at 37°C. The digestion products were separated by electrophoresis on 3.0% agarose gels for 2 hours in 100 V. Gels were stained with ethidium bromide and photographed under UV light [20, 21].

Statistical Analysis: The data for comparison of phosphate solubilization average were subjected to analysis of variance using MSTATC, computer software [22] and by Duncan's Multiple Range Test [23]. The phenotypic data were converted into a binary matrix that was analyzed using the simple matching similarity coefficient. The Dic similarity coefficient [24] matrix was subjected to UPGMA analysis and a phenogram was created using average linkage procedure. The calculation of simple matching similarity coefficients and the

construction of the UPGMA dendograms were conducted using the NTSYS-PC [25]. The Multivariate Statistical Package (NTSYSpc2.0 Package v.2.02e.) software was used for the analysis.

RESULTS AND DISCUSSION

Ability of Phosphate Solubilization by Fluorescent Pseudomonas Strains: Bacterial isolates examined for determination of their ability of phosphate solubilization. The quantity of phosphate solubilization varied greatly within the bounds from 139.25 - 272.03 mg/l.

The rates of phosphate solubilization for each province are presented. The data on phosphate solubilization by rhizospheric *Pseudomonas* species isolated in Mazandaran province are shown quite variations, for *P. aeruginosa* from 140.22 to 237.12 mg/l and average rate of phosphate solubilization was equal to 191.83 mg/l; for *P. putida* from 161.89 to 263.06 mg/l, with average rate equal to 192.70 mg/l; and for *P. flourescens* species this characteristic was 143.83-233.51 mg/l and average rate of phosphate solubilization equal to 185.543 mg/l (Figure1). The data on phosphate solubilization by rhizospheric *Pseudomonas* species isolated in Golestan province are also shown variation as well, for *P. aeruginosa* from 165.8 to 214.25mg/l and average rate of phosphate solubilization was equal to 192.98 mg/l; for *P. putida* from 139.25 to 231.1mg/l, with average rate equal to 182.89 mg/l; and for *P. flourescens* species this characteristic was 142.78 to 272.72 mg/l and average rate of phosphate solubilization equal to

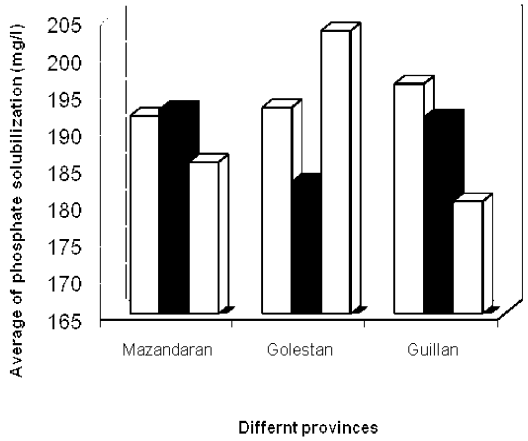

Fig. 1: Average rate of phosphate solubilization by fluorescent pseudomonas strains in 3 provinces paddy fields (Gray column = *P. aeruginosa*, Black column = *P. putida*, White column = *P. fluorescens*)

203.35 mg/l (Figure 1). And so the data for phosphate solubilization by rhizospheric *Pseudomonas* species isolated in Guilan province are shown that phosphate solubilization by several species was varied: for *P. aeruginosa* from 177.57 to 214.85 mg/l and average rate of phosphate solubilization was equal to 196.17 mg/ml; for *P. putida* from 159.48 to 211.24 mg/l, with average rate equal to 191.63 µg/ml; and for various strains of *P. flourescens* species this characteristic was 161.8 to 228.09 mg/l and average rate of phosphate solubilization equal to 180.23 mg/l (Figure 1). Based obtained results, it was concluded that there were significant differences among the isolated strains. The maximum phosphate solubilization rates produced by the desired strains were 263.2 mg/l for the isolate MZ4 strain and the minimum phosphate solubilization rates produced by the selected isolate strains were 207.2 mg/l for the suitable isolate MZ16 strain (Table 1). The presence of phosphate solubilization bacteria in soil has been discussed for rice plants. These investigations were conducted by Bishop and his research team [26] and also by Abd-Alla [27] and many others as well. The results of the present investigation highlighted those phosphate solubilization bacteria from local soils after isolation can be used for inoculation of rice seeds to improve soils fertilities and captivities and production yields. After the comparison of obtained results, the thirty one species with the phosphate solubilization large quantities, more than 205.64 mg/l were selected. The determination phosphate solubilization large quantities were carried out in three replicates and their significantly different means were compared (Table1).

Molecular Analysis: Sixteen isolates with different rates of phosphate solubilization and three type strains contain of *P. aeruginosa* (GPR3), *P. fluorescens* (D. van der Kooij stain P17) and *P. putida* (DSM 291 Type strain) selected for molecular analysis and determination of genotypic diversity.

PCR–RFLP Analyses of 16S-rDNA: The genotypic diversity of bacterial was analyzed by means of RFLP. A 1,200bp fragment was amplified using the universal primers fd1 and rd1 which is consistent with the pre-established size of pseudomonas genus bacterial 1 6S rRNA genes [20, 21, 28]. In general, enzyme digestions of the 1 6S rDNA products resulted in restriction patterns constituted by 3 to 6 bands with molecular size ranging between 130 to 950 bp for enzymes *Hae*III and MspI.

Table 1: Mean values of phosphate solubilization (mg/l) by the most productive strains of fluorescent pseudomonas

Number of strain	Average of phosphate solibulization (mg/l)	Number of strain	Average of phosphate solibulization (mg/l)	Number of strain	Average of phosphate solibulization (mg/l)
MZ1	226.5hi	MZ16	207.2o	GO15	255.1c
MZ3	237.4f	MZ18	210.5lmn	GO17	244.8d
MZ24	212.2klm	MZ34	213.4kl	GU3	208.2no
MZ27	223.9i	MZ47	233.4g	GU10	220.6j
MZ43	207.4o	GO7	214.1k	GU15	208.1no
MZ48	219.5j	GO12	213.6k	GU20	212.0klm
MZ11	220.7j	GO22	231.1g	GU5	213.3kl
MZ4	263.2b	GO2	240.6e	GU6	214.1k
MZ49	220.3j	GO8	237.5f	GU12	228.2h
MZ50	217.9j	GO11	272.0a		
MZ9	209.4mno	GO13	214.3k		

* Values followed by different letters in a column were significantly different ($P < 0.05$); using Duncan's multiple range tests

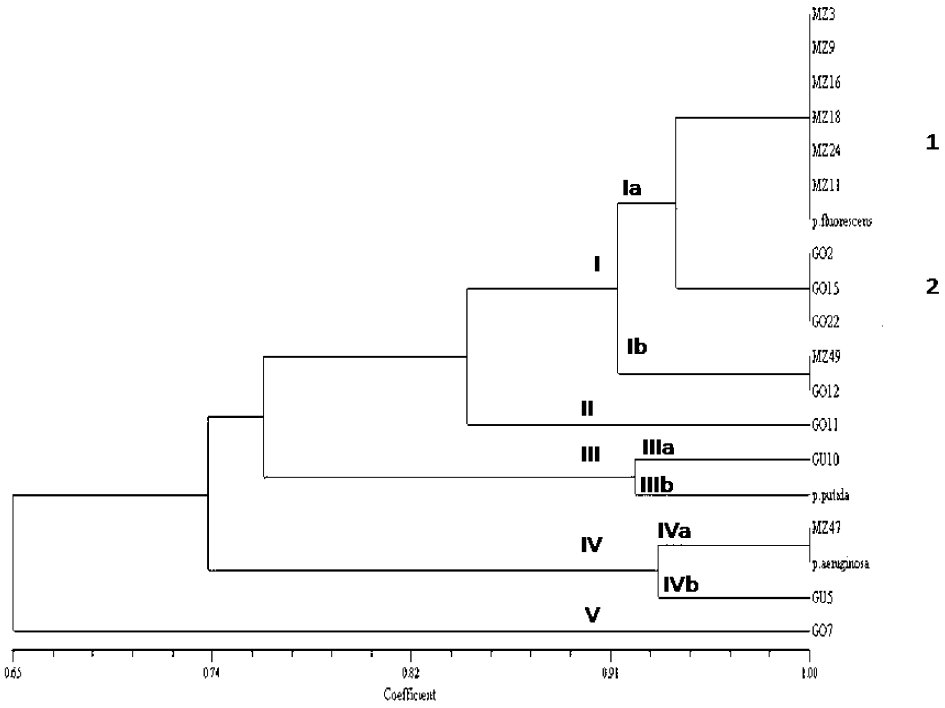

Fig. 2: UPGMA dendrogram of fluorescent pseudomonas strains based on 16S rDNA profiles obtained by using restriction enzymes *Msp*I and *Hae*III and NTSYSpc software (distance calculation with the Dice index). TSF = *P. fluorescens* (HAMBI* 2305), TSP = *P. putida* (HAMB* I7) and TSA = *P. aeruginosa* (GRP3), *HAMBI is the Culture Collection at the Department of Applied Chemistry, University of Helsinki, Finland.

Differences between Genotypic RFLP profiles of isolated strains isolates can be observed for the enzymes *Hae*III and MspI.

Five distinct restriction patterns, with three to four restricted fragments per pattern, were detected for HaeIII restriction enzyme and six distinct restriction patterns with three to five restricted fragments per pattern, were detected for MspI restriction enzyme.

Cluster Analysis of Combined 16S rDNA RFLP: To estimate the relationships between the sixteen rDNA genotypes, cluster analysis was performed based on the combined data sets of the 16S rDNA with two endonucleases restriction patterns using UPGMA (Figure 2). The obtained dendrogram showed that all of the *pseudomonas* strains experimented in this work grouped into five clusters. Cluster I was composed of

two sub cluster designated as sub cluster Ia and Ib. The similarity values for the internal branch of sub cluster Ia and Ib were 92%, although the similarity value for the branch of subgroups Ia was relatively high (94%). Members of group 1 from sub cluster Ia were composed of two groups; group of 1 was contained some isolates that were haplotype and that was composed rDNA isolates MZ3, MZ9, MZ16, MZ18, MZ24,MZ11 and *P. fluorescens* reference. Members of group 2 from sub cluster Ia were composed of rDNA GO2, GO15 and GO22, this group was haplotype too. Sub cluster Ib were composed from two rDNA MZ49 and GO12 as haplotype Strains within these groups were *P. fluorescens* that is related with sub cluster Ia (92% similarity). Cluster of II had one member, rDNA GO11. This cluster related to cluster I with 84% similarity value that concluded GO11 was one biovar of *P. fluorescens*. Cluster III was composed of two sub cluster designated as sub cluster IIIa and IIIb. The similarity values for these sub cluster IIIa and IIIb were 92%. Sub cluster IIIa had one rDNA genotype (GU10) that was related with reference *P. putida* in sub cluster IIIb. This relationship with 92% similarity value acclaimed isolate of GU10 was one biovare from *P. putida*. Cluster IV was composed of two sub cluster designated as sub cluster IVa and IVb. The similarity value for these sub clusters was 93%. Sub cluster IVa had two rDNA genotypes (MZ47) and reference isolate of *P. aeruginosa*, these two rDNA genotype were composed one haplotype group. Sub cluster IVb had one member rDNA genotype, GU5, this isolate can be one biovare from *P. aeruginosa* that was related with sub cluster Iva in 93% similarity value. Finally cluster V, this cluster had one rDNA genotype (GO7), relationship GO7 with *Pseudomonas fluorescent* isolates were studied in this experiment was in 65% similarity value. Results obtained from this dendogram showed the majority of the best isolates with phosphate solubilization ability were *P. fluorescens*. PCR-RFLP based on *16s rRNA* has been used by several authors [9, 4, 11, 12] because this method is useful and rapid for taxonomic and strain-typing purposes. Thakuria and his coworkers [13] have isolated three groups of rhizobacteria from rice rhizosphere, *viz*., phosphate solubilizing, fluorescent bacteria and *Azospirilla* group. RAPD analysis of the *Azospirillum* isolates indicated that they belonged to four distinct genotypes [13].

CONCLUSION

It was concluded that, these results showed fluorescent pseudomonas stains isolated from rhizosphore of rice had phosphate solubilizing ability with high quantities and analyzing genotype diversity some of these isolates, showed that *P. fluorescent* isolates were dominant in this investigation. Ability of phosphate solubilizing of isolates was important factor for the selection of *Pseudomonas fluorescens* as biofertilizer. Further studies for the screening bacteria used for inoculation of rice seed will be conducted based on molecular analysis.

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