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Genetic Diversity Analysis of the *Pseudomonas fluorescent* Isolates in Chhattisgarh Region of India

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Plant growth promoting *Pseudomonas fluorescent* siderophore producing rizobacteria were isolated from the rhizosphere and non-rhizospheric soil of cave, forest, fellow land and agriculture field in Chhattisgarh region. *Pseudomonas fluorescent* single colony was successfully isolated in King's B media under 260 nm wavelength and forty five yellow green isolates were selected for diversity analysis. The genetic diversity analysis was done by 20 RAPD markers. Out of these 5 primers showed clearly scorablebanding pattern. The selected isolates were distributed into two major clusters. RF9 isolates showed most diverged than others. The similarity coefficient of major clusters ranged from 0.02 to 0.91. The objective of this study was to determine a possible correlation between molecular diversity within *Pseudomonas fluorescens* isolates of Chhattisgarh.

Key words: *Pseudomonas fluorescent,* Genetic fingerprinting, Diversity assessment, RAPD.

Pseudomonas fluorescent are common soil bacteria that can improve plant and soil health through nutrient cycling, pathogen antagonism and induction of plant defenses. *Pseudomonas fluorescent* often predominate bacteria of plant rhizosphere and have beneficial effects on plants, either by direct stimulation of plant growth or by exerting antagonism towards soil borne pathogens[1]. It is commonly found on plant surface, as well as in decaying vegetation, soil and water[2]. It can be isolated from soil, water, plants, animals, the hospital environment and human clinical specimens. It is commonly associated with spoilage of foodstuffs such as fish and meat[3]. It is most physiologically diverse species of bacteria found throughout terrestrial habitats. Pure cultures of *Pseudomonas fluorescent* species isolates form King's B medium identified using ultraviolet light illumination by its fluorescence.

Many molecular methods are used to detect the presence of soil-borne pathogens and also to assess the genetic variability among the different isolates[4]. The techniques based on genomic DNA have extensively been used for analysis of genetic diversity and the basis of modern microbial characterization and identification. Random Amplified Polymorphic DNAs (RAPDs) is confidentially used because it is a fast, cheap and easy. One prominent advantage of the RAPD is that it can easily be applied and employed on antagonistic gram - and gram + bacteria[5,6].

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The aim of this study was to characterize the genetic diversity among collected isolates of *Pseudomonas fluorescent isolated* from rhizosphere and non-rhizosphere soil of Chhattisgarh and to determine a possible correlation between and within *Pseudomonas fluorescent*.

MATERIALS AND METHODS

Collection of rhizosphere and non-rhizosphere soil samples and Serial Dilution

Soil samples were collected from various geographical locations of Chhattisgarh from the randomly selected sites (Table 1). Rhizosphere soil samples collected were subjected to serial dilutions to 1000 (10^{-3}) times.

Isolation of *Pseudomonas fluorescent* and designation of isolates

Individual florescent colony was picked up with the help of sterilized loop and inoculated on solidified Kings medium 'B' (KMB) (7) by zig-zag streaking. The plates were incubated at 28°C for 24 hr. The colony growing at the last tip of the zig-zag line was transferred to KMB slants.

RAPD analysis and data interpretation

Pseudomonas grown in King's medium "B (broth) at 28°C with an O.D. of 1.2 and 1.6 at A_{600} wavelength. Young actively growing bacterial suspension (1.5 ml) was dispended in micro centrifuge tube and was then centrifuge at 12000 rpm for 3 minutes and pellet out. The pellet was washed with 0.375µl of 1% NaCl solution to remove EPS (exopolysaccharide) by centrifugation at 12000 rpm for 1 min. The pellet was then re-suspended in 0.375µl of TES (10µM TrisHCl, 10µM EDTA, pH 8.0, 2% SDS) by pipetting and were incubated at 75°C for 5 min to improve cell lysis and genomic DNA yield (1.32-2.8 folds). This was followed by phenol: chloroform (3:1, V/V) treatment to the cell lysate and subsequently centrifuged at 12000 rpm for 3 min. This results in the separation of the layer. Upper chloroform layer (containing DNA) was removed gently by using a pipette to the new eppendorf tube. DNA precipitation was initiated by adding 0.1 volume of 3M sodium acetate (pH-5.2) and 1 volume of isopropanol (RT) and gently inverting the tubes for 20 min. The DNA, which is immediately visible, was either plated by centrifugation at room temperature or pick up with sterile capillary glass hook. Supernatant was discarded and the DNA pellet washed with 70% ethanol dried and resuspeneded in 75µl of TE (10µM Tris HCl, 2µM EDTA, pH-8.0).

Sets of 20 RAPD primers were screened, for PCR based DNA fingerprinting analysis in order to identify polymorphism in 45 *Pseudomonas fluorescent* isolates. Random Amplified Polymorphic DNA primers were obtained from Operon Technology Inc. (Almeda, California). DNA samples (3 ml) containing approximately 60 – 100ng of genomic DNA template were used in a 20 ml reaction mixture for PCR. Amplification was carried out for 35 cycles on PTC 100 (Programmable Thermocycler) of M J Research Pvt. Ltd., USA. The PCR products were separated electrophoretically on 1.5 percent horizontal Agarose in 1X TBE buffer. Gels were visualized under UV-transilliminator and digitally documented using Bio-RAD gel documentation system.

Specific amplification products were scored as present (1) or absent (0) depending on decreasing order of their molecular weights of each DNA sample. The similarity matrix was calculated by UPGMA method and dendrogram was generated using NTSYS.

RESULTS AND DISCUSSION

DNA fingerprinting of *Pseudomonas fluorescent* isolates

Twenty, ten-mer RAPD primers were used to screen the polymorphism among the collected isolates of *Pseudomonas fluorescen* from different geographical locations of Chhattisgarh, out of which five primersOPA- 01, OPA-11, OPA-13, OPA-3, OPA-5 gave the polymorphic and clearly scorablebanding pattern (Fig. 1) and these were selected for further analysis. The number of amplified band per primer varied from 3 to 5 by analysis of 45 *Pseudomonas fluorescent* isolates using 5 RAPD primers generated a total of 23 bands which were scored as present (1) or absent (0) for determining the genetic relationship among isolates. Similarity matrices were calculated using NTSYS (Numerical Taxonomy System Biostatistics) computer programme. Cluster analysis was done within the SAHN program by using UPGMA (unweighted pair-group method with arithmetic averages) method. Similarity

Table 1. Information of Collection site and isolates used for the study

S.No	Collection site	No of samples	Isolates
1.	RF- Research Farm Raipur and Bilaspur	12	RF5, RF6, Rf7, Rf 8, RF3, RF2, RF1, RF10, RF13, RF15, RF4, RF9
2.	M- Mango Plantation Kumrawand Farm	6	M5, M1, M2, M6, M3, M4
3.	Field area: -KCO Kumrawand Farm	3	KCO3, KCO2, KCO1
4.	Jagdalpur Area	2	KM6, KM2
5.	Phankhjore, Kanker	2	P3, P1
6.	K- Kutumsar Cave	8	K1, K5, K7, K8, K6, K4, K2, K3
7.	TG- TirathgarhGhat	7	TG4, TG3, TG1, TG2, TG6, TG7, TG5
8.	NK- Negaraai; kamaraai	5	NK6, NK2, NK4, NK3, NK5

coefficient ranged from 0.02 to 0.91 and average number of polymorphic bands per primer is 4.6. The dendogram indicates that there was a major cluster 'A' consisting of 44 isolates. The other major cluster 'B' consisted of 1 isolate shared 0.02 similarities coefficient with cluster 'A' (Fig.-2).

Major cluster 'A' showed sub clustering near the 0.13 similarity level. The two sub-clusters 'A_1'and 'A_2' consisted of 42 and 2 isolates respectively sharing about 0.13 similarities. The cluster A_1 further showed sub-clustering near the 0.155 similarity levels as sub cluster $A_{1(a)}$ and $A_{1(b)}$ and consisted of 36 and 6 isolates respectively. The cluster $A_{1\,(a)}$ showed sub-clustering near 0.284 similarity level as $A_{1\,(a1)}$ and $A_{1\,(a2)}$, consisted of 23 and 13 isolates respectively. The cluster $A_{1\,(a1)}$ consisted of 23 isolates, which further sub-clustered at 0.328 similarity level into 2, $A_{1\,(a1\text{-}1)}$ and $A_{1\,(a1\text{-}2),}$ consisted of 22 and 1 isolates. The cluster $A_{1\,(b)}$ consisted of 6 isolate. The cluster $A_{1\,(b)}$ showed sub-clustering near 0.199 similarity level as $A_{1\,(b1)}$ and $A_{1\,(b2),}$ consisted of 3 and 3 isolates respectively.

Cluster 'B' was the major cluster and consisted of only one isolate *i. e*. RF 9 and was genetically dissimilar with all the 44 isolates. Clustering of the isolates according to the generated dendrogram, indicated no relationship with the collection sites. All the isolates were variable and only formed similarity groups in subsequent sub-sub clustering at approximately 0.506 to 0.866 similarity coefficient. The candidate isolate P3 collected from Pakhanjore was present in the sub-sub-sub-sub- clusters of $A1_{\,(a1\text{-}1\text{-}1\text{-}1\text{-}2)}$ and was showed similarity with diverse collections of isolates (M3, M4, M2, M1, KCO-1, K8, RF8, P1,

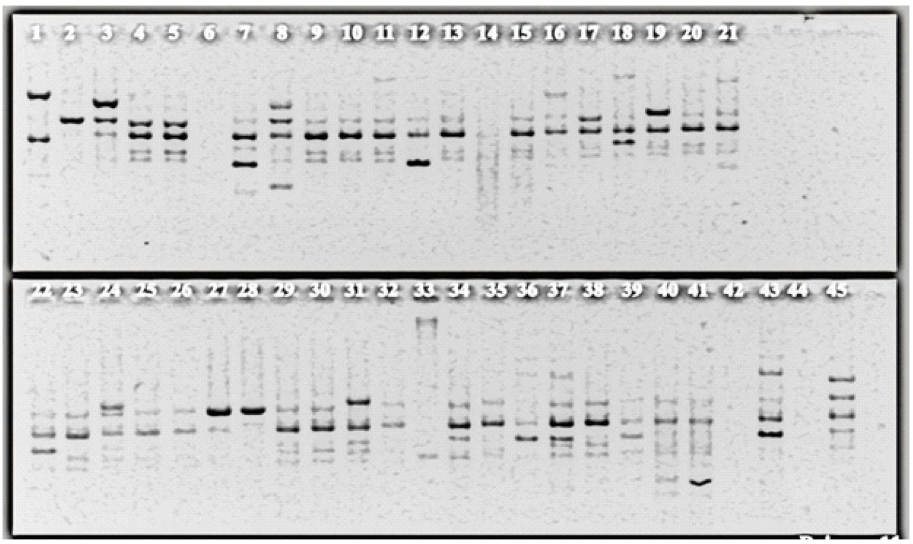

Fig. 1. Amplification pattern of primer OPA-11 on the 45 isolates of *P. fluorescent* Bacteria

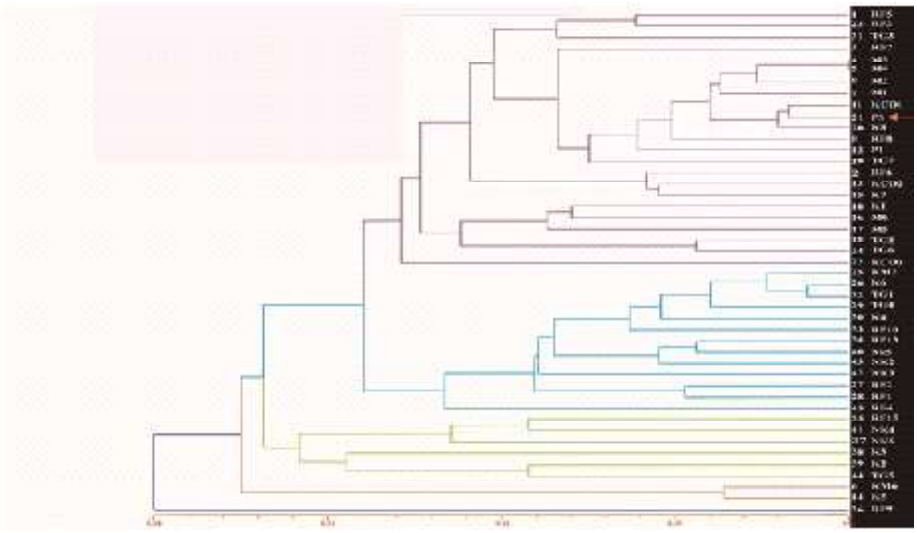

Fig. 2. Dendrogram showing the genetic relationships among 45 *P. fluorescent* isolates based onRAPD analysis

TG7) from different geographical locations of Chhattisgarh.

This study of genetic diversity characterization of *P. fluorescent* isolated from different geographical location of Chhattisgarh showed that *Pseudomonas* sp. were widely diverged in Chhattisgarh soil. Kumar *et al.,*[4] was characterized genetic diversity *among P. fluorescent* strains by RAPD analysis. In summary, the results of the diversity analysis have shown significant level of variation among the isolates. This will helpful for the selecting good candidate isolates for plant growth promoting activity.

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