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Similarity Analysis of Fluorescent Pseudomonas from Apple and Pear Rhizosphere

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Molecular markers have been found useful in assessing the genetic diversity in a precise manner, which is based on naturally occurring DNA polymorphism. Random Amplified Polymorphic DNA analysis was performed using four arbitary decamer primers to observe genetic homogeneity/ polymorphism among 10 isolates of fluorescent *Pseudomonas* spp. isolated from apple and pear rhizosphere from different locations of Mandi and Chamba districts. Out of 52 bands generated with 4 RAPD primers, 13.46% were monomorphic bands, while 86.33% were polymorphic bands. The result showed that the isolates of fluorescent *Pseudomonas* sp. were divided into 2 clusters by RAPD primers. Among all the combinations maximum similarity (90.48%) was found between PN-7-Cha and PN-2-San isolates of Chamba and Mandi districts. However, minimum similarity (36.7%) was observed in PN-11-San and PN-7-Cha isolates of Mandi and Chamba districts. This technique is highly beneficial for testing similarity analysis among fluorescent *Pseudomonas* spp. isolated from different locations.

Key words: PGPR, Fluorescent *Pseudomonas*, RAPD

Plant growth-promoting rhizobacteria (PGPR) improve the plant growth directly or indirectly. *Pseudomonas* is Gram-negative, strictly aerobic, gamma proteobacteria. They are aggressive colonizers of the rhizosphere of various crop plants, and have a broad spectrum antagonistic activity against various phytopathogenic fungi, such as antibiosis (the production of inhibitory compounds), siderophores production (iron-sequestering compounds) and nutrition or site competition (Bull *et al* ., 1991). Some species of *Pseudomonas* can also produce HCN that are toxic to pathogenic fungi (David and O'Gara, 1994). These characteristics make *Pseudomonas* species good candidates for use as seed inoculants and root dips for biological control of soil-borne plant pathogens.

Rapidly evolving technologies in molecular biology and genetics have provided new insights into the underlying mechanisms by which biocontrol agents' function in their natural environments to a degree not previously possible. The application of the polymerase chain reaction (PCR), in particular, have greatly facilitated genomic analyses of microorganisms, provide enhanced capability to characterize and classify strains, and facilitate research to assess the genetic diversity of populations (Louws *et al*., 1999).

The aim of the present study was to standardize simple, reproducible RAPD-PCR protocol for PGPR especially fluorescent Pseudomonas species growing in replant and normal sites of apple and pear orchards of Mid-Himalayan ranges of Himachal Pradesh to check the genetic diversity of native fluorescent *Pseudomonas* species.

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Material and Methods

Collection of soil samples

Rhizospheric soil samples along with roots were collected from different plants of apple and pear from the five normal sites of Chamba and Mandi districts of Himachal Pradesh and also from the five sites of replant problem areas of these two districts.

Isolation, enumeration and identification

The media employed for the isolation of *Pseudomonas* spp. were nutrient agar (NA) and selective King's B medium supplemented with 3 antibiotics *i.e.*, Penicillin-G (75,000 units/l), Cycloheximide (75 mg/l) and Novobiocin (45 mg/l). Plates were incubated at 28 ± 2ºC for 24 – 48 h. Ten isolates were classified on the basis of colony characteristics such as size, colour, shape, texture and type of fluorescent pigment production. The most predominant *Pseudomonas* spp. isolates showing greenish/yellowish fluorescent pigments at 302 nm wavelength in BIO-RAD gel doc system XR were assumed to be fluorescent colonies.

Then they were characterized on the basis of morphological and biochemical tests as per their genera as prescribed in Bergey's Manual of Systematic Bacteriology. The pure culture of ten selected strains were maintained on the nutrient agar slants at 4ºC and were subcultured periodically on the same media at 28 ± 2ºC. They all were also

maintained and preserved in 20% glycerol at -20ºC. All the further experiments were conducted after raising fresh cultures.

In vitro screening of bacterial isolates for plant growth promoting traits

All isolates were screened for the ability for phosphate solubilisation and siderophore HCN and ammonia production.

Available phosphorous (Olsen *et al.,* 1954) was quantified. Bacterial cultures grown in PVK broth supplemented with 5.0 g/l tri -calcium phosphate (TCP) and solubilization of phosphorous was calculated using standard curve of KH_2PO_4 (100-1000 µg/ml). Siderophore production in liquid culture of Pseudomonads in Chrome-azurol-S (CAS) broth was carried out and change in the colour of reaction mixture was observed from dark blue to orange or pink (Schwyn and Neilands, 1987).For estimation of hydrocyanic acid (HCN) production by *Pseudomonas* spp., a color change in the sodium picrate containing filter paper strip was observed from yellow to orange brown to dark brown (Bakker and Schippers, 1987). For ammonia production, the assay method of Lata & Saxena (2003) was followed. Pseudomonas isolates were grown in 5ml of peptone water in tubes at 28±2ºC for 4 days. After 4 days, 1ml of Nessler's reagent was added to each culture. Presence of very light brown color (+) indicates small amount of ammonia production and light brown (++) to orange brown color (++++) indicates large amount of ammonia production.

RAPD Analysis

RAPD analysis was performed on ten different isolates of fluorescent Pseudomonas. The DNA was extracted according to manufacturer's (Banglore GeNei's) specifications using DNA isolation kit. Eluted DNA was quantified spectrophotometrically at 260 nm and stored at -20$_0$C for future use.

RAPD analysis were carried out in 25 ìl reaction, contained 25 ng of genomic DNA **,** 18.8 µl sterilised distilled water, 0.20 µl Taq DNA polymerase, 2.50 µl Taq buffer A, 1.50 µl dNTPs mixture, 1.0 µl random primer, and 1.0 µl genomic DNA. The PCR was carried out in a thermal cycler with a total of 45 cycles. Each cycle consisted of 1 minute denaturation at 94ºC, 1 minute annealing at 36ºC, and 2 minute extension at 72ºC. All the PCR samples were given 3 minutes pre-amplification at 94 ºC and 10 minutes post-amplification at 72 ºC, PCR products were analysed in 1.2% agarose gels stained with ethidium bromide and were visualized and photographed using a BIO-RAD (USA) Gel Documentation System. The primers used were

Primers used	Sequence 5' →3'
OPA-01	CAGGCCCTTC
OPA-02	TGCCGAGCTG
OPA-03	AGTCAGCCAC
OPA-04	AATCGGGCTG

All isolates were used for cluster analysis based on RAPD results. Each amplification band was scored as 1 (present) or 0 (absent) for all isolates. The isolates were clustered by the unweighted average pair group method (UPGMA). Differentiation between the isolates was done by observing readily discernible band patterns.

Results and Discussion

Pseudomonas spp, the most diverse, versatile and indigenous group of microorganisms may have wide applications in agriculture. The development of different strategies using a mixture of PGPR strain especially fluorescent *Pseudomonas* spp. would be an important emerging area in crop growth promotion, protection and also in establishment of newly planted trees on replant sites of apple and pear. It may reduce the losses caused by replant problem in economically important fruit crops especially apple. Hence the genetic diversity of indigenous fluorescent *Pseudomonas* species of those true species is very important. They all produced fluorescent pigment.

Biochemical studies showed that all the isolates were positive for gelatin liquification and lecithinase activity while four isolates i.e. AN-1-Cha, AN-3-Cha, PN-7-cha and PN-8-Cha were found negative for tween hydrolysis. Optimum growth was recorded at 25$_0$C. PN-7-Cha and PN-12-San showed growth at 4$_0$C while all strains showed growth at 42$_0$C except one strain i.e. PR-2-San.

Table 1. Plant growth promoting traits of Pseudomonas isolates from apple and pear rhizosphere

Isolates	Phosphate solubilization (µg/ml)	Siderophore (% siderophore unit)	HCN	Ammonia production
AN-1-Cha	290	28.39	+	+++
AN-3-Cha	305	29.82	+	+++
AR-5- Cha	205	32.14	+	++++
PN-7-Cha	345	40.71	+	+++
PN-8-Cha	225	35.35	+++	+++
PN-11-San	285	47	++++	++++
PN-12-San	300	45	+++	++++
PN-13-San	285	45	+++	+++
PN-2-San	355	39	+	+++
PR-2-San	195	52	+++	+++

Phosphorus is one of the major essential macronutrients for biological growth and development (Fernandez *et al.,* 2007). The concentration of soluble phosphate in soil is usually very low. This pool of immediately available phosphate is extremely small and must be replenished regularly to meet plant requirements. Fluorescent *Pseudomonas* spp. are prevalent in the rhizosphere of plants (Lamanceau *et al.,* 1995). Certain members of this group are called plant growth-promoting rhizobacteria (Schroth and Hancock, 1982) because they are able to promote

plant growth through solubilization of inorganic phosphates present in soil and through protection of plants against diseases caused by phytopathogenic fungi.

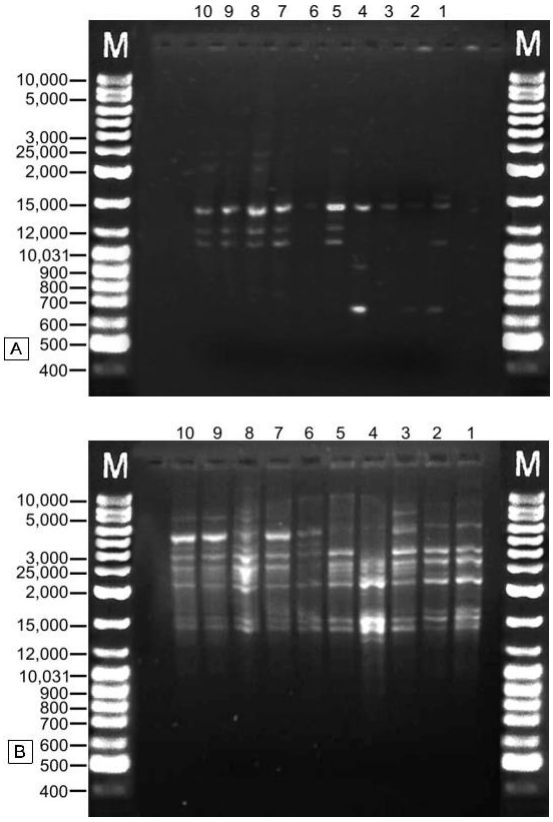

Fig. 1. RAPD banding pattern of OPA-01 (A) and OPA-02(B) primers in apple and pear rhizosphere. The RAPD primers, M-Marker; (1) PN-13-San; (2) PR-2-San; (3) AN-1-Cha; (4) PN-11-San; (5) AN-3-Cha; (6) PN-12-San; (7) AR-5-Cha; (8) PN-8-Cha; (9) PN-7-Cha; (10) PN-2-San; M-marker

The maximum solubilization of inorganic phosphorus and maximum release of ortho phosphate (Pi) in supernatant was shown by PR-2-San (355µg/ml) while minimum was showed by PN-2-San (195 µg/ml) shown in Table 1.

There are number of reports suggesting that plant species are capable of obtaining iron from some microbial siderophores (Park *et al.,* 2005). Cline *et al.* (1984) demonstrated that the iron from the microbial hydroxamates siderophores may become available to plants both in nutrient solution and in soil. Siderophores from fluorescent *Pseudomonas* species have also been implicated in iron uptake by tomato plant (Dass *et al.,* 1986).

Maximum siderophore production, which was estimated by CAS assay, was 52 %SU by strain PN-2-San followed by PN-11-San (147 %SU) (Table 1).

Microbial production of HCN has been reported as an important antifungal trait to control root infecting fungi (Ramette *et al.,* 2003). All bacterial isolates showed positive result for HCN production and ammonia production (Table 1).

RAPD-PCR is a genotypic identification and characterization system that has shown great specificity and sensitivity to define bacterial isolates. Marques *et al.* (2008) studied the phenotypic characteristics and genetic fingerprints of a collection of 120 bacterial strains, belonging to *Pseudomonas syringae* sensu lato group, *P. viridiflava* and reference bacteria were evaluated,

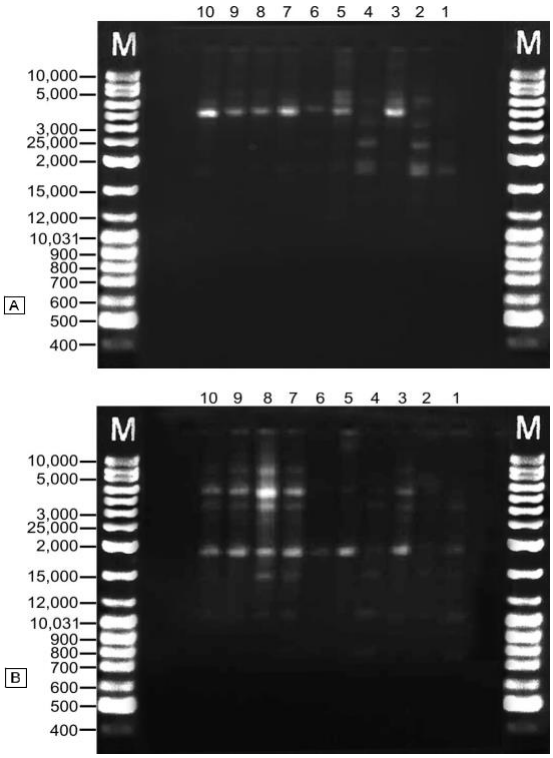

Fig. 2. RAPD banding pattern of OPA-03 (A) and OPA-04(B) primers in apple and pear rhizosphere. The RAPD primers, M-Marker; (1) PN-13-San; (2) PR-2-San; (3) AN-1-Cha; (4) PN-11-San; (5) AN-3-Cha; (6) PN-12-San; (7) AR-5-Cha; (8) PN-8-Cha; (9) PN-7-Cha; (10) PN-2-San; M-marker

with the aim of species identification. Babalola *et al.* (2002) analysed three rhizosphere bacteria, *Pseudomonas* sp., *Enterobacter sakazakii* and *Klebsiella oxytoca*, for genetic variation. Cluster analysis indicated that *E. sakazakii* and *K. oxytoca* are the most closely related of the three.

Total 52 scoreable bands were amplified with 4 primers of these 45 bands were found to be polymorphic (86.53%) and 7 monomorphic bands (13.46%) were found shown in Fig 1 & 2. The similarity coefficient value ranged from 0.36-0.90. Among all the combinations, maximum similarity was found between PN-7-Cha and PN-2-San isolates of Chamba and Mandi district (90.48%) followed by 84.4% between PN-13-San and PR-2-

San isolates of Mandi district. However the minimum similarity of 36.7% was observed in PN-11-San and PN-7-Cha isolates of Mandi and Chamba district.

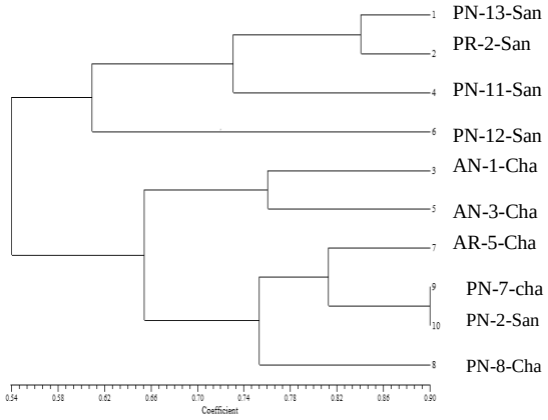

Fig. 3. UPGMA Dendrogram showing similarity coefficients between *Pseudomonas* isolates from apple and pear rhizosphere

The dendogram constructed (Fig. 3) using UPGMA method differentiated ten strains of Pseudomonas into 2 groups. Group 1 includes four isolates (PN-13-San, PR-2-San, PN-11-San, PN-12-San and Group 2 includes six isolates i.e. AN-1-Cha, AN-3-Cha, AR-5-Cha, PN-7-Cha, PN-2-San, PN-8-Cha.In group 1 PN-13-San and PR-2-San showed 84 per cent similarity and in group 2 PN-7-Cha and PN-2-San showed 90 percent similarity.

Conclusion

The present RAPD profiles of phylogenetic analysis suggest that different isolates of Pseudomonas have significant inter and intra species differences and can be of great significance for their exploitation as plant growth promoting bioagents.

References:

Babalola, O.O., Osir, E.O. and Sanni, A.I. 2002. Charaacterization of potential ethylene producing rhizosphere bacteria of Striga-infested maize and sorghum. *African J. Biotech.* **1**(2): 67-69

Bakker and Scippers. 1987. Microbial cyanide production in the rhizosphere in relation to total yield reduction and *Pseudomonas* species mediated plant growth stimulation. *Soil Biol. and Biochem*. **19**: 451-457.

Bray, R.H. and Kurtz, L.T. 1945. Determination of total organic available forms of phosphorus in soil. *Soil Sci.*, **23**: 343-353

Bull, C.T., Weller, D.M. and Thomashow, L.S. 1991. Relationship between root colonization and suppression of *Gaeumannomyces graminis* var. *tritici* by *Pseudomonas fluorescens* strain 2-79. *Phytopathol*. **81**: 950-959

Chaiharn, M., Chunhaleuchanon, S., Kozo, A. and Lumyong, S. 2008. Screening of rhizobacteria for their plant growth promoting activities. *KMITL Science and Technology J.* **8**(1): 18-23

Cline, G.R., Reid, C.P. and Szaniszlo, P.J. 1984. Effect of a hydroxamate siderophore on iron absorption by sunflower and sorghum. *Plant Physiol.* **76**: 36-39

Dass, F., Moazfar, A., Oertle, J.J. and Jaeggi, W. 1986. Effect of bacteria on the iron uptake by axenically-cultured roots of Fe-efficient and Fe-inefficient tomatoes. *J. Plant Nutrition.* **9**: 587-598

David, N.D. and O'Gara, F. 1994.Metabolites of *Pseudomonas* involved in the biocontrol of plant diseases, *Tibtech*. **12**(4): 133-141

Dickman, S.R. and Bray, R H. 1940. Colorimetric determination of phosphate. *Indus and Engineering Chemical Analytical Edition* **12**: 660-665

Fernandez, L. A., Zalba, P., Gamez, M. A. and Sagardoy, M. A. 2007. Phosphate solubilizing activity of bacterial strains in soil and their effect on soybean growth under green house conditions. *Biology and Fertility Soils.* **43**: 805-809

Lata and Saxena A K. 2003. Characterization of plant growth promoting rhizobactria. In:*Training Manual on Biofertilizer Technology* A. K. Saxena (Ed.). IARI Delhi, p. 24-25.

Lemanceau, P., Corberand, T., Garcan, L., Latour, Y., Laguerre, G., Boeufgras, J.M. and Alabouvette, C. 1995. Effects of two plant species, flax (*Linum usitatissinum* L.) and tomato (*Lycopersicon esculentum* Mill.) on the diversity of soilborne populations of fluorescent Pseudomonads. *Appl. & Environ. Microbiol.* **61**: 1004-1012

Louws, F.J., Rademaker, J. L.W. and de Bruijn, F. J. 1999. The three Ds of PCR-based genomic analysis of phytobacteria: diversity, detection and disease diagnosis. *Annl. Rev. Phytopath*. **37**: 81-125

Marques, A.S.A., Marchaison, A., Gardon, L. and Samaon, R. 2008. Box-PCR based identification of bacterial sp. belonging to *Pseudomonas syringae - P. viridiflavia* gp*. Gent. and Mol. Biol.* **31**(1): 106-115

Olsen S. R., Cole, C.V., Whatanable, F. S. and Dean, L. A. 1954. Estimation of available phosphorus by extraction with sodium carbonate. *US Department of Agriculture.* 939 p

Palleroni, N.J. 1984. Genus *Pseudomonas*. In *Bergeys Manual of Systemic Bacteriology*, N.J. Krey and J. G. C. Holt (Eds.). **237**(1): 141-199

Park, M., Kim, C., Yang, J., Lee, H.S., Shin, W., Kim, S. H. and Satong, M. 2005. Isolation and characterization of diazotrophic growth promoting bacteria from rhizosphere of agricultural crops of Korea. *Microbial Res.,* **160**: 127-133

Patten, C.L. and Glick, B.R. 1996. Bacterial biosynthesis of indole-3-acetic acid. *Canadian J. Microbiol*.**42**: 207-220.

Ramette, A, Loccoz, Y. M. and Defago, G. 2003. Prevalence of fluorescent *Pseudomonas* producing antifungal phloroglucinols and hydrogen cyanide in soils naturally suppressive or conductive to tobacco black root rot. *FEMS Microbiol. Ecol*. **44**: 35-43

Schroth, M. N. and Hancock, J. E.1982. Disease suppressive soil and root-colonizing bacteria. *Science.* **216**: 1376-1381

Schwyn, B. and Neilands, J. B. 1987. Universal chemical assay for the detection and determination of siderphores. *Analytical Biochemistry*. **28**(8): 751-759

Winkelmann, G. and Drechsel, H. 1997. Microbial siderophores.In *Biotechnology*. H. J. Rehm and G. Reed (Eds.), VCH, Weinheim. p. 199-246.

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