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Biodiversity Analysis of *Pseudomonas. sp* using 16S r-DNA Genomic Technique

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Abstract

Soil microbial diversity is an important index of agricultural productivity by enhancing soil quality. Present study employed the combination of several methodologies to examine genetic relationships among specific groups of bacteria and to characterize strains at the species level using molecular biotyping techniques. The present study is based upon 16SrDNA analysis for characterisation of bacterial species isolated from Raisen, Bhopal, districts of Madhya Pradesh. On the basis of different biochemical test, 75 strains of *P. aeroginosa, P.putida, P.flourosence a*nd *P.streuzi* , strains were confirmed. Genetic diversity analysis of 75 *Pseudomonas* isolates was done by 16SrDNA-RFLP PCR, and REP PCR Restriction enzyme Alu I, Taq I, Mob I, Hae III, Hind III, Msp I were used for digestion of the amplified 16SrDNA of 75 *Pseudomonas* isolates.16SrDNA -RFLP PCR, REP PCR of S1 to S50 *Pseudomonas* isolates was done . Heterogeneity and variation with in species S27 and S38 shows 67% similarity strains S26 shows 77% dissimilarity with rest of the strain. The dendrogram was prepared by using UPGMA method of NTSYS software .The dendrogram shows grouping at 32% similarity and 42% similarity. Strain P3, P7, P9, P12, P15, P20 and P22 showed negligible amount of phosphate solubilization in test media as compared to rest of *Pseudomonas sp.* All the strains of *P.fluorescens* and *P. aeruginosa* showed production of ammonia. Fluorescent *Pseudomonad* strains reported showed phosphate solubilization potential and combination of *Bradyrhizobium* species as biofertilizer will serve as potential inoculants in promotion of agronomic applications of biological components specially microbes.

Keywords: *Pseudomonas sp.*, Phosphate solubilization, 16SrDNA & REP-PCR

INTRODUCTION

Soil microorganisms play an important role in soil processes that determine plant productivity. For successful functioning of introduced microbial bioinoculants and their influence on soil health, exhaustive efforts have been made to explore soil microbial diversity of indigenous community, their distribution and behaviour in soil habitats **[Hill, *et. al.*, 2000].** Direct plant-growth-promoting rhizobacteria enhance plant growth in the absence of pathogens. Different micro-organisms have been recognized to exude different plant growth promoters **[Patten, *et. al.*, 1996; Arshad and Frankenberger, 1998].** Among the plant growth microorganisms which produce herbal hormones are *Azotobacter*, *Pseudomonas*, *Azosprillium*, *Rhizobium*, *Bacillus*, *Enthrobacter* and Mycorrhiza fungus **[Fett, 1987; Hirsch, 1997].** Microorganism isolated from rhizosphere and rhizoplane of various crops have more potential of producing Auxin than those from root free soil **[Arshad and Frrankenberger, 1998]**.The species of bacteria capable of producing IAA includes *Pseudomonas* sp, *Bacillus* sp, *Klebsiella* sp, *Azosprillum* sp, *Enterobacter a*nd *Serrita* sp **[Frankenberger and Arshad, 1995].** Inoculation of canola seeds with *Pseudomonas putida* GR12-2, which produce low levels of IAA, results in 2 to 3 fold increase in the length of seedling roots .

Phosphorus is the second most important plant nutrient after nitrogen **[Donahue, *et. al.*, 1990]**. Indian soil on an average contains 0.05% phosphorus that constitutes 0.2% of plant dry weight. It is essential part of the cellular activities of living organism**[Khan et.al 2013, and Dhankhar et.al 2013]**.

Conversion of inorganic unavailable phosphate into available form viz., $H_2PO_4^-$and HPO_4^- for the plant uptake, phenomena referred as mineral phosphate solubilization (MPS) the form in which inorganic phosphorus exits also change according to soil pH. Below pH 6.0 inorganic phosphorus is present as monovalent H_2PO_4 species .The plant uptake is also high at the pH range 5.0-6.0 which indicate that phosphorus is primilary taken up as monovalent ions.For many species of *Pseudomonas*, the non phosphorylating oxidation pathway is a primary pathway for aldose sugar utilization **[Lessie and Phibbs, 1984,Vyas, *et. al.*, 2009]**.

Siderophores have been identified in large number of microorganisms, and in few cases, siderophore biosynthesis is been shown to be inhibited by elevated temperatures **[Worsham and Konisky, 1984]**. Some microbes have transport systems that enable them to use other siderophore types in addition to their own. Almost all microorganisms have transport systems for siderophores produced by other bacteria and fungi which may allow them to compete better for Fe in their natural habitat.

Analysis of 16S rRNA genes is now widely used for analysis of bacterial populations, and analysis of 18S rRNA genes and internal transcribed spacer (ITS) regions is increasingly being used to analyse fungal populations. Ribosomal rRNA genes are ideal for this purpose in that they possess regions with sequences conserved between all bacteria or fungi, facilitating alignment of sequences when making comparisons, while other regions exhibit different degrees of variation, enabling distinction

between different groups **[Nobandegani et.al 2015].**

In REP-PCR primers that anneals to these repetitive elements are used. The PCR-products are separated using agarose gel electrophoresis and a species (sometimes strain)-specific pattern is obtained. Applications of the REP-PCR DNA fingerprinting technique to study microbial diversity, ecology and evolution.

The distance methods or distance matrix methods, evolutionary distance is computed for all pairs of taxa a phylogenetic tree is constructed by considering the relationship ship among these distance value. There are many method of constructing trees from distance data one is UPGMA.

Soil microbial diversity is an important index of agricultural productivity by enhancing soil quality. Present study has extensive use in estimating phenotypic and genotypic diversity of phosphate solubilizing rhizobacteria of agricultural importance. Present study employed the combination of several methodologies to examine genetic relationships among specific groups of bacteria and to characterize strains at the species level using molecular biotyping techniques. The 16S or small subunit ribosomal RNA gene is useful for estimating evolutionary relationships among bacteria because it is slowly evolving and the gene product is both universally essential and functionally conserved. The present study is based upon 16SrDNA analysis for characterization of bacterial species isolated from Raisen, Bhopal, districts of Madhya Pradesh

MATERIAL AND METHOD

Isolation of fluorescent *Pseudomonas* 1 g of soil sample and root with just a layer of closely adhering rhizospheric soil were then transferred to 10 ml of autoclaved distilled water, vigorously shaken for 10-15 minutes. After sedimentation of solid particles the suspension from all samples were serially diluted up to 10^{-6} with three replicate for each samples. From 10^{-3} to l0 $^{-6}$ dilutions, 100 µl of each dilution was spread on King's B medium plates **[King, *et. al*., 1954**]@ incubated at 30±2°C for 48 h plates. Plates were screened under UV light for fluorescent colonies. The isolates were varying from fluorescent green, deep fluorescent green and fluorescent yellowish green..The isolated bacteria were subjected to microscopic examination using Gram Staining [Vincent, 1970].

Study of Plant Growth Promoting Properties

(a) Phosphorus Solubilization, (i) Plate assay : Solubilization Index was measured using following formula [Edi-Premono, *et. al.,* 1996].

$$SI = \frac{\textit{Colony diameter + Halo zone diameter}}{\textit{Colony diameter}}$$

Pikovskaya's agar medium was used for Phosphate solubilization **[Pikovskaya's, 1948]** after 6 days of incubation at 30 ±2ºC. Quantitative estimation of P-liberated from inorganic phosphates was done by Ascorbate method **[Ames, 1964]**

Siderophore production

Production of siderophore was estimated qualitatively on chrome Azurol S (CAS) agar medium**[Schwyn and Neilands, 1987]** .The active culture of *Pseudomonas* isolates were spotted on the dark blue agar plate and further incubated for 24-48 hours at 32±1^{0}C. Appearence of dark orange/ yellow zone against the dark blue agar plates indicate the siderophore production **[Seong, 1996]**. The siderophore production was estimated as described by **[Schwyn and Neilands, 1987]**. All positive cultures were compared for Siderophores production ability as described by **[Payne, 1994]** where percent decolorization was calculated by using the following formula.

$$Per\ cent \quad decoloriza\ tion = \frac{Ar - As}{Ar} \quad X100$$

Where; Ar = Absorbance of reference, As = Absorbance of sample at 630 nm.

DNA Isolation

Isolation of DNA was done with slight modification for Marmur's method **[*1961; 1962*]**. Cell pellet was obtained by centrifugation at 10,000 rpm (Remi India). The uppermost layer bearing nucleic acid was pipette out and two volumes of chilled ethanol were added **[Helms *et. al.*, 1985]**. Isolated DNA was preserved in tris-EDTA buffer for further study.

The DNA product was simply analyzed using agarose gel electrophoresis technique **[Walker,1998]**. 2% Percent agarose dissolved in 1X TAE buffer is used as per required in various amplified product of 16SrDNA and REP. The concentration of 50 X TAE buffer is as follows;Tris (free base),242g,Glacial acetic acid 57.1ml,Na_2 EDTA 18.61g,D/W H_2O 1000ml,pH8.3, 1kb DNA ladder (Bangalore Genie, INDIA) was Stored at -20°C in deep freezer.The DNA obtained was estimated at A_{260} for a pure preparation of DNA. Protein content was deduced at A_{280}. A ratio A_{260}/A_{280} up to 1.9 is considered as pure DNA sample **[Henery, 1997,Gorden, 1998].**

16S r DNA- PCR of the isolated DNA:

The 16S r DNA region was amplified using universal forward and reverse primer of 16S r DNA(Reverse primer - (5' AGAGTTTGATCCTGGCTCAG-3') and Forward primer - (5'-AAGGAGGTGATCCAGCC GCA-3'). The PCR reaction mix for a 100µl reaction mixture contained- PCR buffer (10x), 10.00µl; dNTP mix (10mM), 1.0µl; MgCl2 (50mM), 6.0µl; Primer PA (100ng/µl), 1.0µl; Primer PH (100ng/µl), 1.0µl; Taq DNA Polymerase 1.5, 0.5µl; Template DNA (50ng), 10.0µl; and MilliQ

water, 70.5µl. The PCR amplification cycle including four stages- Initial Denaturation, 94 ^{0}C, 5min; Denaturation, 94^{0}C (30cycle), 30 sec; Annealing,50^{0}C (30cycle), 40sec; Extension, 72^{0}C (30cycle), 90sec, and Final extension, 72^{0}C (30cycle), 7 min was followed (Mini Cycler, TM MJ Research PTC-100). The amplified product was run on a 0.8% Agarose gel.

REP –PCR fingerprinting

The 18 nucleotides in the REP primer make it specific primer for genomic DNA analysis **[Louws, *et. al.*, 1998]**. Both reverse primer: REP 1R (5'-IIIICgICgICATCIggC-3') **[Versalovic, *et. al.*, 1991]**and forward primer REP 2I (5'-ICgICTTATCIggCCTAC-3') amplifies the complementary sequence found in the genomic DNA varying in size were later analyzed by gel electrophoresis. The amplification reaction includes Initial Denaturation, 95 ^{0}C, 7min; Denaturation, 94^{0}C (35cycle), 01 min; Annealing,40^{0}C (30cycle) 01 min, ; Extension, 72^{0}C (30cycle), 01 min , and Final extension, 72^{0}C (30cycle), 16 min was followed with final hold at 4^{0}C.

Agarose was melted at 100^{0}C in 1X TAE buffer and gel was casted in 29-welled casting tray. Samples along with 1kb ladder were loaded with 2µl of 6X loading buffer and it was run at 70mV for 1 hr.Bands were visualized using ultraviolet light after Ethidium Bromide staining and image is captured by a video camera as TIFF files for further assay.

Genetic Diversity and Phylogenetic Analysis.

The bands originated in a gel as a result of DNA samples was tabulated in the form of matrix (0-1) where 0 indicates absence of band and 1 presence of bands which was then analyzed NTSYS (software clustering of the data which is based on Unweighted Pair Group Method with Arithmetic Averaging (UPGMA). Similarity was calculating using Jaccard Coefficient. Construction of phylogenetic tree was based on the data produced from the clustering and similarity out put constructed with the help of 0-1 *of the agarose gel with separated DNA bands* **[Rohlf, 1990]** *obtain from various bacterial cultures*

RESULTS AND DISCUSSION:

The soil samples collected from various agricultural field of M.P. were analyzed and Pseudomonas strains were isolated (Table. 1) Cells were grown on Pikovaskya's agar medium and estimation of phosphate liberated in the form of inorganic phosphate was measured by known amount of phosphorus. The entire cultures showed clearing zone (Plate. 1) on incubation at 30^0 C.

The variation in siderophore production between different strains isolated ranged between 25% to the highest value of 85% by strain *P.fluorescens P-15* and *P.*

aeruginosa P-23 (Table 1). It has already being reported that production of siderophore varies amongst various strains of *P. aeruginosa* and *P.fluorescens* due to different level of iron requirement by bacterial species **[Dhanya and Potty, 2007; Somprong, *et. al*., 2010]**.

Conversion of the insoluble forms of phosphorous to a form accessible by plants, like orthophosphate, is an important trait of Phosphate solubilizing rhizobacteria (PSRB) in increasing growth and yield of crop plant. Microorganisms are important components of soil and directly or indirectly influence the soil's health through their beneficial or detrimental activities. Rhizosphere microorganisms mediate soil processes such as decomposition, nutrient mobilization and mineralization, nitrogen fixation and denitrification **[Kang, *et. al*., 2002; Pradhan and Shukla, 2005]**. Furthermore, solubilization of phosphorus in rhizosphere is the most common mode of action implicated in plant growth promoting rhizobacteria (PGPR) that increase the nutrient availability to the host plant**[Richardson, 2001]**

In the present investigation, all the *Pseudomonas* sp. isolated is screened as phosphate solubilizer. Significant decline in the pH of the culture media in all the strains was observed during mineral phosphate solubilization which suggests production of organic acid. There is evidence of direct relationship between phosphate solublization and acidity of medium. The estimation of phosphate solubilization in isolated strain of *P.fluorescens* ranged between 30 -139

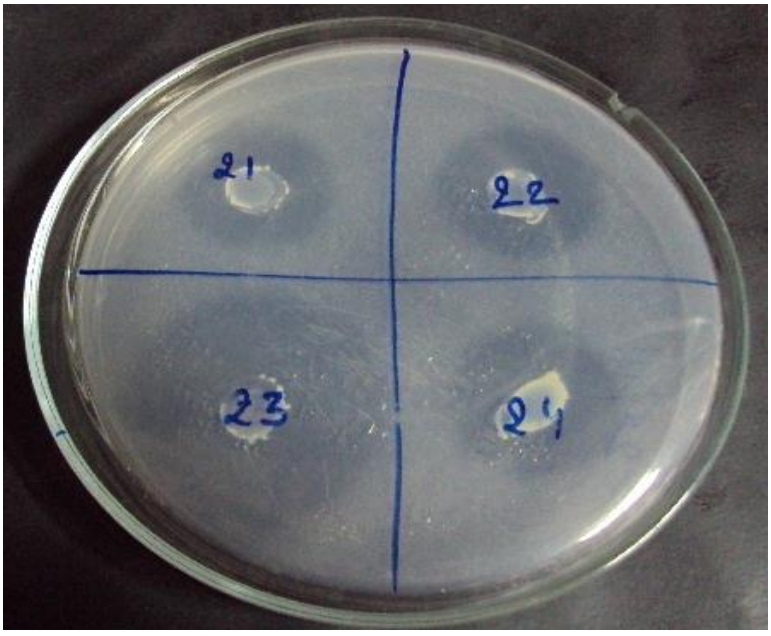

Plate 1: Showing phosphorus solubilization by *Pseudomonas*

Table 1: In *vitro* quantitative phosphorus solubilisation and Siderophore production by *P.fluorescens* and *P. aeruginosa.*

S. No	Strain No	SI	pH	Amount of Phosphorus Solubilised (µg/ml)	Siderophore (%)
1	Control	0.0	7.0	0.0	0.0
2	P-1	2.8	4.6	110.0	83
3	P-2	2.1	6.2	93.0	59
4	P-3	-	-	-	42
5	P-4	2.5	4.4	115.0	60
6	P-5	2.7	4.2	124.0	69
7	P-6	1.7	5.5	62.0	72
8	P-7	-	-	-	33
9	P-8	2.5	5.5	83.0	29
10	P-9	-	-	-	62
11	P-10	1.8	5.4	55.0	57
12	P-11	2.6	6.0	30.0	78
13	P-12	-	-	-	80
14	P-13	3.2	4.1	139.0	39
15	P-14	2.4	5.6	107.0	68
16	P-15	-	-	-	25
17	*P16	2.8	4.1	127.0	46
18	P17	1.8	5.4	59.0	78
19	P18	2.4	4.5	125.0	50
20	P19	3.1	3.9	146.0	57
21	P20	-	-	-	86
22	P21	3.7	3.2	160.0	45
23	P22	-	-	-	74
24	P23	2.9	4.2	133.0	85
25	P24	2.2	4.7	96.0	69
26	P25	2.3	4.6	98.0	43

P-1 to P-15 *P.fluorescens* * P-16 to P-25 *P.aeruginosa* , SI – Solubilization index, P-3; P -7; P 9; P -12; P -14 to P-15 *P.fluorescens* and P-21 and P-22 *P.aeruginosa* were not effective phosphate solubilizer *in vitro*

µg/ml whereas, in case of *P. aeruginosa*, the value stands between 59-160 µg/ml phosphorus released. Strain P-21 of *P. aeruginosa* was found to be effective

phosphate solubilizer in contrast to *P.fluorescens* strain P-13 which resulted in 139 µg/ml of phosphorus release when incubated in Pikovskay's medium. The corresponding value of solubilization index also ranged between 3.2-1.7 in case of *P.fluorescens* in contrast to *P. aeruginosa* where SI ranged between 1.8- 3.7. The correlation coefficient SI and pH value of media showed negative correlation (-0.739; $p \leq 0.0003$). The correlation value of SI and phosphate solubilization as well as pH and phosphate solubilization showed positive and negative correlation, respectively (Table.1). Present study shows capability of *P. aeruginosa* P-21 and P-19 as efficient phosphate solubililizer and could be recommended as bioinoculant. *P.fluorescens* strains P-5, P-13 and P-4 showed enhanced efficiency under similar experimental conditions.

On the basis of different biochemical reaction, 75 strains of *P. aeroginosa, P.putida, P.flourosence a*nd *P.streuzi*, and 20 *Azatobacter* strains were confirmed. DNA isolation of, 75 *Pseudomonas* isolates was done by modified Marmur method **[Marmur.,1961;1962]** and estimation of DNA was done by obtaining the ratio of absorbance at 260 nm/280nm. Genetic diversity analysis of 75 *Pseudomonas* isolates was done by 16SrDNA-RFLP PCR, and REP PCR.Restriction enzyme, Msp I was used for digestion of the amplified 16SrDNAof 75 *Pseudomonas* isolates. 50 *Pseudomonas* sp represented as P1 to P25 were used for microbial diversity study after biochemical characterization.

16SrDNA -RFLP PCR, REP PCR of P1 to P25 *Pseudomonas* isolates was done. DNA content of all microbial isolates from various districts of Madhya Pradesh was examined using standard methods of Murmur's **[1961:1962]**. DNA isolated from P-1 to P-15 strains of *P.fluorescens*, P-16 to P-25 isolates of *P.aeruginosa* species was further analyzed on 0.8% agarose gel to observe the purity of DNA (Fig.1). The results of these observations were further confirmed by performing PCR reactions to established diversity of microbes. The PCR results generates artifacts rather than detecting true genetic variations if low stringent PCR conditions are employed, as far as variety of PCR conditions viz., rep PCR, ERIC PCR, BOX PCR RAPD and RFLP etc.

The purified DNA segments from *P.fluorescens, P. aeruginosa* species were used to study phylogenetic relationship using described primer for amplification of most eubacterial 16S rRNA. The hybridization is measured by performing amplification that works virtually with all bacteria. The primers used for e.g **5'AGAGTTTGATCCTGGCTCAG-3'** and **5'-AAGGAGGTGATCCAGCC GCA-3'** is capable of amplifying wide variety of bacterial taxa Replacing **5'AGAGTTTGATCCTGGCTCAG-3'** primer with **5'-AAGGAGGTGATCCAGCC GCA-3'** primer extends the diversity of species even further, but from the perspective of amplifying the maximum number of nucleotides specific primer are used for 16S rDNA, .

The cells isolated were taken and DNA was isolated from different strains and subjected to polymorphism analysis using restriction enzyme Msp I for all fluorescent *Pseudomonas* strains and amplification was carried out under standard conditions.

Dissimilarity is calculated by subtracting similarity data from 1. The construction of similarity as well as dissimilarity of species thus isolated is based on upon the standard use of software by UPGMA (Unweighted Pair Group Method with Arithmetic Mean) using phylogenetic interface package or NTYSYS software. The evolutionary history of thus resultant strains of *P.fluorescens* and *P.aeruginosa* is present in Fig 2. The observation pattern and representation of dendrogram Fig 2 as reproduced using electrophoresis picture of Fig 1 with 16S rDNA shows grouping at 32% similarity and 42% similarity. Similar *P.fluorescens* P- 2, P-6, P-4, P-5 and *P.aeruginosa* P- 17, P-18,P-19 ,P-21 ,P-25, P-24 and P-22 shows similar grouping when dendrogram is prepared using Jaccard's similarity coefficient. The evolutionary distance between bacterial strains distinguishes the rate of base substitution during evolutionary history of organism.

The availability of sensitive and accurate PCR-based genotyping methods of differentiate among closely related bacterial strains and the detection of high rhizobial diversity has been greatly considered **[Vinuesa *et al.*, 1998; Doignon-Bourcier *et al.*, 2000; Tan *et al.*, 2001]**. The *Rhizobium* strains were studied by using randomly amplified polymorphic DNA (RAPD) fingerprinting, as the technique is frequently used for exploring genetic polymorphisms **[Versalovic *et al.*, 1994; Teaumroong & Boonkerd, 1998]**. In order to fully exploit RAPD results for studies of the genetic structures of populations, it is necessary to utilize a method of data analysis that permits identification of variations within.

Widely accepted parameter of polymerase chain reaction is now days preferred to distinguish different biotypes existing in nature. Technically, repetitive element based PCR (REP-PCR) has shown considerable potential as a DNA typing tool in the laboratory. REP-PCR asses to utilize primer targeting highly conserved repetitive sequence element in bacterial genome. Two such group of repetitive element are enterobacterial repetitive intergenic consensus (ERIC) sequences common to gram negative enteric bacteria, and the BOX elements, originally detected in *Streptococcus pneumonia.*

REP-PCR was carried to see the amplification pattern in case of all 25 strain of *P.aeruginosa* and *P.fluorescens (*Fig 3) in the presence of REP 1R (5'-IIIICgICgICATCIggC-3') and REP 2I (5'-ICgICTTATCIggCCTAC-3'). The size of DNA fragment using REP-PCR primer range between 200 to 800 bp in case of fluorescent *Pseudomonas*. The REP PCR analysis is a simplified amplification process as compared to BOX and ERIC PCR; the benefits of REP PCR method have now been widely recognized in the research of bacterial diversity of clinical isolates as well as strains of industrial, agricultural and environmental importance. REP PCR has previously been applied in most of the disciplines for the study of variation in genomic pattern as reflected by mathematical analysis using NTSYS software. The biotyping analysis of *Pseudomonas* on calculating the dissimilarity index using software reflect divergence of first group at 25% similarity another at 70% dissimilarity and third group *35%* similarity *P.fluorescens* P-3, P-4 and P-23, *P.aeruginosa P-24 s*howed similar groups with *P.fluorescens P-5, P-6* falls similar at dissimilarity index of 48% and P-10 P-12 and P-13 P-11 had highest similarity value

of 83% (Fig 4).

The field of biological control as well as biofertilizer attracts attention of phosphate solubilizing flourescent *Pseudomonas* bacteria in the present scenario. Present study revealed the diversity of *Pseudomonas* sp. on genetic basis and also makes the importance of bacterial strains in view to mineralize phosphate, PGPR and biocontrol properties. The study related to biodiversity of phosphate solubilizing bacteria will be useful to design strategies to apply these bacterial isolates as inoculants in sustainable and organic agriculture. Fluorescent *Pseudomonad* strains reported in this study with phosphate solubilization potential and combination of *Bradyrhizobium* species as biofertilizer will serve as potential inoculants in promotion of agronomic applications of biological components specially microbes.

The detection of these effective microbial strains from various other soil microbes will be helpful for farmers and scientist to formulate effective microbial management strategies and evaluation of genetically improved microbial strains in field validation. The probe could also be used for early detection of efficient microbial strains in the field as bioinoculants. Simultaneously the use of DNA (developed from 16SrDNA and SCARs unique sequences) probe for direct detection of microbial strains from soil and nodules will be carried out The formulation of methods used for direct extraction and purification of DNA from soil/ infected plant parts together with PCR amplification of the DNA, to monitor survival of microbial strain will be performed, which cannot be detected by any conventional technique. Small-scale procedure for DNA sample preparation to support rapid monitoring in the field PCR analysis for sensitive detection of target microbial strain from the environmental samples (soil) will be established.

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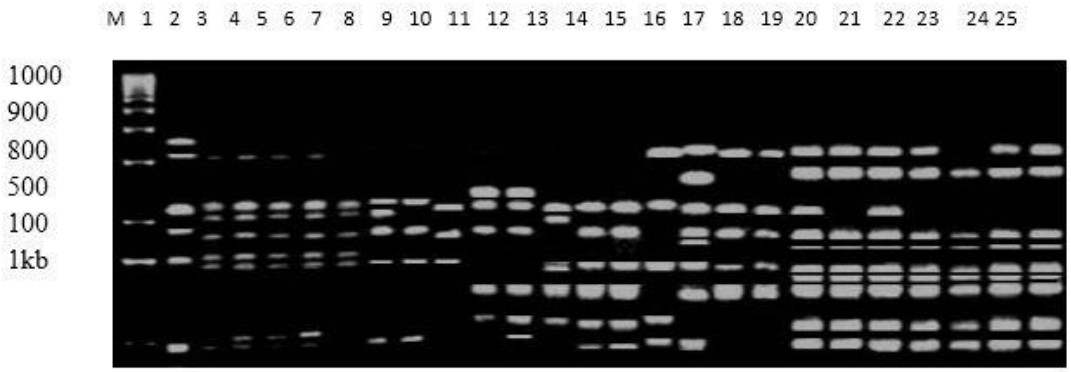

Fig.1: Restriction enzyme *Msp I* treated 16S rDNA segments of isolated strains of *Pseudomonas sp.* on 2% agarose gel

1-15 P-1 to P-15 *P.fluorescens;* 16 -25 P-16 to P-25 P.aeruginosa

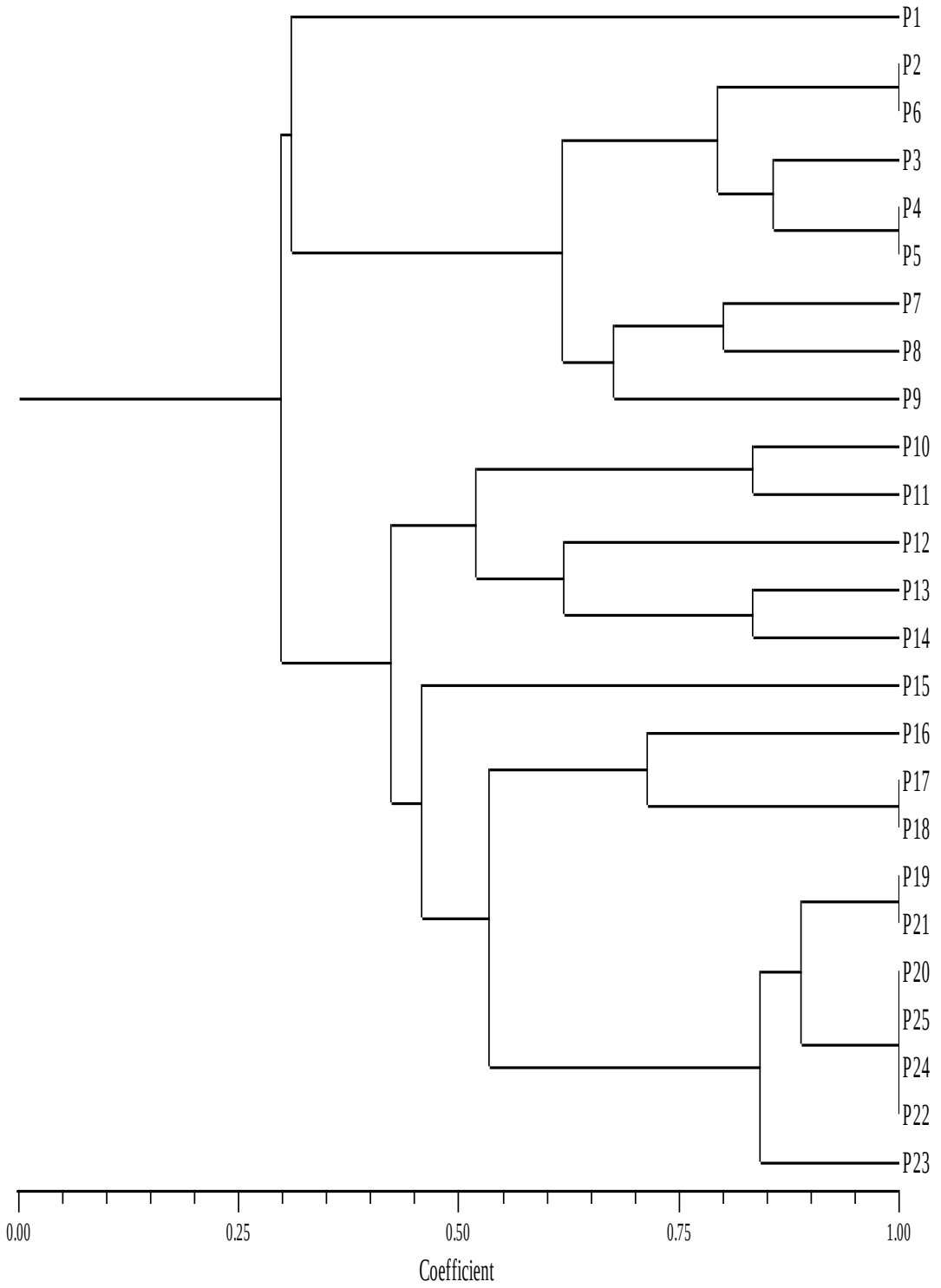

Fig.2: Dendrogram based on *Msp I* treated 16S rDNA segment of *Pseudomonas* isolates showing genetic relatedness using Jaccard's similarity coefficient and UPGMA cluster method.

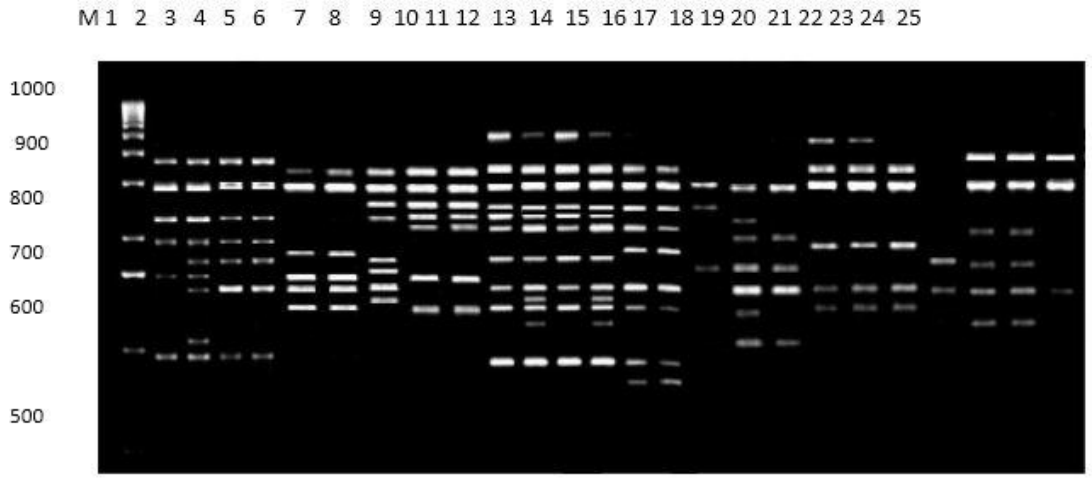

Fig.3: REP-PCR fingerprinting of *Pseudomonas* strains isolated from agricultural field of M. P. on 2% agarose gel

1-15 P-1 to P-15 *P.fluorescens,* 16 -25 P-16 to P-25 P.aeruginosa

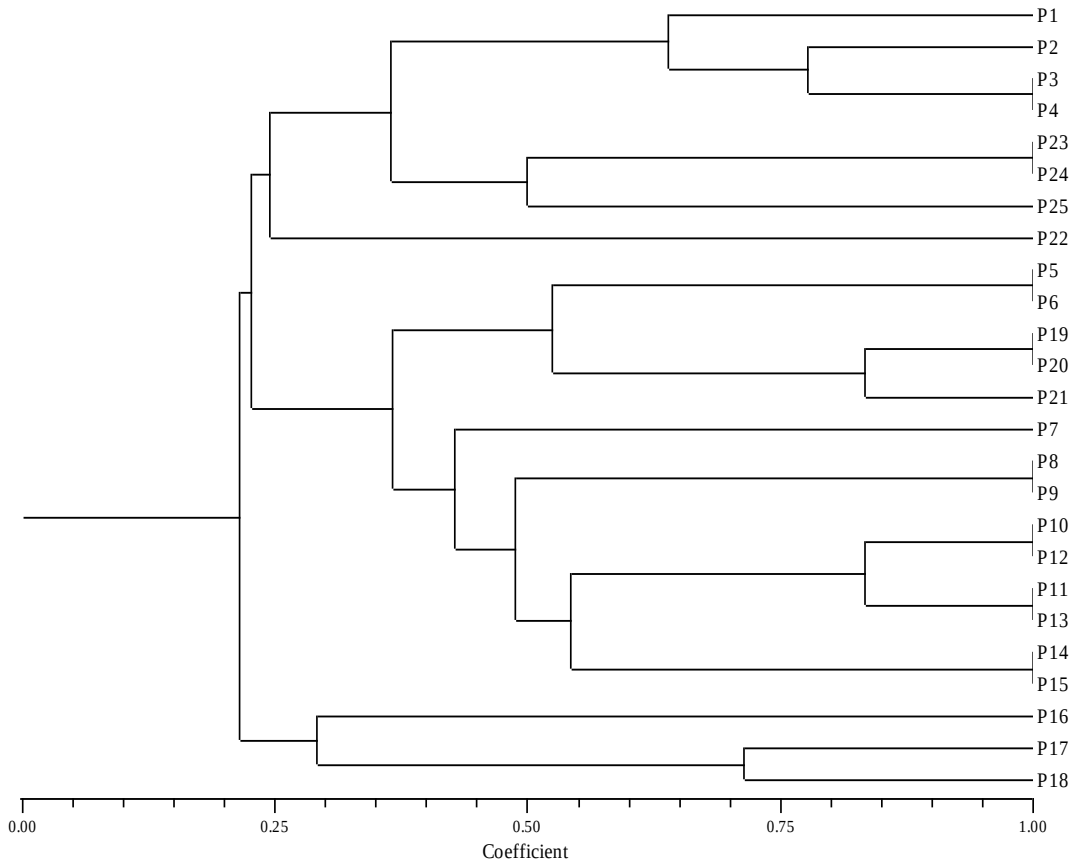

Fig.4: Dendrogram based on REP-PCR of *Pseudomonas isolates* showing genetic relatedness using Jaccard's similarity coefficient and UPGMA cluster method

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