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ISOLATION AND CHARACTERIZATION OF NATIVE FLUORESCENT *Pseudomonas* FROM RICE FIELDS OF ASSAM (INDIA)

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ABSTRACT

In present study ten isolate of *Pseudomonas,* isolated from rice rhizosphere, were characterized on the basis of morpho-biochemical and molecular characteristics. Screening of their plant growth promoting activities *viz*., siderophore production, proteolytic activity, ammonia production, IAA production and HCN production revealed that the isolate *Pf*As1 was most promising one with highest siderophore production (6.18 cm dia. of yellow halo) and proteolytic activity (5.85 cm dia. halo zone). The results showed that none of the isolates possessed all the tested PGP activities. IAA and ammonia production was determined quantitatively for all the isolates. The IAA and ammonia production in tested *Pf* strains ranged from 15.66 to 48.58 and 179.05 to 394.72 mg L^{-1}. The phylogenetic analysis using 16S rRNA gene sequences showed phylogenetic position of different fluorescent *Pseudomonads*. The results revealed that the isolated strains were *Pseudomonas fluorescens* and *P. putida.*

Key words: PGPR, proteolytic activity, *Pseudomonas,* rhizosphere, rice, siderophore

INTRODUCTION

The rhizosphere autochthonous microbes have attracted much interest in recent years due to their habitat and involvement in several biologically important processes. In rhizosphere, a large number of microorganisms exist and among them bacteria are the most abundant. Soil bacteria actively participate in biogeochemical cycles. Plant bacterial interactions in rhizosphere are determinants of plant health and soil fertility. The root colonizing bacteria exert beneficial impact on plant growth and development via direct or indirect mechanisms, so are called as plant growth promoting rhizobacteria (PGPR) (Saxena, 2003; Nelson, 2004). The PGPR have widely been exploited in agriculture fields as it offers an attractive strategy to improve crop growth and development by replacing or supplementing fertilizers and pesticides. One of the dominant PGPR *Pseudomonas fluorescens* and *P. putida* acts as active biocontrol agent against soil-borne diseases in many crop plants by preventing the deleterious effects of phytopathogenic organisms through production of antibacterial and anti-fungal compounds (Hayat *et al.,* 2010). Fluorescent *Pseudomonas* is uniquely capable of synthesizing many antibiotics, not only to enhance its own fitness but also to help in the maintenance of soil health and bio-protection of crops against pathogens (Gaur *et al*., 2004; Sharma and Bora, 2016). *P. fluorescens* and *P. putida* as a biocontrol agent have drawn wide attention as it produces secondary metabolites such as siderophore, antibiotics, volatile compounds, HCN,

enzymes and phytohormones (Wohler, 1997; Zhang *et al.*, 2000; Nagarajkumar *et al*., 2004). *P. fluorescens* isolated from rice rhizosphere reportedly suppressed bacterial blight caused by *X. oryzae* pv. *oryzae* (Jeyalakshmi *et al.,* 2010), but level of control depends on the status of soil micronutrient, predominant type of clay mineral, their effect on the pathogen or host or host pathogen interactions. Hence anattempt was made to isolate indigenous *Pseudomonas* from different location of Jorhat district of Assam (India) and screen them for multiple plant growth promoting characters so as to develop bio-inoculants for crop production.

MATERIALS AND METHODS

The rhizosphere soil samples along with roots were collected randomly from 55 rice fields located in Jorhat district of Assam lying at an altitude of 116 m masl. The GPS coordinate was 26°44' N latitude, 94°10' E longitude. The sampling done in the year 2014 and the samples were immediately brought to the laboratory for gradient dilution using autoclaved water and aliquots were transferred to appropriate growth medium. Rhizosphere soil samples (10 g) were dispensed in 90 mL water blanks and serially diluted and then streaked on King's B (KB) medium. The culture plates were incubated at 27±1ºC until individual pure bacterial colonies of *Pseudomonas* were observed after 2-4 days. Isolated colonies were picked up and streaked onto KB medium in test tubes. The appearance of green fluorescence on irradiation under UV light indicated *P. fluorescens* and *P. putida*. Of the 55 isolates, 10 named as *Pf*As1, *Pf*As3, *Pf*As14, *Pf*As18, *Pf*As20, *Pf*As24, *Pf*As32, *Pf*As38 and *Pf*As46 were morphologically characterized on the basis of colony shape, elevation, margin, colour, staining behaviour and microphotography (Vincent, 1970).

Biochemical characterization

Ammonia production: For ammonia production, each isolate was inoculated in 50 mL peptone water in 100 mL conical flask and incubated at 30ºC. Broth culture was centrifuged at 10,000 rpm for 10 min and the cell free supernatant (CFS) was used to record the production of ammonia in culture medium. The intensity of yellow/brown colour was measured using UV-VIS spectrophotometer at 410 nm. The quantity of ammonia produced was calculated from standard curve prepared with known concentration of ammonium chloride and expressed as mg L^{-1}. The Proteolytic activity was determined by using skimmed milk agar (Kumar *et al*., 2005). After 2 days incubation at 30±1°C, a clear zone around the well indicated positive proteolytic activity. Proteolytic activity was expressed in terms of diameter of clear zone (in mm) produced around the well. The IAA production was assayed using qualitative method of Bric *et al.* (1991). All isolates were spot inoculated into L-tryptophan amended Luria-Bertani agar medium (LB). Then each inoculated Petri-dish was overlaid with an 82 mm-diameter disk of nitrocellulose membrane. Petri-dishes were inversely incubated in an incubator (at 30°C) for 2-4 days, until the colony diameter reached 2 mm dia. At this time, the membrane was removed from the plates and treated with Salkowski reagent (2% of 0.5 M $FeCl_3$ in 35% perchloric acid). Isolates of *P. fluorescens* producing IAA were identified by the formation of a characteristic red halo within the membrane immediately surrounding the colony. Quantification IAA was determined following the method described by Wohler (1997). The selected *P. fluorescens* isolates were grown for 72 h in KB media. Each culture was centrifuged at 7000 rpm for 5 min. The intensity of pink colour was measured using UV-VIS spectrophotometer at 535 nm wavelength. The quantity of IAA present in the extract was calculated from the standard curve prepared with known concentration of IAA and expressed the quantity of IAA in mg L^{-1}.

HCN production: Screening of bacterial isolates for hydrogen cyanide (HCN) production *vis-a vis* the biocontrol properties were performed following the method of Bakker *et al.* (1993). The production of HCN was checked on tryptic soy agar (TSA) slants streaked with the test isolates.

These slants were sealed properly in order to contain gaseous metabolites produced by the test isolates and to allow chemical reaction to take place with picric acid present in the filter padding. After 4 days of incubation at 30±1°C, the appearance of dark brown to yellow coloration of paper indicated HCN production.

Siderophore production: Production of siderophore by rhizobacterial strains was assayed by chrome azurol-S (CAS) plate assay method (Schwyn and Neilands, 1987) with several modifications. This assay was carried out based on the competition for iron between the ferric complexes of an indicator dye CAS and the siderophores produced by bacteria which apparently have a higher affinity for chelating Fe^{+3} of CAS. The assay was performed by using CAS agar medium containing the ternary complex CAS/Fe^{+3}/hexadecyltrimethyl-ammonium bromide as an indicator. The rhizobacterial inoculum was placed at the centre of solid medium. The plates were incubated in dark at 30°C for 24-48 h. The change of colour from blue to yellow/orange/purple or dark purplish red surrounding a bacterial colony was regarded as positive for siderophore production.

Molecular characterization

Isolation of genomic DNA of selected *Pseudomonas* species was done as per the modified method of Cardinal *et al.* (1997). A loop full culture of each of the isolates was inoculated in 20 mL LB broth and incubated at 37°C until the absorbance became 1.0 at 600 nm. From each culture, 1.5 mL was taken in 2 mL vials and centrifuged at 12000 rpm for 5 min. The supernatant was discarded and pellets vortexed in the remaining supernatant. To that, 200 µL lysis buffer (100 mM tris-acetate pH = 7.8, 20 mM sodium acetate and 1 mM EDTA, 1% SDS) was added and mixed thoroughly. To that, 100 µL of 5 M NaCl was added and thoroughly mixed by vortexing. The mixture was kept at -20°C for 20 min. The mixture was then centrifuged at 12000 rpm for 10 min at room temperature, and to the supernatant equal volume of phenol: chloroform: iso-amyl alcohol [(25:24:1] solution was added. The mixture was thoroughly mixed till the formation of milky emulsion. The mixture was centrifuged at 12000 rpm for 10 min at room temperature and top aqueous phase collected to which an equal volume of chloroform: isoamyl alcohol solution was added and centrifuged at 12000 rpm for 10 min at 4°C. Again, the top layer was taken to which double volume of 100% chilled ethanol (Merck, Germany) was added and kept at -20°C overnight. The vial was centrifuged at 12000 rpm for 10 min at 4°C. The pellet obtained was washed with 70% ethanol and air-dried for 30 min. Then, to the pellet 20 µL TE buffer (1 mM tris-HCl and 1 mM EDTA) was added, followed by the addition of 3 µL RNase and incubation at 35°C for 30 min. The genomic DNA was visualized *via* agarose gel electrophoresis using 0.8% agarose gel stained with 1 mg mL^{-1} ethidium bromide.

The isolated DNA was quantified and its purity estimated with the help of Nanodrop 1000 (Thermo Scientific). The absorbance of genomic DNA was measured at 260 nm to find out the concentration of DNA in solution. The absorbance at 280 nm was also taken to assess the extent of protein contamination in extracted DNA. The quality of isolated DNA was accessed based on A_{260}/A_{280} and A_{280}/A_{260} ratios.

PCR amplification of DNA

PCR was performed with a total volume of 20 µL reaction mixture containing 1X Taq buffer (Genei, India), 0.25 mM dNTP mix (Genei), 1U Taq DNA polymerase (Genei), 10 pM of both forward and reverse primers (Operon Technologies, Germany) and 50 ng genomic DNA. DNA amplification was performed on a thermocycler (Applied Bio-System). After initial denaturation at 95°C for 5 min, a PCR protocol was followed for 35 cycles each consisting of 95°C for 1 min, annealing temperature ranging from 48 to 56°C for different primers for 1 min, and 72°C for 1 min. The final extension was done at 72°C for 5 min. The amplification of the gene was observed by running the PCR sample in 1.5% agarose gel stained with ethidium bromide and electrophoresis was done at 10-15 V/cm. The gel was observed in gel documentation system.

In silico identification of species and phylogenetic analysis

The PCR products were sent to Xcelris Labs. Ltd, Ahmedabad, India, for sequencing. The resultant sequences were aligned with the already available sequences in the database of NCBI Gene Bank by BLAST analysis. Based on maximum identity score, E-value and percentage coverage, the sequence were identified at genus and species level. The characterized sequences were subjected to multiple sequence alignment (MSA) in MEGA v.6.0 tool. Based on MSA result, a 2-dimensional phylogenetic tree was established implementing Dayhoff model of substitution using Neighbor-Joining method in MEGA v6.1.0 with a bootstrap value of 1000 iterations.

Statistical analysis

Completely randomized design and Fisher's method of analysis of variance were employed for statistical analysis of the data. The significance of variance was calculated by 'F' values and comparing them with appropriate values of 'F' at 5% probability level. The data was transformed into corresponding angular values or log values wherever necessary. To compare the different treatments, critical differences were calculated. Standard error was calculated by using the formula:

$$CD = S.E. \times t_{0.05} \text{ for error degrees of freedom}$$

$$S.E.\pm = \sqrt{\left[\frac{2 \times \text{Error mean square}}{\text{No. of replications}}\right]}$$

Where, S.E. = Standard error difference; $t_{0.05}$ = "t" value at 5% probability level.

RESULTS AND DISCUSSION

Soil and rice root samples were collected from farmer's fields of 55 different locations in Jorhat district of Assam (India). Rhizospheric bacteria were isolated using standard protocol (White *et al.,* 2015) and purified in KB medium. From the 55 soil samples collected, 10 *Pseudomonas fluorescens* and *P. putida* isolates were identified on the basis of their colour, elevation, margin, shape, staining behaviour, green fluorescence pigment and growth characters. Meera and Balabaskar (2012) isolated 35 *P. fluorescens* from rice rhizosphere and on testing against *Sclerotium oryzae* by dual culture method 7 isolates (*Pf*4, *Pf*6, *Pf*8, *Pf*11, *Pf*13, *Pf*15 and *Pf*20) showed bright fluorescence under UV light.

Morphological study

All the selected isolates were rod shaped, Gram negative and fast growing. The colonies were opaque, creamy, convex shaped and produced yellow green pigment on KB medium. Biochemical characterization of *Pseudomonas* isolates revealed them positive to gelatin liquefication, catalase, oxidase, citrate utilization test, H_2S production, denitrification and dextrose utilization but were negative to starch hydrolysis test (Table 1). Lukkani and Reddy (2014) identified 55 isolates of fluorescent pseudomonad from the rhizosphere of ground nut in Andhra Pradesh (India), based on colony morphology and biochemical tests.

Qualitative evaluation of different plant growth promoting activity of Pseudomonas isolates

All the selected isolates of *Pseudomonas* showed the development of brown to yellow colour after 96 h incubation in peptone water, thus indicated their ability to produce ammonia. However, the intensity of colour varied from isolate to isolate (Table 2). On incubation, all the isolates released considerable amount of ammonia as compared to control (83.6 mg L^{-1}) with significantly high production by isolate *Pf*As1 (394.7 mg L^{-1}), followed by isolate *Pf*As3 (350.2 mg L^{-1}) and *Pf*As14 (348.1 mg L^{-1}). Least ammonia was produced by isolate *Pf*As18 (179.1 mg L^{-1}) [Table 3]. Noori and Saud (2012) isolated 20 Pseudomonad strains from paddy rhizosphere which were positive for the production of siderophores, HCN, ammonia and IAA. Similarly, Sakthivel and Karthikeyan

Table 1: Biochemical characteristics of different *Pseudomonas* isolates

Isolates	Biochemical characters							
	OT	GL	CT	SH	DN	CU	HS	DT
*Pf*As1	+ve	+ ve	+ve	-ve	+ve	+ ve	+ve	+ ve
*Pf*As3	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	+ ve	+ ve
*Pf*As14	+ve	+ ve	+ve	- ve	+ve	+ ve	+ve	+ ve
*Pf*As18	+ ve	- ve	+ ve	+ ve	+ ve	- ve	+ ve	+ ve
*Pf*As20	+ve	-ve	-ve	+ ve	+ve	- ve	+ve	+ ve
*Pf*As24	+ ve	+ ve	- ve	+ ve	- ve	- ve	+ ve	+ ve
*Pf*As32	+ve	- ve	+ve	- ve	-ve	+ ve	+ve	+ ve
*Pf*As38	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	+ ve	+ ve
*Pf*As46	+ve	+ ve	+ve	- ve	+ve	+ ve	+ve	+ ve
*Pf*As54	+ ve	+ ve	+ ve	- ve	+ ve	+ ve	+ ve	+ ve

–ve = negative reaction; SH = starch hydrolysis; GL = gelatin liquefication; CU = citrate utilization; DT = dextrose test; CT = catalase test; OT = oxidase test; HS = H_2S production; DN = denitrification test

Table 2: Plant growth promoting activities shown by different *Pseudomonas* isolates

Isolates	Ammonia production	HCN production	Siderophore production	Proteolytic activity	IAA production
*Pf*As1	+ve	+ ve	+ ve	+ ve	+ ve
*Pf*As3	+ ve	+ ve	+ ve	+ ve	+ ve
*Pf*As14	+ ve	+ ve	+ ve	+ ve	+ ve
*Pf*As18	+ ve	+ ve	- ve	+ ve	+ ve
*Pf*As20	+ ve	+ ve	+ ve	+ ve	+ ve
*Pf*As24	+ ve	- ve	- ve	- ve	+ ve
*Pf*As32	+ ve	+ ve	+ ve	+ ve	+ ve
*Pf*As38	+ ve	+ ve	+ ve	+ ve	+ ve
*Pf*As46	+ ve	+ ve	+ ve	+ ve	+ ve
*Pf*As54	+ ve	- ve	+ ve	- ve	+ ve

(2012) reported ammonia production by 92% isolates of *Pseudomonas*. Proteolysis leads to the breakdown of proteins into smaller polypeptides or amino acids through hydrolysis of peptide bonds by the enzyme protease. Bacterial proteases act as exotoxin against bacterial pathogens while exotoxic protease destroys extra-cellular structure of pathogenic bacteria. In present study, 8 isolates of *Pseudomonas viz*., *Pf*As1, *Pf*As3, *Pf*As14, *Pf*As18, *Pf*As20, *Pf*As32, *Pf*As38 and *Pf*As46 formed clear halo zones in SMA medium so indicated protease enzyme production (Table 3). Maximum halo zone diameter was observed in isolate *Pf*As1 (5.85 cm), followed by isolate *Pf*As3 (5.30 cm) and isolate *Pf*As14 (5.18 cm) while minimum halo zone was noticed in isolate *Pf*As20 (1.38 cm). Kumar *et al*. (2005) reported protease activity in all the 26 isolates collected from pear and apple rhizosphere.

All the tested isolates produced IAA with varied intensity of IAA production. The highest IAA production was found in isolate *Pf*As1 (48.58 mg L^{-1}), followed by isolate *Pf*As3 (39.32 mg L^{-1}) and *Pf*As14 (36.33 mg L^{-1}). Various *Pseudomonas* species stimulate phytohormone production like IAA, cytokinins, gibberellins, etc. which are known plant growth promoting hormones. The quantity of IAA production differed significantly amongst the isolates. The extent of IAA production by various isolates ranged from 16.34 to 48.58 mg L^{-1}. Kannapiran and Ramkumar (2011) reported IAA production of 26.5 and 19.8 mg L^{-1} by *P. aeruginosa* and *Bacillus* sp., respectively. Yasmin *et al*. (2009) observed IAA production by PGPR isolates in the range of 4.94 to 46.66 mg L^{-1}.

The HCN production by PGPR inhibitsthe growth of soil/root borne phytopathogens. The present study revealed that 8 isolates of *Pseudomonas* produced HCN with variable colour intensity. Isolates *Pf*As1, *Pf*As3, *Pf*As14, *Pf*As46, *Pf*As38 and *Pf*As18 depicted prominent colour change from yellow to orange/brown as compared to other isolates. The isolates *Pf*As32 and *Pf*AS20 were weak HCN producers, whereas isolates *Pf*As54 and *Pf*As24 did not produce HCN (Table 2). HCN produced by many rhizobacteria plays role in biological control of pathogens (Defago *et al*., 1990) as it inhibits electron transport so disrupts energy supply to the cell thereby leads to the death of pathogens. In present study, 8 isolates were HCN positive, which might have induced rice plant resistance. Rani*et al*. (2012) reported that only 5 out of 16 PGPR isolates as HCN producers. Kumar *et al*. (2012) found only 2 out of 5 isolates as HCN producers.

Siderophores are small, low molecular weight high affinity Fe-chelating organic compounds secreted by bacteria andfungi. Siderophores act as strong soluble Fe^{3+} binding agents. These Fe-chelating agents play vital role in nutrient immobilization and renders Fe unavailable to the pathogens. The microbes having siderophore producing ability are considered efficient biocontrol

Table 3: *In vitro* ammonia production, proteolytic activity, indole acetic acid (IAA) and siderophore production by *Pseudomonas* isolates

Isolates	Ammonia (mg ammonia produced L^{-1})	Proteolytic activity (halo zone dia. in cm)	IAA production (mg IAA produced L^{-1})	Siderophore activity (diameter of yellow/ purple halo in cm)
*Pf*AS 1	394.72	5.85 (13.94)	48.58 (44.14)	6.18 (14.30)*
*Pf*AS 3	350.22	5.30 (13.31)	39.32 (38.82)	4.83 (12.66)
*Pf*AS 14	348.12	5.18 (13.05)	36.33 (37.05)	4.35 (11.97)
*Pf*AS 18	179.05	1.88 (7.71)	15.66 (23.26)	0.00 (4.05)
*Pf*AS 20	299.69	1.38 (6.55)	19.88 (26.42)	2.10 (8.33)
*Pf*AS 24	238.11	0 .00 (4.05)	16.46 (23.89)	0.00 (4.05)
*Pf*AS 32	224.85	1.78 (7.49)	16.34 (23.81)	3.40 (10.63)
*Pf*AS 38	329.01	2.50 (9.10)	34.38 (35.85)	3.03 (9.98)
*Pf*AS 46	301.93	1.53 (7.04)	28.82 (32.46)	1.48 (6.80)
*Pf*AS 54	276.81	0.00 (4.05)	18.18 (25.18)	2.55 (9.10)
Control	83.64	0.00 (4.05)	0.00 (4.05)	0.00 (4.05)
SEd ±	0.268	0.205	0.574	0.258
$CD_{0.05}$	0.461	0.348	0.986	0.439

*Data in the parenthesis are angular transformed value
Data are mean of four replications

agents. *Pseudomonas* isolates *Pf*As1, *Pf*As3, *Pf*As14, *Pf*As20, *Pf*As32, *Pf*As38, *Pf*As46 & *Pf*As54 produced siderophore evidenced through the formation of yellow halo. The intensity of halo varied from isolate to isolate with isolate *Pf*As1 showing maximum halo (6.18 cm dia.) and isolate *Pf*As46 showing minimum halo (1.48 cm) [Table 3].

Molecular characterization of Pseudomonas isolates

The genomic DNA was subjected to agarose gel electrophoresis in 0.8% agarose gel with ethidium bromide. The gel was visualized under UV radiation using gel documentation system. The genomic DNA of *Pseudomonas* isolates were >10000 bp (Fig. 1). The isolated DNA was quantified and its purity estimated with the help of NanoDrop spectrophotometer technique. The absorbance of genomic DNA was measured at 260 nm to find out the concentration of DNA in solution. The absorbance at 280 nm was taken to check the extent of protein contamination in the extracted DNA. NanoDrop readings are tabulated in Table 4. The DNA concentration was found optimum for sequencing. The extracted DNA was subjected to PCR amplification using gene specific primers (forward US16F and reverse US16R). Agarose gel (1.2%) electrophoresis showed clean amplification of ~1500 bp fragments of 16S rRNA genes (Fig. 2). Sequencing results were obtained for 10 PCR products of 16S rRNA of different of *Pseudomonas* isolates. The sequences were analyzed for homology using BLAST (NCBI) to obtain possible identities. From BLAST search results, isolates were determined based on highest homology (Table 5). The analysis using partial 16S rRNA gene sequences and

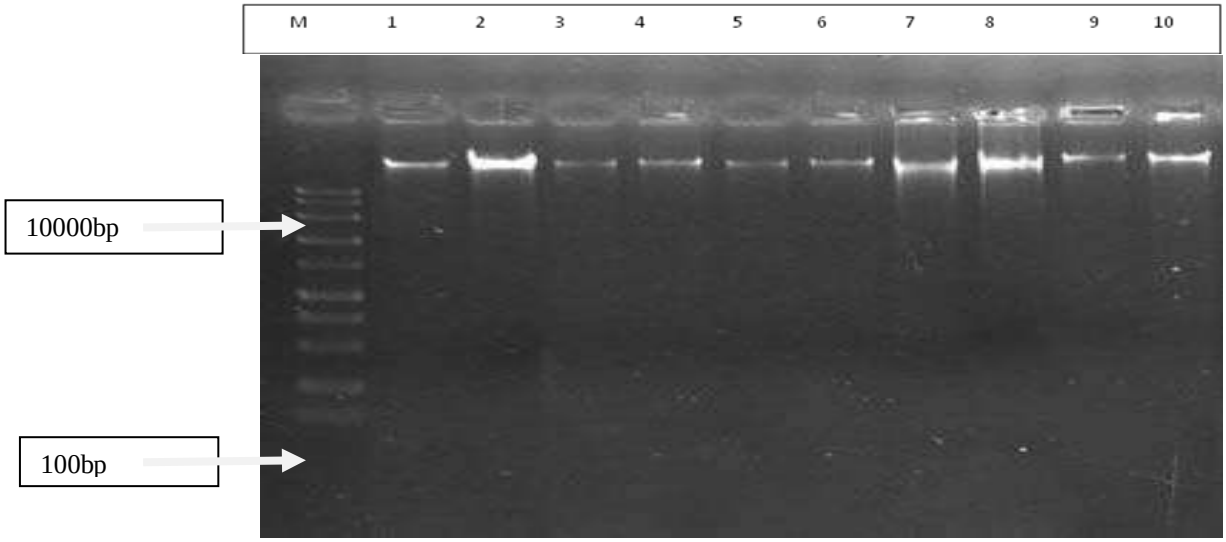

Fig. 1: Qualitative analysis of genomic DNA of *Pseudomonas* isolates. Lane M: 1 Kb ladder (Himedia), Lane 1-10: genomics DNA of the *Pf*-isolates; 1: As1, 2: As 3, 3: As14, 4: As18, 5: As 20, 6: As24, 7: As 32, 8: As 38, 9: As 46 and 10: As54

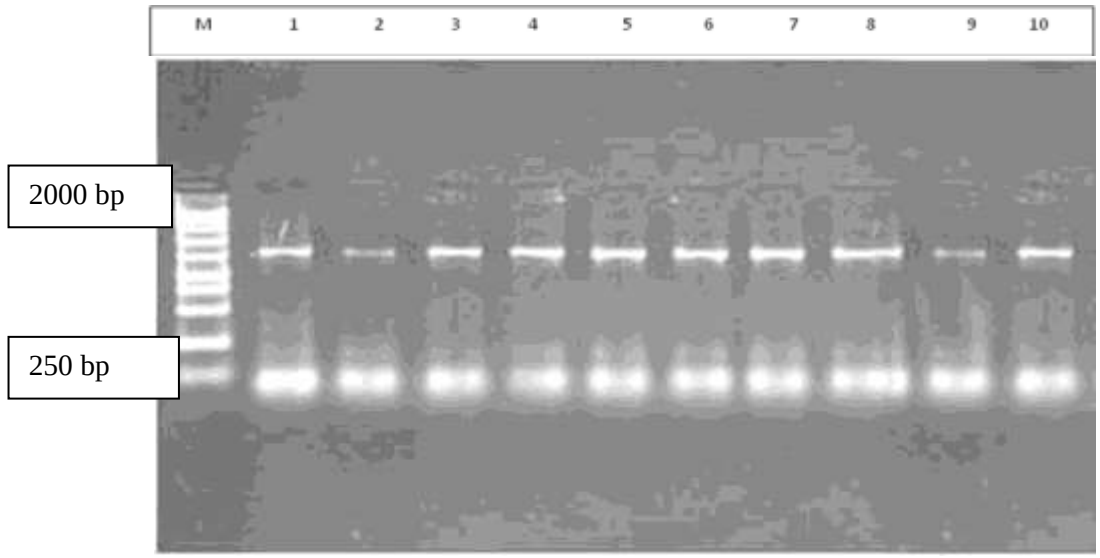

Fig 2: 16S rRNA amplified product of different isolates of *Pseudomonas* species; Lane M: 1 kb ladder (Farmentous), Lane 1-10: genomic DNA of *Pf*-isolates; 1: As 1, 2: As 3, 3: As 14, 4: As 18, 5: As 20, 6: As 24, 7: As 32, 8: As 38,9: As 46 and 10: As 54

Table 4: Concentration of genomic DNA extracted from different of *Pseudomonas* isolates at 260 nm

Isolates of *Pseudomonas*	Ng μL^{-1}	260/280 ratio
*Pf*As 1	2060.01	1.94
*Pf*As 3	2437.82	1.90
*Pf*As 14	3230.18	1.85
*Pf*As 18	4503.47	1.03
*Pf*AS 20	204.33	1.20
*Pf*As 24	4403.73	1.09
*Pf*As 32	4075.31	1.83
*Pf*As 38	1045.29	1.81
*Pf*As 46	453.13	1.72
*Pf*As 54	4441.32	1.25

molecular, cultural & biochemical studies revealed the isolated cultures to be *Pseudomonas fluorescens* and *P. putida*. 16S ribosomal RNA (16S rRNA) sequencing is widely and most popularly used in microbiological studies to identify the diversities in prokaryotic organisms. Woese and Fox (1977) proposed the use of ribosomal RNA genes for molecular taxonomic research. Manjunatha and Naik (2013) amplified a 1316 bp fragment of 16S rDNA gene of isolates from chilli and brinjal rhizosphere using primers fD1 and rP2. The strains RPF-13 and RPF-81 were identified as *P. putida* and *P. fluorescens*, respectively, on the basis of 16S rDNA sequence homology.

Phylogenetic analysis by unweighted pair group method with arithmetic mean (UPGMA) method produced similar tree topologies which were well supported by the bootstrap values within their nodes. The 16S rRNA gene sequences of the isolated 10 *P. fluorescens* formed 2 clusters. Moreover, the sub-clusters within the clusters were well supported by their strong bootstrap values within their nodes. The phylogenetic analysis using 16S rRNA gene sequences showed phylogenetic position of different fluorescent *Pseudomonads*.

Table 5: Homology of different*Pseudomonas* isolates by BLAST analysis

Isolates	Identification	Similarity (%)	Accession No.
*Pf*As 1	*Pseudomonas fluorescens* SBW25	100	AM181176.4
*Pf*As 3	*Pseudomonas fluorescens*, F113	100	CP003150.1
*Pf*As 14	*Pseudomonas* sp. lip23	99	EU439250.1
*Pf*As 18	*Pseudomonas putida* strain311807	96	KF956613.1
*Pf*As 20	*Pseudomonas* sp. SL35 (*Pseudomonas putida*)	97	HQ264095.1
*Pf*As 24	*Pseudomonas* sp. EU102 (*Pseudomonas putida*)	95	JF681295.1
*Pf*As 32	*Pseudomonas* sp. lip35	98	EU439251.1
*Pf*As 38	*Pseudomonas* sp. GN66 (*Pseudomonas putida*)	100	KJ719381.1
*Pf*As 46	*Pseudomonas* sp. PCWCW2 (*Pseudomonas putida*)	97	GQ284471.1
*Pf*As 54	*Pseudomonas putida* strain ZA22	98	KJ728678.1

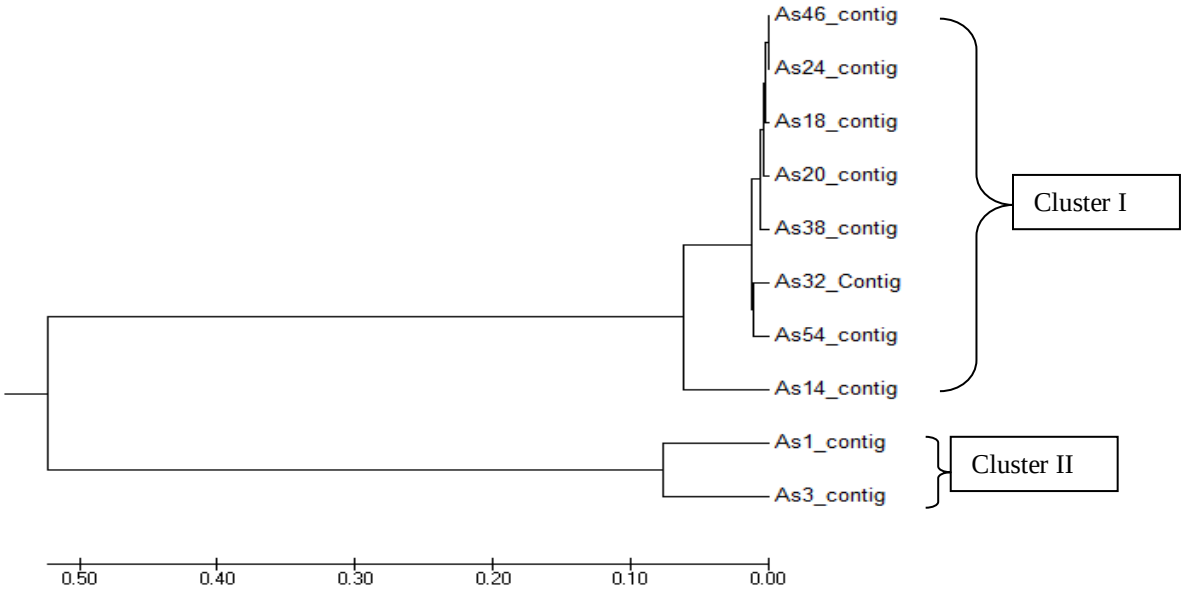

Fig. 3: Cluster analysis showing phylogenetic position of different isolates of *Pseudomonas* isolates

The results revealed that the isolated strains were *P. fluorescens* and *P. putida* (Fig 3). The cluster analysis showed that the isolate *Pf*As1 and *Pf*As3 belong to the same cluster and isolate *Pf*As14 in other cluster but distinctly different from other isolates. It was also found that the isolates *Pf*As18, *Pf*As20, *Pf*As24, *Pf*As32, *Pf*As38, *Pf*As46 and *Pf*As54 were closely related and distinctly different from isolates *Pf*As1, *Pf*As3 and *Pf*As14. The biochemical characterization, plant growth promoting activity and cluster analysis revealed that the isolates *Pf*As1 and *Pf*As3 were different from other isolates. The present investigation indicated that *Psudomonas fluorescens* and *P. putida* isolates are able to induce the production of IAA, ammonia, siderophores activities and thereby have the ability to improve plant growth. The isolate *Pf*As1 was most efficient isolate. Molecular characterization is inevitable considering the present scenario of *P. fluorescens* diversity.

REFERENCES

Bakker, P.A.H.M., Raajmakers, J.M. and Schippers, B. 1993. Role of iron in the suppression of bacterial plant pathogens by fluorescent pseudomonads. pp. 269-282. **In**: *Iron Chelation in Plants and Soil Microorganisms*. (eds. L.L. Barton and B.C. Hemming). Academic Press, San Diego, Calif, USA.

Bric, J.M., Bostock, R.M. and Silverstone, S.E. 1991. Rapid *in situ* assay for indoleacetic-acid production by bacteria immobilized on a nitrocellulose membrane. *Applied and Environmental Microbiology*, **57**: 535-538.

Cardinal, M.J., Meghrous, J., Lacroix, C. and Simard, R.E. 1997. Isolation of *Lactococcus lactis* strain producing inhibitory activity against Listeria. *Food Biotechnology*, **11**: 129-146.

Defago, G., Berling, C.H., Borger, U., Keel, C. and Voisard, C. 1990. Suppression of black rot of tobacco by a *Pseudomonas* strain: Potential applications and mechanisms. pp. 93-108. **In**: *Biological Control of Soil Borne Plant Pathogen* (eds. D. Hornby, R.J. Cook and Y. Henis). CAB International, Surrey, UK.

Gaur, R., Shani, N., Johri, B.N., Rossi, P. and Aragno, M. 2004. Diacetyl-phloroglucinol-producing pseudomonads do not influence AM fungi in wheat rhizosphere. *Current Science,* **86**: 453-457.

Hayat, R., Ali, S., Amara, U., Khalid, R. and Ahmed, I. 2010. Soil beneficial bacteria and their role in plant growth promotion: A review. *Annual Review on Microbiology,* **60**: 579-598.

Jeyalakshmi, C., Madhiazhagan, K. and Rettinassababady, C. 2010. Effect of different methods of application of *Pseudomonas fluorescens* against bacterial leaf blight under direct sown rice. *Journal of Biopesticide,* **3**: 487-488.

Kannapiran, E. and Ramkumar, V. 2011. Inoculation effect of nitrogen-fixing and phosphate-solubilizing bacteria to promote growth of black gram (*Phaseolus mungo* Roxb; Eng). *Annual Review of Biological Research,* **2**: 615-621.

Kumar, A., Kumar, A., Devi, S., Patil, S., Payal, C. and Negi, S. 2012. Isolation, screening and characterization of bacteria from rhizospheric soils for different plant growth promotion (PGP) activities: An *in vitro* study. *Recent Research in Science and Technology,* **4**: 1-5.

Kumar, R.S., Ayyadurai, N., Pandiaraja1, P., Reddy, A.V., Venkateswarlu, Y., Prakash, O. and Sakthivel, N.N. 2005. Characterization of antifungal metabolite produced by a new strain *Pseudomonas aeruginosa* PUPa3 that exhibits broad-spectrum antifungal activity and biofertilizing traits. *Journal of Applied Microbiology,* **98**: 145-154.

Lukkani, N.J. and Reddy, E.C.S. 2014. Evaluation of plant growth promoting attributes and biocontrol potential of native fluorescent *Pseudomonas* sp. against *Aspergillus niger* causing collar rot of ground nut. *International Journal of Plant, Animal and Environmental Sciences,* **4**: 256-262.

Manjunatha, H. and Naik, M.K. 2013. Biological and molecular characterization of fluorescent pseudomonas isolates from crop rhizosphere soil. *Indian Journal of Scientific Research and Technology,* **1**: 18-22.

Meera, T. and Balabaskar, P. 2012. Isolation and characterization of *Pseudomonas fluorescens* from rice fields. *International Journal of Food, Agriculture and Veterinary Science,* **2**: 113-120.

Nagarajkumar, M., Bhaskaran, R. and Velazhahan, R. 2004. Involvement of secondary metabolites and extracellular lytic enzymes produced by *Pseudomonas fluoresces* in inhibition of *Rhizoctonia solani*, the rice sheath blight pathogen. *Microbiological Research,* **159**: 73-81.

Nelson, L.M. 2004. Plant growth promoting rhizobacteria (PGPR): Prospects for new inoculants. *Plant Management Network* [online doi: 10.1094/CM-2004-0301-05-RV].

Noori, M.S.S. and Saud, H.M. 2012. Potential plant growth-promoting activity of *Pseudomonas* sp. isolated from paddy soil in Malaysia as biocontrol agent. *Journal of Plant Pathology and Microbiology,* **3**: 221-226.

Rani, M.U., Arundhathi and Reddy, G. 2012. Screening of rhizobacteria containing plant growth promoting (PGPR) traits in rhizosphere soils and their role in enhancing growth of pigeon pea. *African Journal of Biotechnology,* **11**: 8085-8091.

Sakthivel, U. and Karthikeyan, B. 2012. Isolation and characterization of plant growth promoting rhizobacteria (PGPR) from the rhizosphere of *Coleus forskohlii* grown soil. *International Journal of Recent Scientific Research,* **3**: 288-296.

Saxena, L. 2003. Characterization of plant growth promoting rhizobacteria. **In**: *Biofertilizer Technology* (ed. A.K. Saxena). New Delhi, India.

Schwyn, B. and Neilands, J.B. 1987. Universal assay for the detection and determination of siderophores. *Analytical Biochemistry,* **160**: 47-56.

Sharma, P. and Bora, L.C. 2016. *In vitro* evaluation of native fluorescent *Pseudomonas* against virulent strain of *Xanthomoas oryzae* pv. *oryzae,* A causal agent of bacterial blight of rice. *Biopesticide International,* **12:**157-164.

Vincent, J.M. 1970. *A Manual for Practical Study of Root Nodule Bacteria*. IBP Handbook No. 15, Oxford Blackwell Sci. Publ., Oxford, UK.

White, L.J., Brozel, V.S. and Subramanian, S. 2015. Isolation of rhizosphere bacterial communities from soil. *Bio-protocol,* **5**(16): 1569.

Woese, C.R. and Fox, G.E. 1977. Phylogenetic structure of the prokaryotic domain: The primary kingdoms. *Proceedings of the National Academy of Sciences (USA),* **74**: 5088-5090.

Wohler, I. 1997. Auxins-indole derivatives in soils determined by a colorimetric method and by high performance liquid chromatography. *Microbiological Research,* **152**: 399-405.

Yasmin, F., Othman, R., Sijam, K. and Saad, M.S. 2009. Characterization of beneficial properties of plant growth-promoting rhizobacteria isolated from sweet potato rhizosphere. *African Journal of Microbiology Research,* **3**: 815-821.

Zhang, L., Tosa, Y., Nakayashiki, H. and Mayama, S. 2000. Antibiotic 2, 4-diacetylphloroglucinol played an important role in biocontrol ability of *Pseudomonas fluorescens* FPT9601. *Annual Report on Interdisciplinary Research, Institute of Environmental Sciences,* **19:** 151-163.