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# Molecular and functional diversity of PGPR fluorescent Pseudomonads based on 16S rDNA-RFLP and RAPD markers

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**Abstract**

The genetic and functional diversity of plant growth promoting rhizobacterial (PGPR) fluorescent pseudomonads associated with chickpea (*Cicer arietinum* L.) rhizosphere was analyzed. In total, 34 isolates along with two reference isolates were screened for various plant growth promoting traits (phosphorous solubilization, ACC deaminase, HCN, IAA and siderophore productions) and antagonist activity against four fungal phytopathogens and three bacterial pathogens. Most of the isolates, that showed PGPR activity, also showed antagonistic activity against all the three fungal pathogens. The genetic relationship was assessed by using random amplification of polymorphic DNA (RAPD) and PCR-restriction fragment length polymorphism (16S rDNA-RFLP). Relationship between both the markers was analyzed based on mantel test and a negative correlation was observed. The study concluded that PGPR traits appeared to be strain specific rather than specific to any phylogenetic group. The study also reported that 16S rDNA based profiling differentiated PGPR fluorescent *Pseudomonas* on the basis of location rather than biological trait. RAPD profiling could be useful to differentiate among the closely related isolates. The genetic and functional diversity of fluorescent pseudomonads, associated with the chickpea rhizosphere, has useful ecological role and potential utilization in sustainable agriculture.

**Keywords**

Fluorescent pseudomonads, Phosphate solubilization, 16S rDNA-RFLP.

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## Introduction

Agriculture and other natural resource-based enterprises are the foundation for economic growth in many Asian countries. Excessive and indiscriminate use of agrochemicals has indisputably resulted in negative impact and sometimes permanent damage to the environment. In the same way, reliability on chemical pesticides to manage pest problems has aggravated environmental ruins (Stark *et al*., 2007; Bhattacharyya and Jha 2012). It is in this context that bio-alternatives or bio-agents, such as bio-fertilizers and bio-pesticides are considered as viable options. Fluorescent pseudomonad is one of the important groups of bacteria which have been well accepted as plant growth promoting rhizobacteria (PGPR). Reasons could be suppression of plant pathogens or production of certain growth hormones. Fluorescent pseudomonads have been studied for their capability to enhance plant growth by suppressing soil borne pathogens through well known mechanism of Induced systematic resistance (ISR) against phytopathogens (Naik *et al.,* 2008; Adesemoye and Kloepper, 2009; Adesemoye *et al.,* 2009). Fluorescent pseudomonads are widely distributed which suggests their range of adaptability with nature. This group of bacteria is preferably matched as soil inoculants due to aggressive colonization with plant roots. This feature alone is suggested as a disease control mechanism by preventing the invasion of soil pathogens onto the root surface (Martinez-viveros *et al*., 2010; Berg, 2009; Mishra *et al.,* 2013). In this direction, PGPR fluorescent Pseudomonad offer an attractive alternative as bio-inoculants and bio-control agents for sustainable agriculture (Jatiyanon *et al.,* 2003; Dardanelli *et al.,* 2010; Bhattacharyya and Jha 2012; Perez-Brandian *et al.,* 2014).

Conventionally, morphological characters have been used for identification of bacteria, but these methods are not precise enough to distinguish between similar organisms and to

understand the molecular evolution (Dastager *et al.,* 2010). Henceforth, use of molecular markers can be a reliable and authenticate method for identification and to understand the evolution.

Molecular and functional diversity of *Pseudomonas* spp. is not only important to understand their ecological role, but also to know the type of interaction taking place in soil rhizosphere for various applications. Likewise reports are now available correlating the genotype of an isolate with its biological activity (Landa *et al.,* 2002; Berg *et al*., 2002; Kwon *et al*., 2005). Diversity of fluorescent *pseudomonas* is being reported from rhizosphere of many important crops like rice, cotton, banana and wheat (Juliastuti *et al.,* 2003). However, a comprehensive analysis of functional activities has yet not been carried out in chickpea rhizosphere. Therefore, the present study determined the relationship between the genotype of indigenous fluorescent pseudomonads against phytopathogens isolated from chickpea rhizosphere. The potential isolate could be used to develop site specific bio-fertilizer for chickpea crop.

## Materials and Methods

**Isolation and *in-vitro* screening of *PGPR pseudomonas:*** Soil samples were collected from the rhizosphere of chickpea plants grown in agricultural fields from seven different locations in India and fluorescent pseudomonads were isolated. The soils were chemically analyzed by measuring pH, total nitrogen, organic matter, sand, silt and clay compositions. Roots along with the adhering soil were homogenized in phosphate-buffered solution and subsequently serially diluted and plated on King`s B medium (Protease peptone: 20.0g, Glycerol: 10.0 mL, $K_2HPO_4$ (anhydrous): 1.5g; $MgSO_4.7H_2O$: 1.5g, Agar-agar: 16.0g, Adjust the pH to : 7.2-7.4) (Johnson and Curl, 1972). The plates were incubated at $30^0$ C for 24 hrs, and colonies that fluoresced under UV (λ=356 nm) were selected. Pure cultures were obtained by repeated streaking on the same medium.

**Siderophore production assay:** Semi-quantitative analysis of siderophore production by the cultures was determined by Alexander and Zuberer (1991). The CAS agar medium (Chrome Azurol S 60.5mg/50ml of distilled water, Hexadecyltrimethyl ammonium bromide 72.9mg/40ml of distilled water, Kings Medium B base 42.23g, Distilled water 900ml, adjust the pH to 6.8±0.2) was prepared according to Alexander and Zuberer's protocol. For each bacterial isolate, 10 µl of log phase bacterial suspension was spotted on the plate. Change in medium color was observed after 3 days, discoloration of medium (blue to orange) indicated siderophore-producing ability. Two perpendicular diameters of discoloration area were measured.

**Inorganic phosphorus solubilization:** All the isolates were screened for phosphate solubilization activity after growth on Pikovaskaya`s (PKV) agar (Glucose: 10.0g, Tricalcium phosphate: 5.0g; $(NH_4)_2SO_4$: 0.5g; KCl: 0.2g; $MgSO_4.7H_2O$: 0.1g; $MnSO_4$ and FeSO: Traces; Yeast extract: 0.5g; Agar: 18.0g; adjust the pH to: 7.0) amended with tri-calcium phosphate as a source of insoluble phosphorus in the medium. Bacterial cells at log phase were collected by centrifugation and spotted on PKV medium. The plates were incubated for 5 days at $30^oC$ and solubilization of mineral phosphate was characterized by forming a clear halo zone around the bacterial isolates (Pikovskaya, 1948).

**HCN production:** Isolates were grown on HCN induction medium (containing 30 g Tryptic soy broth, 4.4 g glycine, 15 g agar per liter) and incubated at 30°C for 4 days (Castric, 1977). For each bacterial isolate, 10 µl of the inoculum was dropped at the center of plate (5.5 cm of diameter, 10 µl of HCN induction medium). Then, a disk of Whatman paper (5.5 cm diameter), previously dipped in HCN revealing solution (0.5% picric acid and 2% $Na_2CO_3$), was placed the middle of Petri dish and the plate was sealed with an air-tight tape. After 4 days of incubation at 30°C, a yellow to brown colour indicated HCN producing bacterial isolates.

**Indole acetic acid (IAA) production:** Production of indoles was assayed as described by Patten and Glick (1996). Tryptone broth was prepared and transferred into the test tubes. After sterilization, these test tubes were then inoculated with the culture and one of the tubes was kept un-inoculated as control. These inoculated tubes were incubated at 30°C for 24 hr. After 24 hr of incubation, 1 ml of Kovac's reagent was added to each tube including control. Tubes were shaken gently after 10-15 min interval at and allowed to stand. Development of cherry red colour the top layer of the tube indicated a positive result by *In vitro* test for antagonism.

**Antifungal activity:** To determine the antifungal activity, agar plugs (5 mm) of fully grown fungal culture was taken by using the sterilized crock borer**.** Four phytopathogens namely *Fusarium udum* (MTCC-2755) *Fusarium oxysporum (CABI 293942)*, *Fusarium culmorum* (MTCC 2090) were used in the present study. Simultaneously, bacterial cultures were streaked at a distance of 4 cm from the agar plug at the sides towards the edge of the petriplate. Control plate was kept without any bacterial culture and all the plates were incubated at 28 °C until control plate grew fully (Ayyadurai *et al.,* 2007).

**Morphological and biochemical characterization of PGPR *pseudomonas*:** Morphological characteristics (Gram stain, shape, motility and sporulation) and biochemical characterization (fluorescent pigment production on King`s B medium, oxidase, gelatin hydrolysis, catalase, citrate utilization, indole test, Arginine and alanine decarboxylation and gelatin liquefactions) were recorded by following the keys and protocols of Bossis *et al.,* (2000).

**Genetic relationship study:** Genomic DNA was extracted by using Hi-Pure[ATM] Bacterial and Yeast Genomic DNA Purification Spin Kit (HI-MEDIA, Mumbai, India) according to the



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manufacturer's instructions. 16S rRNA gene was amplified as described earlier by using universal *E. coli* primers, forward from position 7 to 26 (5'-AGAGTTTGATCCTGGCTCAG-3') (Gamaleroa et al., 2003) and reverse primer from position 1,513-1492 (5'-ACGGCTACCTTGTTACGACTT-3') (Bossis et al., 2000). Amplification was performed in 20µl reaction mixture consisting of 2µl of PCR assay buffer (10X), 2.0µl of dNTPs (2.5mm), 0.6µl primers (10 pmol), 0.2µl of Taq polymerase (1U/µl), 1.5µl of template DNA (50ng/ µl), and remaining of millipore distilled water. Amplification was conducted in Veriti Applied Bio-system thermal cycler with an initial denaturation step at 95°C for 5 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min 20 sec, followed by final extension at 72°C for 6 min. An expected amplicon of 1.4-1.5 kb was obtained and subsequently digested with three (*Alu*I, *Msp*III and *Hae*III) tetra cutter restriction endonucleases (MBI Fermentas). Restriction was carried out in a 15µl reaction mixture containing 1.0µl of restriction enzyme (10U/µl), 1.5µl of dilution buffer (10X), 10 µl of PCR product and 2.5µl of autoclaved distilled water followed by incubation at $37^{0}$C for 3 hours in water bath. Digested fragments were separated on 2.0% agarose gel electrophoresis, containing ethidium bromide (0.5µg/ml) and photographed under ultraviolet light. Fragments sizes were compared with low range DNA ladder (Fermentas).

In total of 40 random primers were screened, 18 of which produced clear and unambiguous bands were selected. PCR amplification was carried out in 25 uL volumes containing 2.5 µL of 10X reaction buffer (with 2.5 mM $MgCl_2$) 2.5 µL of 2mM of dNTP mixture, 5 µL of 50 pmol of each primer, 1 µL of 3U Taq DNA Polymerase (Fermentas), 13 µL of HPLC grade water and 1 µL 25ng template DNA. DNA amplifications was performed with one cycle of initial denaturation ($94^{0}$C, 5 min), followed by 40 cycles of denaturation ($92^{0}$C, 4 min), annealing ($28^{0}$C, 1 min) and extension ($72^{0}$C, 2 min) with a final extension at $72^{0}$C for 10 min. The PCR products were visualized as earlier. All RAPD-PCR were repeated three times and regenerative bands were included in scoring for genetic relationship analysis.

Calculation of pair-wise coefficient of similarity was based on presence and absence of bands. The character state '1' was given for the presence and character state '0' was assigned if the band was absent. The data matrix thus generated was used to calculate Jaccard's similarity coefficient bye unweighted pair group method with arithmetic mean (UPGMA). Similarity matrix was calculated with the Dice coefficient (Kumar et al., 2002). In order to test the goodness of fit of cluster analysis, cophenetic value matrices were calculated and compared with the original similarity matrices that were UPGMA clustered by using the NTSYS-pc analysis package (Satou and Nei, 1987).

Correlation between the matrices generated from ITS-RFLP and RAPD analysis was compared using the product moment correlations (r) derived from the normalized Mantel Z test

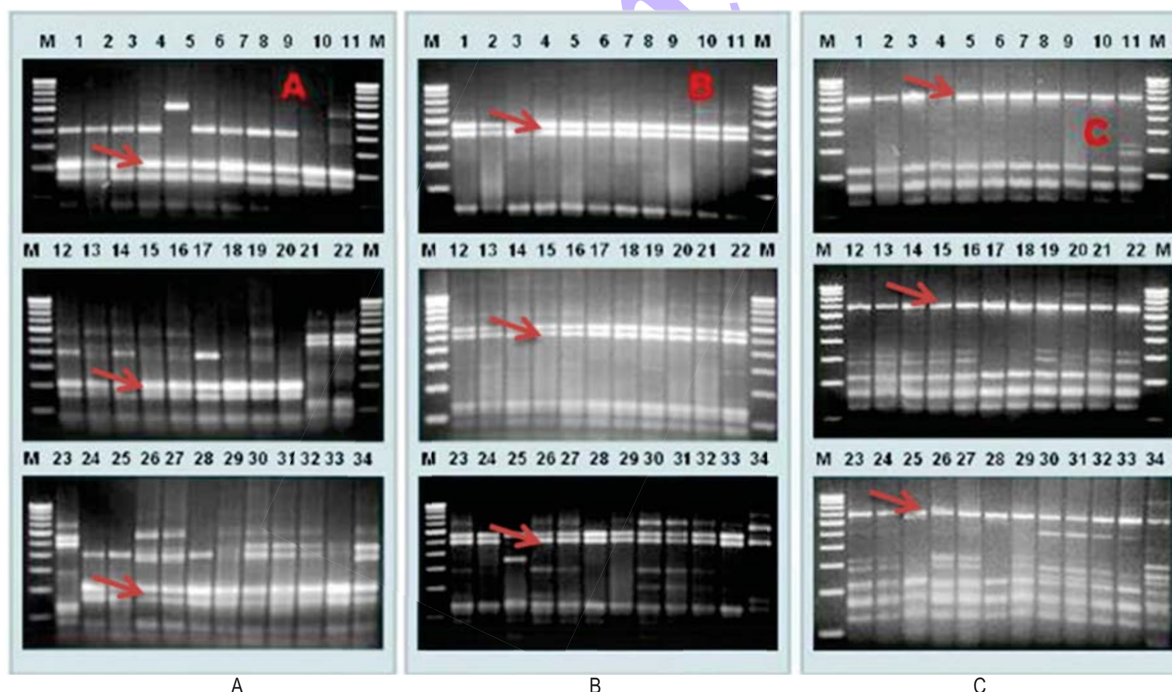


**Fig. 1 :** ARDRA banding pattern obtained from deferent restriction endonucleases. (A) Digested with *Alu* I; (B)Digested with *Msp* I; (C) Digested with *Hae* III. Isolates numbers are shown as 1-34, M-1KB molecular marker. Monomorphic bands obtained are marked with arrows

**Table 1** : Production of plant growth promoting traits by selected PGPR fluorescent *Pseudomonas*

| Isolate | Siderophore production | IAA production | HCN production | P-Solubilization (in mm) | Antifungal Activity (mm*) | | |
|---|---|---|---|---|---|---|---|
| | | | | | FO | FM | FS |
| 1 | + | + | + | + | + | + | + |
| 2 | + | + | + | + | + | + | + |
| 3 | + | + | + | + | + | + | + |
| 4 | + | + | + | + | + | + | + |
| 5 | + | + | + | + | + | + | + |
| 6 | + | + | + | + | ++ | + | + |
| 7 | + | + | + | + | + | ++ | ++ |
| 8 | - | + | - | - | - | - | - |
| 9 | + | + | + | + | ++ | + | +++ |
| 10 | + | + | + | + | + | + | + |
| 11 | + | + | + | + | + | + | + |
| 12 | + | + | + | + | ++ | ++ | ++ |
| 13 | + | + | + | + | + | ++ | + |
| 14 | + | + | + | + | + | + | + |
| 15 | + | + | + | + | ++ | + | + |
| 16 | + | + | + | + | + | + | + |
| 17 | + | + | + | + | ++ | + | + |
| 18 | + | + | + | + | + | + | + |
| 19 | ++ | + | + | ++ | ++ | ++ | + |
| 20 | + | + | + | + | + | + | + |
| 21 | + | + | + | + | + | + | + |
| 22 | + | + | + | + | + | + | + |
| 23 | ++ | + | + | + | + | + | + |
| 24 | - | + | + | - | - | - | - |
| 25 | + | + | + | + | + | + | + |
| 26 | ++ | ++ | + | +++ | +++ | ++ | +++ |
| 27 | + | + | + | + | + | + | + |
| 28 | + | + | + | + | ++ | + | + |
| 29 | - | + | - | - | - | - | - |
| 30 | +++ | + | + | + | ++ | + | + |
| 31 | + | ++ | + | + | + | + | + |
| 32 | + | + | + | + | + | + | + |
| 33 | + | + | + | + | ++ | + | + |
| 34 | + | + | + | NS | + | ++ | + |

*FO- Fusarium oxysporum ; FM- Fusarium udum; FS-Fusarium culmorum; *+= 5-10mm inhibition; *++- 11-15 mm; *+++-16-20 mm*

(Mantel 1967). Matrices comparisons were carried out using the Mantel test feature of PopTools Microsoft Excel add-in, version 2.6.9 (Hood 2011). The estimated Z test criterion was compared to the randomised distribution of Z obtained from 1000 random permutations of the matrices (excluding the observed comparison) to determine the probability of obtaining a random Z greater than the estimated Z.

## Results and Discussion

Physico chemical analysis of soil sample revealed that the pH of soil samples were ranged from 5.2 to 7.2 percentage of organic matter ranged from 3.0 to 8.8%, percentage of total nitrogen was from 0.11% to 0.35% whereas the percentage composition of sand, silt and clay was 43-79%, 12-37% and 10-25% respectively. In total, 121 isolates showing pigmentation under UV light were consider as fluorescent pseudomonads. All the isolates were screened for functional properties of PGPR like IAA production, phosphate solubilization, siderophore production and antifungal activity. Among all the screened isolates, 32 isolates which were positive for more than two PGPR traits were selected for the study and designated as isolate No.1 to isolate No 32. Two reference isolates were also obtained from NCIM, Pune and included in the study with demarcation as isolate No. 33 and isolate No. 34. Studies of plant growth promoting traits for the selected 34 isolates revealed that all the isolates were positive for IAA, siderophore and HCN production. Among them, 29 isolates were able to solubilize tricalcium phosphate. The radius of clearing zone ranged between 6.0 mm to 18 mm with isolate no 26 showing maximum solubilization (Table 1).



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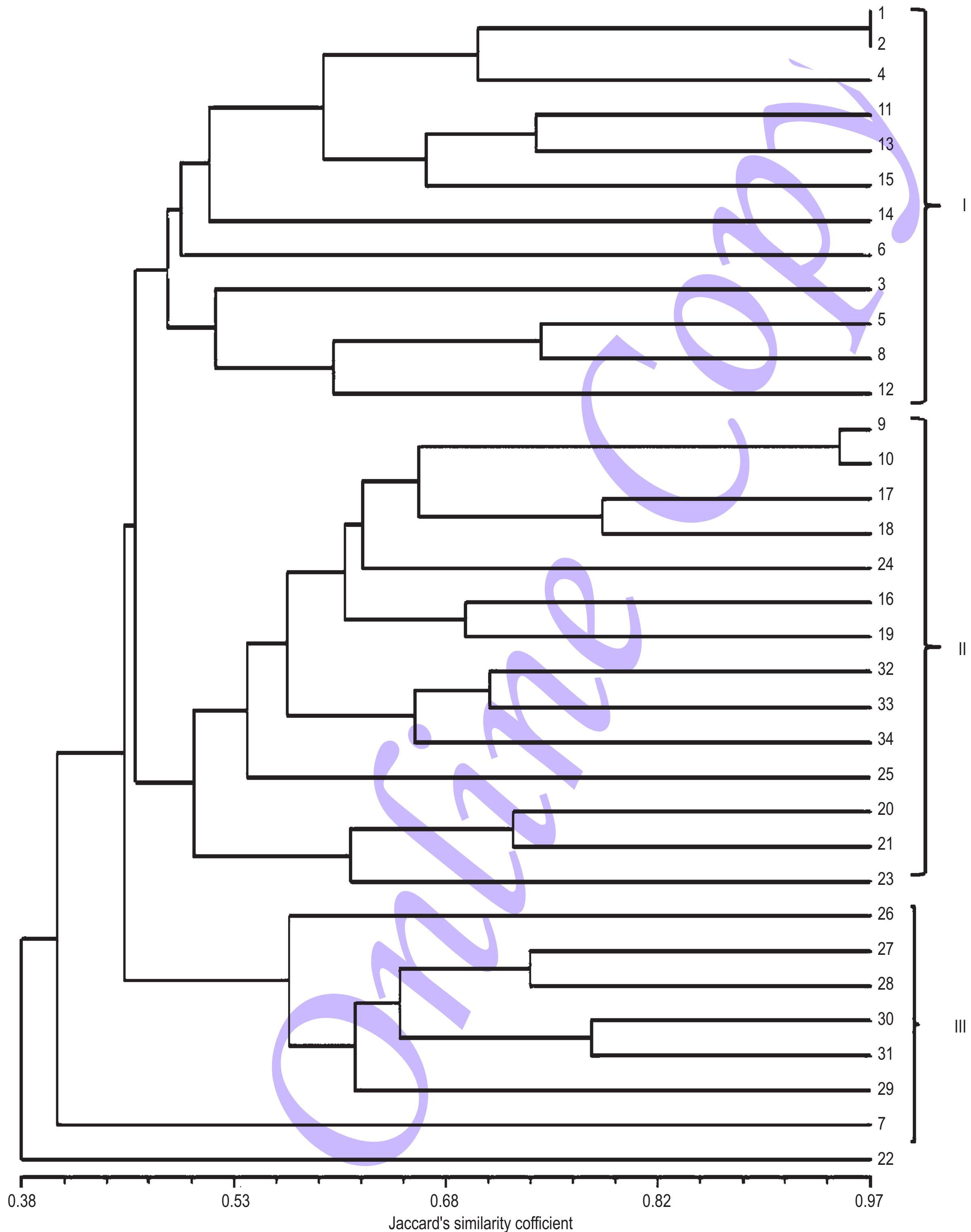


**Fig. 2 :** Dendrogram showing the genetic relationship between the 34 isolates of PGPR fluorescent *pseudomonas* strains derived from 16S rDNA-RFLP analysis using the restriction endonucleases *Hae*III, *Alu*I and *Msp*I.

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**Table 2** : Distribution of PGPR fluorescent *Pseudomonas* among RAPD based genotypes and 16S rDNA-RFLP based genomic groups

| Isolate | Place of collection | RAPD-based genotype | ARDRA-based genotype |
|---|---|---|---|
| 1 | Visakhapatnam | I | I |
| 2 | Visakhapatnam | I | I |
| 3 | Visakhapatnam | V | I |
| 4 | Visakhapatnam | I | I |
| 5 | Varanasi | II | I |
| 6 | Varanasi | III | I |
| 7 | Varanasi | III | III |
| 8 | Varanasi | II | I |
| 9 | Varanasi | IV | II |
| 10 | Varanasi | IV | II |
| 11 | Varanasi | I | I |
| 12 | Varanasi | IV | I |
| 13 | Varanasi | II | I |
| 14 | Varanasi | I | I |
| 15 | Varanasi | II | I |
| 16 | Allahabad | II | II |
| 17 | Aligarh | II | II |
| 18 | Aligarh | II | II |
| 19 | Aligarh | III | II |
| 20 | Aligarh | II | II |
| 21 | Aligarh | II | II |
| 22 | Tamil Nadu | II | IV |
| 23 | Tamil Nadu | IV | II |
| 24 | Tamil Nadu | II | II |
| 25 | Tamil Nadu | I | II |
| 26 | Maunath Bhanjan | III | III |
| 27 | Maunath Bhanjan | I | III |
| 28 | Maunath Bhanjan | I | III |
| 29 | Maunath Bhanjan | V | III |
| 30 | Maunath Bhanjan | I | III |
| 31 | Maunath Bhanjan | II | III |
| 32 | Maunath Bhanjan | III | II |
| 33 | NCIM, Pune | II | II |
| 34 | NCIM, Pune | IV | II |

Furthermore, all the isolates were screened for antagonistic activity against three fungal phytopathogens and all the isolates exhibited antifungal activities except three isolates *viz.*, 8, 24 and 29, which did not showed P-solubilization efficiency either. Siderophore activity among the isolate numbered 8, 24 and 29 found to be very low which indicated that antagonistic activity was siderophore mediated in all PGPR *pseudomonas*. This finding also shows the similar results, the isolates which could not siderophore production activity were also not able to solubilize inorganic phosphorous (Hu *et al.,* 2005; Nishimori *et al.,* 2000; Joshi and Bhat, 2011). All the isolates, including reference isolates, were able to produce IAA at varying concentration. Siderophore production was shown by 91% isolates on CAS medium, similar number of isolates showed P-solubilization efficiency along with antifungal activity. The results indicated that the isolates showed more than one plant growth promoting traits, which could promote plant growth directly or indirectly. Similar to this finding multiple PGP traits among PGPR is reported by many investigators (Dey *et al.,* 2004). The potential of microorganisms to produce siderophore is also reported as one of the important factor of iron availability to the crops (Sharma *et al.,* 2003). Recent focus is on the development of formulation of fungi and fluorescent pseudomonads to fight against deadly crop pathogens, which can also be implemented for the development of crop specific bio-fertilizers for the studied regions (Kumar *et al.,* 2012; Perez-Brandan *et al.,* 2014)

Morphological and biochemical characterization of the isolates associated with chickpea rhizosphere revealed that all were Gram-negative, rod-shaped, non-spore forming, motile organisms showing oxidase, catalase and arginine hydrolysis potential. All the isolates were able to produce fluorescent pigment and able to utilize citrate but were found negative for the indole test (Table 1). Genus fluorescent pseudomonad consists of several species and phenotypic characters can be used for confirmation of the genus pseudomonas but not till the species level.



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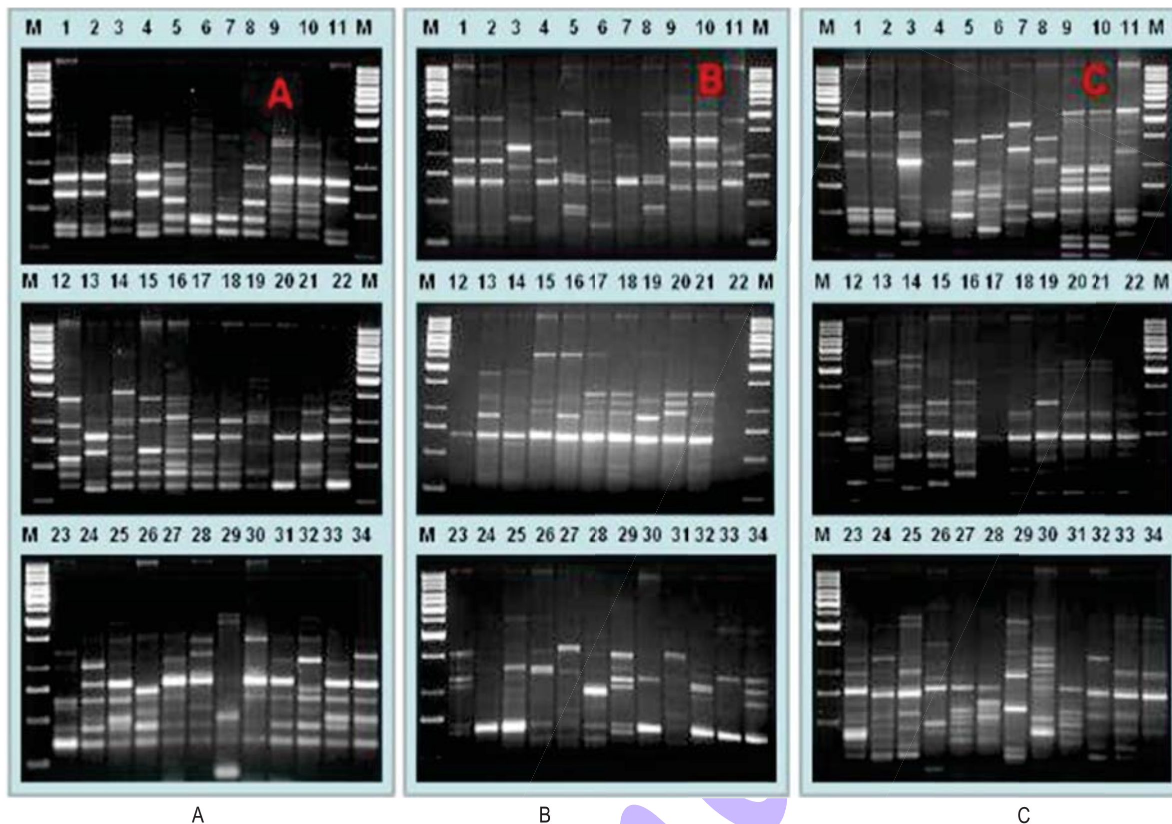


**Fig. 3 :** RAPD profiles of PGPR fluorescent *pseudomonas* using different primers (A) OPV-11; (B) PGS2; (C) PGS4. Lane M-1-kb ladder, Lane 1-34 shows the different isolates

ARDRA analysis of the isolates results in different fingerprinting patterns based on three restriction endonucleases used. By using restriction endonucleases AluI and MspI, 3-5 bands were seen among all the isolates, but all the 34 isolates were showed monomorphic pattern with HaeIII. The MspI pattern was also same in most of the isolates except two isolates which showed polymorphic bands (Igual *et al.,* 2001; Poonguzhali *et al.,* 2008). Therefore, a single restriction endonucleases could not differentiate among the isolates. The combined pattern obtained with three endonucleases defined 3 distinct genomic groups among 34 isolates at 50% similarity coefficient (Fig. 2). Cluster I and II were largest and contained 12 and 14 isolates respectively. Recently, molecular techniques have allowed a rapid and easy way to identify and detect PGPR fluorescent pseudomonas till species level. By seeing the RFLP pattern as compared to the reference isolates, it can be predicted that the chickpea rhizosphere was also dominant in *P. fluorescence*. 16S rRNA gene profiling very clearly differentiates the isolates based on location. All the isolates selected from Visakhapatnam, Aligarh and Maunath Bhanjan fell into clusters I, II and III respectively (Table 2). Interestingly, isolates obtained from Visakhapatnam and Varanasi were clustered together in cluster I. It may be concluded that ARDRA based profiling can be used to differentiate PGPR *pseudomonas* based on location.

ARDRA was not able to differentiate among the closely related isolates so, attempts were made to use whole genome fingerprinting by using random primers. In total, 40 random primers were selected based on previous reports, among which 18 primers was showed reproducible and interpretable results when PCR was repeated three times. RAPD pattern revealed the banding pattern range from 200 to 3,000 bp. Variations were clearly seen among the RAPD pattern in most of the isolates. Among all the 18 primers selected, three primers (OPV-11, PGS2 and PGS3) showed most predominant and reproducible banding pattern. Henceforth, all the samples were analyzed using these three primers to generate RAPD profile of PGPR *pseudomonas* (Fig. 3). Based on the combined profiling of the three primers all the 34 isolates were defined into 5 different genome clusters at 50% similarity level (Fig. 4).

Clusters I and II were the major groups and contained 9 and 13 isolates respectively. These clusters included isolates from different locations. For example, cluster I included isolates from Varanasi, Aligarh, Mau and Tamil Nadu. Interestingly, all the

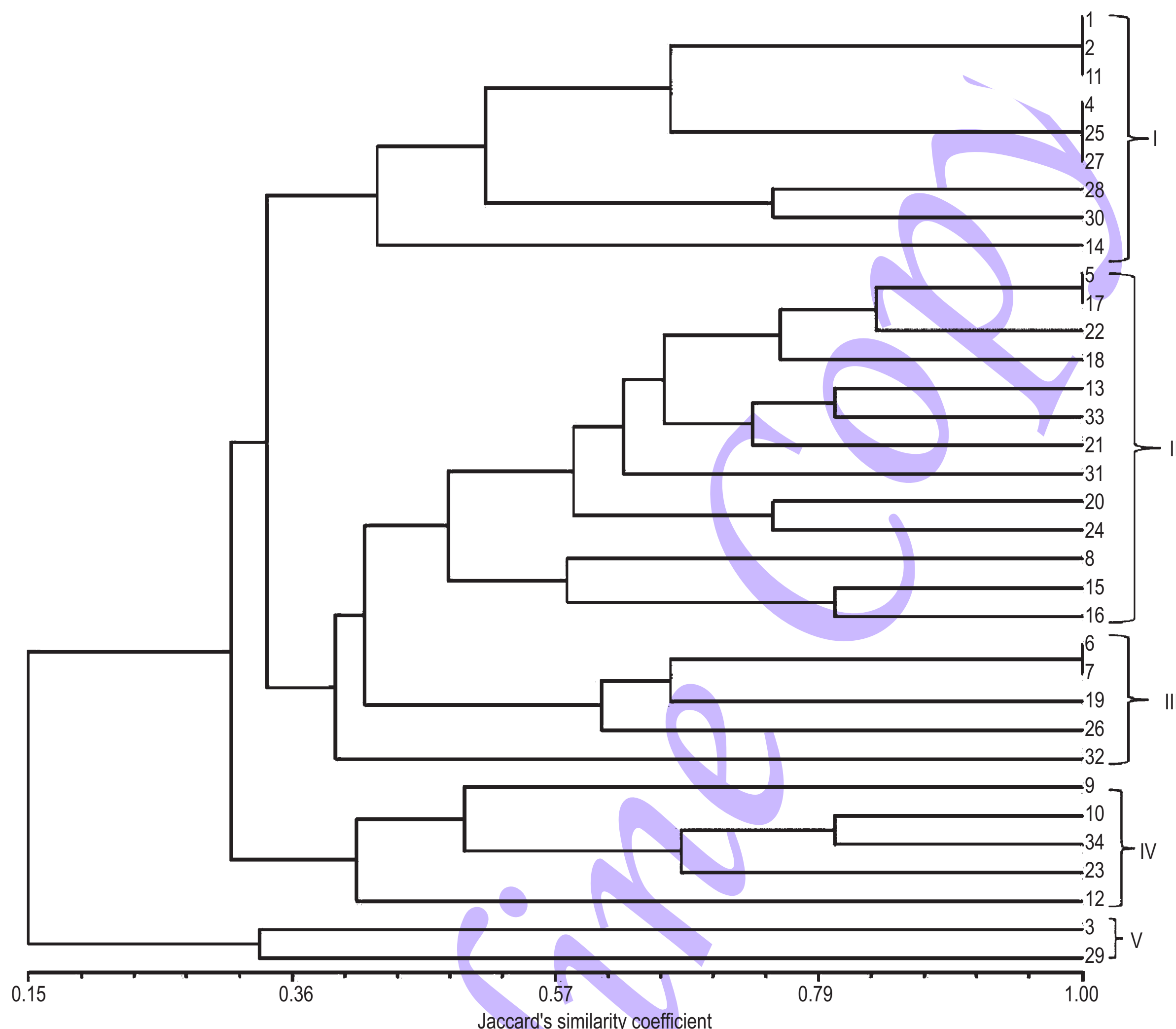


**Fig. 4 :** Dendrogram showing the genetic relationship between the 34 isolates of PGPR fluorescent *pseudomonas* strains derived from RAPD analysis

isolates of this cluster could solubilize phosphorus. Conversely isolates from single location were distributed in different clusters. For example, the 11 isolates obtained from Varanasi were distributed in clusters I, II and IV. Many isolates from the same place have showed 100% homology with each other like the isolates collected from Varanasi (10, 25 and 26). RAPD banding pattern can be used to differentiate the closely related organisms as compared to 16S rDNA-RFLP which was not able to, differentiate closely related organisms showing monomorphic banding pattern. Further it may be concluded that RAPD profiling can distribute isolates on the basis of biological trait *i.e.,* P-solubilization rather than soil collection place. RAPD analyses have given a better understanding of PGPR diversity from same as well as from different environments (Picard *et al.,* 2000). Workers used a collection of 18 strains of *Pseudomonads fluorescens,* involved in the suppression of *Rhizoctonia bataticola* and *F. oxysporum* and showed that bacteria isolated from entirely different geographical location can share some genetic relationship (Ellis *et al.,* 2002). Due to their multifunctional properties, PGPR fluorescent *pseudomonas* strains reported in the present study may be used as plant growth promoting bacteria and biocontrol agents in agriculture. The level of congruence between ITS-RFLP coefficient matrix and RAPD generated similarity matrix was analysed by calculating the Pearson product-moment correlation between the two matrices. Mantel test showed positive significant correlation (r= - 0.651) of the matrices.

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