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Phenotypic and genotypic characterization of inhabitant PGPR strains of *Pseudomonas* from apple orchards

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Abstract: Aim of present research was to isolate and characterize the *Pseudomonas* strains phenotypically and genotypically from the rhizospheric soil of apple orchard at Maggota (Shimla district) Himachal Pradesh. Phenotypic identification of the test isolates was based on morphological, physiological and biochemical characterization of the bacteria followed by genotypic analysis using rRNA gene sequencing and RAPD-PCR analysis. The fourteen *Pseudomonas* sp. isolates were screened out for various plant growth promoting activities such as siderophore production, antifungal activity, phosphate solubilisation, HCN and ammonia production, production of plant growth regulators and lytic enzymes. Isolates showed production of plant growth regulators (auxins, gibberellins and cytokinins) in the range of 19.67-83.33µg/ml, 21.00-58.67 µg/ml and 12.33-43.33 µg/ml respectively. *Pseudomonas* strains showed phosphate solubilising activity in the range of 12.33-63.33 Pi µg/ml, 53.66-93.44 % SU siderophore production and 11.33-96.33mm (diameter) protease activity in plate assay. Five *Pseudomonas* isolates i.e. An-16-kul, An-1 -mag, An-2-mag, An-3-mag and An-6-mag showed maximum antifungal activity against plant pathogenic fungi. Therefore, the aim of present investigation was to study multifarious plant growth promoting qualities of *Pseudomonas* sp. and to select more efficient PGPR strain of fluorescent *Pseudomonas* sp. which can be further used as biofertilizer.

Keywords: Genotype, PGPR, Phenotype, Phosphate solubilization, *Pseudomonas sp.,* Siderophore

INTRODUCTION

Root colonizing bacteria, or rhizobacteria, that exert beneficial effects on plant development are defined as plant growth promoting rhizobacteria (PGPR). The enhancement of plant growth by many PGPR has been utilized for several decades, and the mechanism has been extensively studied since the 1990s (Vessey, 2003; Lucy *et al.,* 2004). Plant growth promoting rhizobacteria are soil bacteria that when applied to seeds or roots are able to colonize plant roots and stimulate plant growth**.** PGPR can exhibit a variety of characteristics responsible for influencing plant growth (Ahmad *et al.,* 2005). PGPR are considered to promote plant growth directly or indirectly. Indirect effects are related to production of metabolites, such as antibiotics, siderophores, or HCN, that decrease the growth of phytopathogens and other deleterious microorganisms. Direct effects are dependent on production of plant growth regulators or improvements in plant nutrients uptake (Bai *et al.,* 2003). In the last decades research on PGPR has been increasing at an ever increasing rate since the term was first used by Kloepper and coworkers in the late 1970s (Vessey, 2003). This scientific interest is related to the PGPR potentiality in agriculture which is steadily increased as it offers an attractive way to replace the use of chemical fertilizers, pesticides and other supplements. Pseudomonads possess many traits that make them well suited as biocontrol and growth-promoting agents. These include the ability to (i) grow rapidly *in vitro* and to be mass produced; (ii) rapidly utilize seed and root exudates; (iii) colonize and multiply in the rhizosphere and spermosphere environments and in the interior of the plant; (iv) produce a wide spectrum of bioactive metabolites (i.e. antibiotics, siderophores, volatiles, and growth promoting substances); (v) compete aggressively with other microorganisms; and (vi) adapt to environmental stresses (Glick, 1995). In addition, pseudomonads are responsible for the natural suppressiveness of some soils to soil borne pathogens (Weller *et al.,* 2002). 16S rDNA gene sequencing is a powerful tool that has been used to trace phylogenetic relationships between bacteria, and to identify bacteria from various sources, such as environmental or clinical specimens. Identification based on the 16S rRNA sequence is of interest because ribosomal SSU exists universally among bacteria and includes regions with species specific variability, which makes it possible to identify bacteria to the genus or species level by comparison with databases in

the public domain (Vandamme *et al.,* 1996). According to the remarkable PGPR characters of *Pseudomonas* sp, therefore in this study we isolated *Pseudomonas* sp. from the rhizosphere of apple and its phenotypic and genotypic identification (16S r RNA and RAPD). It was further investigated for its possible plant growth promoting traits.

MATERIALS AND METHODS

Isolation and screening of fluorescent *Pseudomonas* strains: Bacteria isolated from the rhizospheric soil of apple orchard at Maggota (Shimla district), root samples were shaken vigorously to remove loosely adhering soil and 4.5 ml of sterile physiological water was added to 0.5 g of rhizospheric soil and the mixture was shaken at 120 rpm for 2 min. Serial ten-fold dilutions were prepared from the extract and 0.1 ml of each dilution was seeded onto King's B medium (Kings *et al.,* 1954), supplemented with 100 µg/ml of cycloheximide to suppress fungi. After 28 ± 2°C for 48 h incubation, well separated individual colonies with yellow green and blue white pigments were marked and detected by viewing under UV light. All isolates of fluorescent *Pseudomonas* sp. were morphologically i.e. colony morphology and biochemically characterized for Gram staining, spore staining, catalase, oxidase, denitrification, gelatin liquification test, lecithinase activity, arginine hydrolysis and growth at 4ºC, 25ºC, 37 ºC and 41ºC (Aneja,2003)

Plant growth promoting potential of isolates of fluorescent *Pseudomonas* species for multifarious activities: All the fluorescent *Pseudomonas* sp. were characterized for plant growth promoting activities *viz.*, phosphate solubilizing activity, siderophore activity, production of HCN, ammonia, hydrolytic enzymes, plant growth regulators (auxins, gibberellins and cytokinins) and antifungal activity .

Detection of phosphate solubilizing activity: Bacterial isolates were screened on Pikovskaya's agar plates for phosphate solubilization index with known amount of inert phosphorus (Ca_3 $(PO_4)_2$) (Pikovskaya's, 1948). Phosphate solubilisation was expressed in terms of mm diameter of yellow colored zone produced around well. Quantitative analysis of solubilization of tricalcium phosphate in liquid medium was made as described by Bray and Kartz (1945). The absorbance of the developing blue color was read at 600 nm. The amount of soluble phosphorus was detected from standard curve of Potassium dihydrogen orthophosphate (KH_2PO_4).

Siderophore activity: Siderophore production was tested by growing *Pseudomonas* sp. in the universal siderophore detection medium CAS agar (Schwyan and Neilands, 1987).

HCN and Ammonia production: For the production of hydrogen cyanide (HCN), the *Pseudomonas* sp. was screened out according to Baker and Schippers (1987) and ammonia production was detected by the method given by Lata and Saxena (2003).

Lytic enzymes: Protease activity: All *Pseudomonas* sp. strains were screened out for proteolytic activity by well plate assay method on 1% skim milk agar plates (Kaur *et al.,* 1989) and proteolysis i.e. clear zone (mm dia.) produced around the well was observed. The proteinase liquid assay was based on casein digestion and employed in five minute assay time period. The casein substrate was 2% solution in 0.05M Tris buffer (tris hydroxyl methylaminomethane), pH 8.0. One milliliter of substrate solution was incubated at 37°C with 1ml of enzyme dilution in same Tris buffer (0.05M) for 5 minutes. The mixture was then precipitated with 3 ml of 5%TCA (trichloroacetic acid). The unit of activity was defined as hydrolysis of one equivalent milligram of protein (casein) per milliliter of enzyme per minute under standard assay conditions.

Screening of isolates for the production plant growth regulators (PGRS): *Pseudomonas* sp. isolated from the rhizosphere soil of apple orchards were screened out for the production of plant growth regulators viz., auxins, gibberellins and cytokinins. Quantitative estimation of auxins was done by colorimetric method (Gordon and Weber, 1951) with slight modifications i.e. 2 to 3 drops of orthophosphoric acid was added to 2 ml supernatant and 4 ml of salper reagent (1 ml of 0.5 M $FeCI_3$ in 50 ml of 30 % $HCIO_4$ freshly prepared). The gibberellins were estimated calorimetrically by the method of (Holbrook *et al.,* 1961). Radish cotyledons expansion bioassay test was employed (Letham, 1971) for estimation of cytokinins like substances the radish seeds (*Raphanus sativus* L. cultivars Japanese white) were germinated in total darkness for 48 h at 28° C.

***In vitro* antifungal activity:** Antifungal activity of each test strain of *Pseudomonas* species isolate against different indicator fungal pathogens *viz., Alternaria solani*, *Dematophora necatrix*, *Fusarium oxysporum*, and *Pythium ultimum* checked by well plate assay method (Vincent, 1947).

Genotypic characterization of *Pseudomonas* sp. Isolates: Nine isolates fluorescent *Pseudomonas* sp. *viz.*, Pn-13-San, An-16-kul, An-1-Mag, An-2-Mag, An -3-Mag, An-5-Mag, An-6-Mag, Ar-3-Mag and An-6-Shr were selected on the basis of overall plant growth promoting and disease suppressing activities for their genotypic characterization which was done by two methods: a). 16S rRNA-PCR and DNA sequencing and b). Random Amplification Polymorphic DNA-PCR (RAPD-PCR).

Molecular characterization of fluorescent *Pseudomonas* sp. by 16S rRNA technique: Genomic Deoxyribonucleic acid (DNA) was extracted with DNA isolation kit (Bangalore GeNei), and the 16S rDNA gene was amplified by PCR using the set of primers FP 1 (GGTCTGAGAGGATGATCAGT) and RP 1 (TTAGCTCCACCTCGCGGC) in MJ Mini BIORAD personal thermal cycler-100 (PTC-100). The PCR amplification was carried out in MJ Mini BIORAD per-

sonal thermal cycler-100 (PTC-100) with a total of 35 cycles. Amplification was as follows: denaturation at 94°C for 1 min, annealing at 55°C for 2 min, and elongation at 72°C for 2min. For DNA sequencing, eluted amplified DNA products of two selected *Pseudomonas* sp. (Pn-13-San and An-16-Kul) was first purified followed by sequencing in Bioserve Private limited (Hyderabad, India). Similarity searches of the Genbank database were performed with BLAST (Widmer *et al.*, 1998).

Random Amplified Polymorphic DNA-PCR (RAPD -PCR): Amplification of genomic DNA was carried out using four random 10-mer oligonucleotide primers: OPA-05, OPA-06, OPA-07 and OPA-08 (M/S Operon Tech. Inc., Alameda, USA) in MJ Mini BIO-RAD personal thermal cycler-100 (PTC-100) (Altschul *et al.*, 1990).

The amplified DNA was viewed under the UV transilluminator and the image was taken through BIO-RAD gel documentation system XR using quantity one software and saved in computer.

Primers used	Sequence 5' → 3'
OPA-05	AGGGGTCTTG
OPA-06	GGTCCCTGAC
OPA-07	GAAACGGGTG
OPA-08	GTGACGTAGG

RAPD analysis: The amplified products of seven selected isolates Pn-13-San, An-16-Kul, Ar-3-Mag, An-1 -Mag, An-2-Mag, An-5-Mag and An-6-Shr. *Pseudomonas* sp. isolates were scored with four primers for the presence and absence of bands. Presence of an amplified product was designated as '1' and absence was marked as '0'. A pair wise similarity matrix was constructed by using dice similarity index. NTSYS-pc, version 2.02 (Numerical Taxonomy System, Exeter Software) was used to perform cluster analysis of the complete RAPD data. Molecular weights of amplified bands of all *Pseudomonas* sp. isolates under study were analyzed by using quantity one.

Statistical analysis: Experiments were analyzed under completely randomized (CRD) design with three replications. Statistical significance of the data was determined using analysis of variance (ANOVA).

RESULTS AND DISCUSSION

Morphological, physiological and biochemical characteristics of bacterial isolates: All the selected fourteen isolates from the rhizosphere of apple were found to be fluorescent, with transparent colonies (irregular to circular colonies with entire edge and flat elevation on nutrient agar plate), pigmented (bluish and yellowish pigmentation on King's B agar), The pigment produced by the bacterial isolate Pn-13-San was fluorescent green and An-16-kul, An-3-cha, An-6-shr and An-3-Mag were bluish green while rest of the isolates produced yellow green pigment on King's B medium (Table 1). In biochemical characterization (Table 2), all isolates were found to be gram negative rods with no ability to form endospores and all the isolates were found to be positive for catalase and oxidase tests. All isolates were negative for indole, methyl red and Voges-Proskaur negative and positive for citrate test. All *Pseudomonas* isolates showed aerobic growth. Out of fourteen isolates, nine isolates showed growth at 4˚C and other five isolates shown growth at 41˚C.In physiological characterization (Table 3), all isolates were found to liquefy gelatin and have the ability to produce gas (cracking of agar medium). Out of fourteen isolates only seven gave opaque zone around growth of bacteria in lecithinase test. Ten isolates showed presence of chitinase as showing chitin hydrolysis on chitin agar plate. All isolates produced dark brown to orange brown color in arginine hydrolysis showed positive results. All isolates showed cultural, morphological similarities with *Pseudomonas* sp. that was also observed by Sharma *et al.*, (2014).

Plant growth promoting attributes of fluorescent *Pseudomonas* sp.

Phosphate solubilization assay: All the strains are identified as potential phosphate solubilizer based on their capacity to solubilize tricalcium phosphate [$Ca_3(PO_4)_2$] by the formation of clear halo zone on medium. Maximum phosphate solubilization was expressed by *Pseudomonas* isolates on Pikovskaya agar plate in the range 14.33-21.33 mm diameter by formation of yellow zone on Pikovskaya's agar medium by 100µl of 72h old supernatant of culture (Table 4) The ability of bacteria to solubilize mineral phosphates has been shown of interest to agricultural microbiologists as it can enhance the availability of phosphorus and iron for plant growth. Gopalakrishanan *et al*. (2011) reported that free living P-solubilizing bacteria release phosphate from spare soluble inorganic and organic phosphate compounds in soil and so contribute to increase available phosphate for the plants.

Siderophores production assay: Almost all the isolates showed production of siderophores which was estimated by Chrome Azurol S blue agar (CAS) plate assay and liquid assay. Fourteen isolates of *Pseudomonas* sp. showed yellow to orange coloured zone in the range of 12.33-13.67 mm diameter followed by another isolates which showed slightly less production i.e. 10.00-11.67 mm diameter of pinkish or orange zone on Chrome Azurol S blue agar plates. Siderophore production was also quantified by liquid assay in terms of reduction in blue color as percent siderophore units (% SU). All the isolates showed maximum production of siderophore units in the range of 56.44- 93.44%. Two isolates An-4-Mag and An-6-Mag are the high producers of siderophore activity in the range of 80.94 and 93.44% (Table 4). Chaiham *et al.*, (2009) reported that siderophore producing microorganisms suppress some soil borne fungal pathogens through direct role of siderophore-mediated iron com-

Table 1. Morphological colony characterization of different isolates of fluorescent *Pseudomonas* sp.

Pseudomonas Isolates	Colony Morphology					Pigment / Fluorescence
	Form	Elevation	Margin	Opacity	Surface	
Pn-13-San	Irregular	Flat	Entire	Transparent	Smooth	Fluorescent green
An-16-Kul	Irregular	Flat	Entire	Transparent	Smooth	Bluish green
An-3-Cha	Irregular	Flat	Entire	Transparent	Smooth	Bluish green
An-6-Shr	Irregular	Flat	Entire	Transparent	Smooth	Bluish green
An-1-Mag	Irregular	Flat	Entire	Transparent	Smooth	Yellow green
An-2-Mag	Irregular	Flat	Entire	Transparent	Smooth	Yellow green
An-3-Mag	Irregular	Flat	Entire	Transparent	Smooth	Bluish green
An-4-Mag	Irregular	Flat	Entire	Transparent	Smooth	Yellow green
An-5-Mag	Irregular	Flat	Entire	Transparent	Smooth	Yellow green
An-6-Mag	Irregular	Flat	Entire	Transparent	Smooth	Yellow green
An-7-Mag	Irregular	Flat	Entire	Transparent	Smooth	Yellow green
Ar-3-Mag	Irregular	Flat	Entire	Transparent	Smooth	Yellow green
Ar-4-Mag	Irregular	Flat	Entire	Transparent	Smooth	Yellow green
Ar-5-Mag	Irregular	Flat	Entire	Transparent	Smooth	Yellow green

petition in the biocontrol ability.

Protease activity: The production of proteolytic enzyme in 72 h old culture supernatant was detected by using 1% skim milk agar. Enzyme activity was expressed in terms of mm diameter of clear zone produced around bit/well at 28±2ºC after 48 h of incubation. The presence of proteolytic activity was shown by almost all the isolates of *Pseudomonas* sp. from the rhizosphere of apple growing in normal and replant sites in the range of 12-16 mm diameter of clear zone (Table 4).

Production of HCN and Ammonia: Isolates of *Pseudomonas* sp. showed HCN production on King's B medium amended with glycine. Out of fourteen isolates of *Pseudomonas* sp. maximum production of HCN (**+++)** was shown by five isolates An-16-Kul, An -3-Cha, An-6-Shr, An-3-Mag and An-6-Mag followed by six isolates i.e. Pn-13-San, An-1-Mag, An-2-Mag, An-4-Mag. The production of ammonia in peptone water after inoculation with test isolates and incubation for four days at 28±^{0}C was observed by using Nessler's reagent. Color change in the broth tubes after growth from yellow to brown after addition of Nesseler's reagent showed presence of ammonia that was soluble in the media. Out of fourteen, eight isolates i.e. Pn-13-San, An-3-Cha, An-6-Shr, An-1-Mag, An-2-Mag, An-4-Mag, An-6-Mag and Ar-3-Mag showed maximum production of ammonia (+++) while other isolates showed slightly less production of ammonia (++) (Table 4). Cyanide acts as a general metabolic inhibitor to avoid predation or competition. Ramette *et al.* (2003) reported that microbial production of HCN has been as an important antifungal feature to control root fungi pathogen. The host plants are generally not harmfully affected by inoculation with HCN production bacteria and host specific rhizobacteria can operate as biological control agents.

Production of PGRs: The result showed that all the strains of *Pseudomonas* species produce plant growth regulators (Table 4). The maximum production of auxins was shown by four *Pseudomonas* isolates An-3-Cha, Pn-13-San, An-6-Shr and An-3-Mag in the range of 62.33-83.33 µg/ml followed by two isolates i.e. An-1-Mag and An-4-Mag in the range of 52-57.33 µg/ml. Out of fourteen, seven isolates i.e. An-16-Kul, An-4-Mag, Pn-13-San, An-1-Mag, An-5-Mag, An-6-Shr and An-6-Mag showed maximum production of gibberellins in (50.33-58.67) µg/ml range followed by three isolates An-2-Mag, An-7-Mag and An-3-Mag i.e. in the range of 32.67-46.67 µg/ml for gibberellins production. Cytokinins production by all isolates of *Pseudomonas* sp. was measured indirectly by radish cotyledons bioassay. Maximum production of cytokinins (30.67-43.33µg/ml) was detected in four *Pseudomonas* isolates An-3-Cha, An-5-Mag, An-4-Mag and An-3-Mag. Production of IAA by PGPR generally affects the root system, increasing the size and number of adventitious roots and also the root subdivision, enabling a bigger soil amount to be exploited by the roots, thus providing large amounts of nutrients accessible to the plant (Ribeiro and Cardoso, 2011). However, IAA production by PGPR can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability (Sajjad *et al.*, 2001).

Antifungal activities: All isolates of *Pseudomonas* species showed antifungal activity against one or the other plant pathogenic fungi. Overall antifungal activity against *Alternaria solani* has shown by nine isolates out of fourteen isolates of fluorescent *Pseudomonas* species in the range of 16.25 to 31.25% inhibition. Ten isolates of fluorescent *Pseudomonas* sp. have shown antifungal activity against plant pathogen *Dematophora necatrix* in the range of 9.33 to 22.6 %I. The maximum percent growth inhibition was shown by two isolates i.e.An-6-Shr and An-6-Mag which was in the range of 20.00-22.6 %I followed by three isolates An-2 -Mag, An-3-Cha and An-7-Mag in the range of 16.00-17.33 %I. Only eight of the isolates of fluorescent *Pseudomonas* as well as from normal site of apple at

Table 2 Biochemical characterization of different isolates of fluorescent *Pseudomonas* sp.

Pseudomonas Isolates	Oxidase (blue color)	Catalase (effervescence)	Gram staining	Spore Staining	Indole production	Methyl red	Voges Proskaur	Citrate	O/F	Starch hydrolysis	Growth (^{0}C)			
											4^{0}C	25^{0}C	37^{0}C	41^{0}C
Pn-13-San	+	+	-	-	-	-	+	+	+/-	-	-	+	+	+
An-16-Kul	+	+	-	-	-	-	+	+	+/-	-	-	+	+	+
An-3-Cha	+	+	-	-	-	-	+	+	+/-	-	-	+	+	+
An-6-Shr	+	+	-	-	-	-	+	+	+/-	-	-	+	+	+
An-1-Mag	+	+	-	-	-	-	+	+	+/-	-	+	+	+	-
An-2-Mag	+	+	-	-	-	-	+	+	+/-	-	+	+	+	-
An-3-Mag	+	+	-	-	-	-	+	+	+/-	-	-	+	+	+
An-4-Mag	+	+	-	-	-	-	+	+	+/-	-	+	+	+	-
An-5-Mag	+	+	-	-	-	-	+	+	+/-	-	+	+	+	-
An-6-Mag	+	+	-	-	-	-	+	+	+/-	-	+	+	+	-
An-7-Mag	+	+	-	-	-	-	+	+	+/-	-	+	+	+	-
Ar-3-Mag	+	+	-	-	-	-	+	+	+/-	-	+	+	+	-
Ar-4-Mag	+	+	-	-	-	-	+	+	+/-	-	+	+	+	-
Ar-5-Mag	+	+	-	-	-	-	+	+	+/-	-	+	+	+	-

(-) Indicates negativity of test (+) indicates positivity of test

Maggota showed antifungal activity against *Fusarium oxysporum* in the range of 10.7-27.6 % of mycelial growth. The maximum percent growth inhibition (23.07-27.6 %I) was shown by two isolates i.e. Pn-13-San and An-3-Mag followed by isolate An-6-Mag i.e. (18.4 %I).Ten isolates of fluorescent *Pseudomonas* sp. out of fourteen showed antifungal activity against *Pythium ultimum* in the range of 5.71-22.8 % inhibition of mycelial growth on malt extract agar (MEA) plates after 5 days of incubation at 25±^{0}C. The maximum percent inhibition of mycelial growth was shown by An-16-Kul (22.81 %I) followed by five other isolates i.e. An-3-Cha, An-4-Mag, An-6-Mag, An-3-Mag and An-6-Shr in the range of 14.28-17.14 %I. Overall all the fourteen isolates showed antifungal activity against one or other species each of fungal pathogen. Five isolates showed broad range of antifungal activity as inhibited all the four tested fungal pathogen. Antifungal activities of *P. fluorescence* against phytopathogenic fungi were screened out by Shalini and Srivastava (2008). Secondary metabolites produced by fluorescent pseudomonads have been reported antifungal activity inhibiting *Rhizoctonia solani* (Mina *et al.*, 2013). It is evident from the present studies that the fluorescent pseudomonads under investigation are capable of producing plant growth promoting substances and antifungal substances. Hence they are potential candidates for the development of bioinoculants for crop plants.

Molecular characterization

16S rRNA sequencing: Using the universal primer set, DNA fragment of the 16S rDNA gene was amplified by PCR. The PCR amplified 16S rDNA region was sequenced and sequence data were analyzed by BLAST and the nearest match from the GenBank data was reported. DNA sequencing and phylogenetic analysis revealed that strain Pn-13-San showed 99% homology with *Pseudomonas aeruginosa* M18 with accession number (NC_017548.1), and An-16-Kul the isolate An-16-Kul showed 99% homology with *Pseudomonas aeruginosa* PAO1 with accession number (NC_002516.2) and *Pseudomonas aeruginosa* DK2 with accession number NC_018080.1. Similarity searches of the GenBank database were performed with BLAST. The following sequences of *Pseudomonas* sp. isolate Pn-13-San and An-16-kul with universal primers:

Pn-13-San sequences

C T T G G A A C T G A G A C C G G T C C A - GACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA CAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTG TGTGAAGAAGGTCTTCGGATTGTAAAGCACTTTAAG TTGGGAGGAAGGGCAGTAAGTTAATACCTTGCTGTT TTGACGTTACCAACAGAATAAGCACCGGCTAACTTC GTGCCATCAGCCGCGGTAATACGAAGGGTGCAAGC GTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAG GTGGTTCAGCAAGTTGGATGTGAAATCCCCGGGCTC AACCTGGGAACTGCATCCAAAACTACTGAGCTAGA GTACGTTAGAGGGTGGTGGAATTTCATGTGAAGTG

Table 3. Physiological characterization of different isolates of fluorescent *Pseudomonas* sp.

Pseudomonas Isolates	Denitrification[1] (gas production)	Gelatin[2] liquification	Lecithinase[3] (opaque zone)	Chitinase[4] (Clear zone)	Arginine[5] Hydrolysis
Pn-13-San	+	+	-	+	+++
An-16-Kul	+	+	-	+	+++
An-3-cha	+	+	-	+	+++
An-6-shr	+	+	-	+	+++
An-1-Mag	+	+	+	-	++
An-2-Mag	+	+	+	+	++
An-3-Mag	+	+	-	+	++
An-4-Mag	+	+	+	+	+
An-5-Mag	+	+	+	+	+
An-6-Mag	+	+	+	+	++
An-7-Mag	+	+	-	+	+
Ar-3-Mag	+	+	+	-	+
Ar-4-Mag	+	+	+	-	++
Ar-5-Mag	+	+	-	-	+

[1]Denitrification test expressed positive(+) in terms of gas production and cracking of agar medium.
[2]Gelatin test expressed positive(+) in terms of liquification of gelatin.
[3]Lecithinase test expressed positive(+) in terms of opaque zone produced around the growth of streaked bacterial culture.
[4]Chitinase test expressed positive(+) in terms of clear zone on chitin agar.
[5]Arginine test expressed positive(+) in terms of color change of culture broth from dark brown to orange brown.

GTGAAATGCGAATAAATAGGAACGAACACCAGTTG
CGGGGCGGTAAGGGAAATTGATGGGACACTGACCT
TTCAATCCTGTTCCTTTTCTTGACTAAATACCAAGGT
ATTCCTCCGCCCTAATGATTTCTTTTCCCGTTACGCT
ATTTATAGATCATTTTTTCCCATCCATCGAAAACTG
CAAACCACCCAGGTGCGTAATAGTCGCCAGGTCTTT
CTCTCCATATTTTTTTTATATATATCTCTTTACCACC
CCCTGGCTCTCTGATCGTTTCCATGTATATATTTCAC
CGTGAAAACGTTTGACCCTACGCCGAGGATGTAAA
AAAATTACTCTCACAAAAAG

An-16-Kul sequences

CGGGGAAAGTAAGACCGGTCCAGACTCCTACGG-
GAGGCAGCAGTGGGGAATATTGGACAATGGGCGAA
AGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGG
TCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAG
GGCAGTAAGTTAATACCTTGCTGTTTTGACGTTACC
AACAGAATAAGCACCGGCTAACTTCGTGCCAGCAG
CCGCGGTAATACGAAGGGTGCAAGCGTTAATCGGA
ATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGC
AAGTTGGATGTGAAATCCCCGGGCTCAACCTGGGA
ACTGCATCCAAAACTACTGAGCTAGAGTACGGTAG
AGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGC
GTAGATATAGGAAGGAACACCAGTGGCGAAGGCGA
CCACCTGGACTGATACTGACACTGAGGTGCGAAAG
CGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAATCGATGTCCACTATCCGTTGGGATC
CTTGAGATCTTATTGGCGCAGCTCACGCGATAAGTC
GACCGCCTGGGGGAGTACGGCCGCATGGTTCAAAC
TTATATGTATTGACAGGTGCCCGCCCACCTCGGTGT
AGCATGTGGTTTATATTAAATTCGCCGCGAAAAACC
TTAAATGGACTTTACATGTATATACTTACCCATAGA
AGCTTTTGAGATTTGGGGTATTCTCACTTAAGGTGG
CTGTATGACTGCACACAAGATCTCGACATCATAATA
GTGGCCTAGCTCTCCACTTCCTGCTCAACCCTTGGA
AAATGTGTGTCGCTCTCTCTCCTCAT

Random amplified polymorphic DNA-PCR analysis (RAPD-PCR): All the selected *Pseudomonas* sp. isolates were used for amplification of genomic DNA using RAPD primers (OPA series).The RAPD (Random Amplified Polymorphic DNA) profiles of seven strains of *Pseudomonas* sp. with four random 10 -base oligonucleotide primers: OPA-05, OPA-06, OPA -07 and OPA-08.The primer OPA-05 amplified genomic DNA of only one strain of *Pseudomonas* i.e., Ar -3-Mag and generated 4 reproducible DNA fragments ranging from 200 to 1000bp. All the amplified bands with primer OPA-05 were found to be polymorphic. With primer OPA-06, there is no amplification of *Pseudomonas* strains. The primer OPA-07, amplified genomic DNA of three strains of *Pseudomonas* i.e., Ar -3-Mag, Pn-13-San and An-1-Mag and generated 3 reproducible DNA fragments ranging from 200 to 500bp. All the amplified bands with primer OPA-07 were found to be polymorphic. The primer OPA-08 amplified the genomic DNA of only one strain of *Pseudomonas* i.e., An-5-Mag and generated 3 reproducible DNA fragments ranging from 100 to 400bp. All the amplified bands with primer OPA-08 were found to be polymorphic (Tables 6 and 7). The dendrogram analysis showed that all the seven isolates could be categorized into 2 distinct clusters. Group one includes An-3-Mag and group two was further divided into two subgroups group 2a which include Pn-13-San and group 2b which include An-6-Mag, An-1-Mag, An -2-Mag, An-16-kul and An-6-Shr (Fig 1). PCR-RAPDs has proved to be a very efficient tool to define strains within the same bacterial species (Gutierrez Mañero *et al.,* 2002; Lucas Garcia *et al.,* 2001). The international Committee for Bacterial Systematic determined that 30% divergence in DNA estimated by DNA hybridization defines the taxonomic range of bacterial species, while divergences of 40-50% define bacterial genera. The arrangement obtained by PCR-RAPDs and UPGMA and Pearson coefficient may be understood as

Table 4. Potential of fluorescent *Pseudomonas* isolates for production of multifarious PGP activities

Pseudomonas isolates	PGP Activities									
	Phosphate Solubilization		Siderophores		Proteolytic	Ammonia	HCN	Plant Growth regulators (µg/ml)		
	Change of color (yellow to brown)									
	Yellow zone (mm dia.)	Available (Pi µg/ml)	Orange/pinkish (mm dia.)	%SU (Ar =1.8) at A_{630}	Clear zone (mm dia.)	Culture broth	paper strip	Auxins	Gibberellins	Cytokinins
Pn-13-San	20.33	23.00	11.33	62.05(62.05)	13.67	+++	++	73.67	52.33	23.00
An-16-Kul	17.00	20.67	13.00	58.16(58.49)	13.67	++	+++	26.33	50.33	22.67
An-3-Cha	19.67	36.33	13.00	58.11(57.88)	14.67	+++	+++	62.33	48.33	30.67
An-6-Shr	21.33	52.33	13.30	63.94(63.12)	13.33	+++	+++	74.33	54.67	23.00
An-1-Mag	15.33	31.00	11.33	56.77(56.57)	14.33	+++	++	52.00	52.33	15.00
An-2-Mag	18.33	42.67	11.33	65.38(65.31)	13.33	+++	++	34.00	32.67	13.67
An-3-Mag	18.00	22.33	13.67	59.94(59.87)	12.67	++	+++	83.33	46.67	43.33
An-4-Mag	17.67	52.67	11.00	80.94(90.79)	14.67	+++	++	57.33	51.33	36.67
An-5-Mag	14.33	26.33	13.67	56.44(57.40)	14.67	++	++	33.33	52.67	32.67
An-6-Mag	17.67	63.33	12.67	93.44(93.45)	13.67	+++	+++	35.33	58.67	26.67
An-7-Mag	17.00	32.00	12.33	58.55(58.70)	16.00	++	-	36.67	34.33	15.67
Ar-3-Mag	20.67	13.33	11.67	60.11(61.57)	11.33	+++	++	23.67	26.67	25.63
Ar-4-Mag	15.00	15.00	10.33	53.66(52.60)	14.33	++	-	19.67	21.00	12.33
Ar-5-Mag	16.67	12.33	10.00	56.05(55.27)	12.00	++	-	22.00	22.67	15.33
CD_{05}	**2.37**	**3.32**	**0.12**	**2.58**	**3.84**			**3.09**	**3.04**	**2.81**

genetic distances obtained by nucleotide substitutions (Clark and Lanigan, 1993; Nei and Miller, 1990). Molecular diversity of ten isolates was analyzed by RAPD -PCR.

Conclusion

The world over is changing from inorganic conventional farming towards organic ecofriendly farming methods. This not only requires the isolation of bioinoculants with high potential for use as biofertilizers but also several other factors right from appropriate application procedures to correct marketing practices also being economically cheaper. These results proved that plant growth activities produced by Pseudomonas species could also play a critical role in plant growth promotion. The fluorescent *Pseudomonas* strains Pn-13-san and An-16-Kul isolated from apple rhizosphere have possible potential for the plant growth promotion under field condition due to their multifarious plant growth promoting traits. However, the new techniques recently developed over tremendous potential for improving our understanding of the traits that are involved in the root colonization process, and also provides information on the life of the inoculated PGPR in the rhizosphere.

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Table 5. Potential of fluorescent *Pseudomonas* isolates for production of antifungal activities against different plant pathogenic fungi

***Pseudomonas* isolates**	**Indicator fungi**								
	***Alternaria solani* mm of control (C=80mm)**		***Dematophora necatrix* mm of control (C =75mm)**		***Fusarium oxysporum* mm of control (C=65mm)**		***Pythium ultimum* mm of control (C = 70mm)**		**Antifungal activity against no. of patho-gens**
	mm dia. of test (Z)	**% inhibition**	**mm dia.of test (Z)**	**% inhibition**	**mm dia. of test (Z)**	**% inhibition**	**mm dia. of test (Z)**	**% inhibi-tion**	
Pn-13-San	65	18.75(29.57)	0	0(0.00)	50	23.07(31.60)	62	11.42 (20.53)	3
An-16-Kul	55	31.25(40.12	65	13.32(4.045)	58	10.70(18.56)	54	22.81 (34.50)	4
An-3-Cha	0	0(0.00)	62	17.33(4.350)	55	15.38(26.11)	60	14.28 (22.42)	3
An-6-Shr	0	0(0.00)	60	20.00(4.295)	57	12.30(19.21)	58	17.14 (27.39)	3
An-1-Mag	65	18.75(27.41)	67	10.66(3.747)	59	9.23(19.17)	63	10.00 (17.80)	4
An-2-Mag	56	30.00(38.97)	63	16.00(4.188)	56	13.20(22.53)	65	7.14(17.22)	4
An-3-Mag	62	22.5(32.17)	66	12.00(3.973)	47	27.60(35.43)	59	15.71 (27.05)	4
An-4-Mag	57	28.75(35.69)	64	14.66(4.406)	0	0(0.00)	60	14.28 (23.85)	3
An-5-Mag	59	26.25(35.23)	0	0(0.00)	0	0(0.00)	0	0(0.00)	1
An-6-Mag	67	16.25(25.27)	58	22.60(5.302)	53	18.40(26.83)	60	14.28 (22.58)	4
An-7-Mag	0	0(0.00)	62	17.30(4.959)	0	0(0.00)	0	0(0.00)	1
Ar-3-Mag	60	25.00(30.88)	0	0(0.00)	0	0(0.00)	0	0(0.00)	1
Ar-4-Mag	0	0(0.00)	0	0(0.00)	0	0(0.00)	66	5.71(19.66)	1
Ar-5-Mag	0	0(0.00)	68	9.33(3.948)	0	0(0.00)	0	0(0.00)	1
$CD_{.05}$	0.17	6.43(3.89)	2.93	4.01(0.48)	0.18	4.24(3.05)	2.48	5.17(4.13)	

%I = C-T/ C× 100) Where, C: Growth of mycelia in control. T: Growth of mycelia in test.

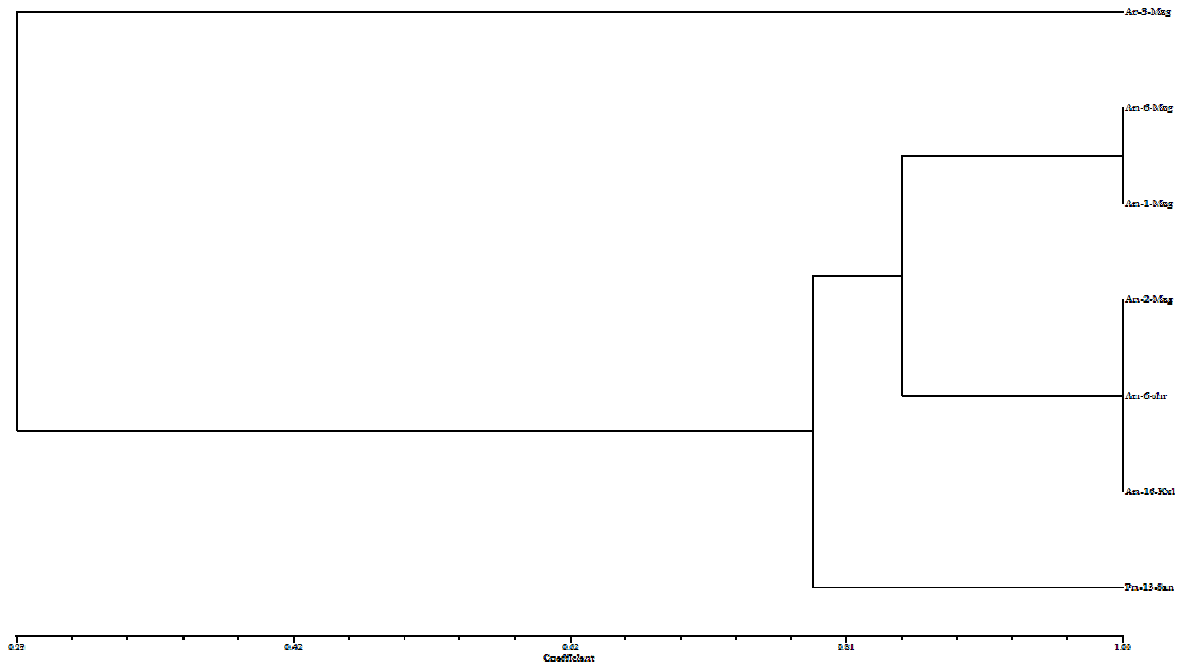

Fig. 1. *Phylogenetic tree of Pseudomonas isolates from apple in replant and normal site of orchard at Maggota (Jubbal) with RAPD primers.*

Table. 6. Total number of amplified fragments and number of monomorphic and polymorphic fragments generated by PCR using 4 random decamer oligonucleotide primers.

S. N.	Primer name	Total number of amplified bands	Total number of monomorphic amplified bands	Total number of polymorphic
1	OPA-05 (AGGGGTCTTG)	4	0	4
2	OPA-06 (GGTCCCTGAC)	0	0	0
3	OPA-07 (GAAACGGGTG)	5	0	3
4	OPA-08 (GTGACGTAGG)	3	0	3
	TOTAL	12	0	10

Table 7. Similarity matrix showing relationship among *Pseudomonas* sp. isolates from the rhizosphere of apple at Maggota

Ar-3-Mag	An-6-Mag	An-1-Mag	An-2-Mag	An-6-Shr	Pn-13-San	An-16-Kul
1.0000000						
0.1538462	1.0000000					
0.1538462	1.0000000	1.0000000				
0.3076923	0.8461538	0.8461538	1.0000000			
0.3076923	0.8461538	0.8461538	1.0000000	1.0000000		
0.1538462	0.6923077	0.6923077	0.8461538	0.8461538	1.0000000	
0.3076923	0.8461538	0.8461538	1.0000000	1.0000000	0.8461538	1.0000000

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