BACTERIAL DIVERSITY OF PHOSPHATE AVAILABILITY ON GROWTH AND NUTRIENT UPTAKE OF CHICKPEA (*CICER ARIETINUM L.)* CROPS

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***ABSTRACT* : In the present study phosphate Solubilizing bacteria were isolated from soil and their effect on germination of *Cicer arietinum* seeds as well as seedling growth was studied with an objective to develop a biofertilizer and its multifunctional properties will attract more attention in the field of biofertilization and biological control potentiality of *Pseudomonas* and its ability to excrete phytohormone and antimicrobial metabolites may be used as biofertilizer and biocontrol agents in sustainable agriculture.**

Genetic content exhibit molecular diversity to evaluate and compare the new Miniprimer-PCR to the two DNA-based typing techniques and the phenotypic characteristics for the purpose of assessing the diversity of microbial community isolated as collection of fluorescent pseudomonads. All the 50 strains of pseudomonas sp such as *Pseudomonas aeruginosa, P.putida, P. fluroscence*, and *P. streuzi* were shown to have genetic similarity with variation in arrangement of nucleotides in genome. Expression of species within the genus is regulated by reading frame of organism, therefore, with common concentration of DNA most of the species shows architectural motive once NTSYS prediction are used to made a dendrogram UPGMA .Ornamentation of genome represent base substitution thus defining evolutionary distance along with constancy of character within species. All the Pseudomonas isolated looking into their biochemical profile can be used as PGPR and biocontrol agents.

Keywords*: PGPB, IAA, ACC, PGPR phytohormone, Biofertilizers, *Cicer arietinum Pseudomonas.

I. INTRODUCTION

Rhizosphere microorganism can have marked effect on plant performance in agricultural and marginal soils by influencing growth and development of root, improving nutrient availability in the rhizosphere (Setiawati and Mutmainnah, 2016; Jitendra Malviya *et al*; 2012 and Vikram A; hamzehzarghani 2007). Soluble forms of phosphorous (P) fertilizers applied to the soil are easily precipitated as insoluble promoting forms (Chen YP *et al*., 2006). Improving soil fertility is one of the most common tactics to increase agricultural production and maintaining high levels of available phosphorus (P) and nitrogen (N), the two most limiting nutrients in soil (Jitendra Malviya, 2011) remains a major challenge to ecologist and land managers. The green revolution in agriculture has been one of most successful human achievements in this century. These revolutions resulted in global food security and played an important role in transforming developing countries, such as India, from being food -deficient to having a food surplus. However, increases in food production are being offset with a constant increase in human population and now the world food security is again being threatened. Fertilizer played a significant role in the green revolution but excessive use of them as led to reduction in soil fertility and to environmental disbalance. Phosphorus (P) is an essential nutrient for plants, participating as a structural component of nucleic acids, phospholipids and adenosine triphosphate (ATP), as a key element of metabolic and biochemical pathways, important particularly for BNF and photosynthesis (Khan *et al*., 2009; Richardson and Simpson, 2011). Plants absorb P in two soluble forms: the monobasic ($H_2PO_4^-$) and the dibasic (HPO_4^{2-}) .P is rapidly fixed to unavailable forms and accounts for low P use efficiency by biological immobilization and chemical precipitation which in turn would soon deplete the available phosphate supply, leaving very little available for plants (Kamlesh K. Meena *et al*.,2010). Plant growth promoting rhizobacteria PGPR which belongs to diverse genera such as *pseudomonas* are rhizosphere inhabitation bacteria that have a positive influence on plant growth (Jitendra Malviya *et al*., 2017), they are called phosphate solubilizers (PSB) and they convert the insoluble phosphates into soluble forms by acidification, chelation, exchange reactions and production of gluconic acid (Hameeda *et al*., 2006). Current trends in agriculture are focused on reduction in the use of chemical pesticides and inorganic fertilizers, compelling the search for alternatives that enhance environmental quality. The management of soil organic matter requires inputs of composts, crop residues, green manure, and other organic wastes. (N. Wani *et al*., 2009)

The present study samples were collected from agriculture land of Betul, Raisen Hoshangabad district of Madhya Pradesh. 100 samples were collected rhizospheric region of leguminous plants. Isolation and identification of samples was done. These bacteria were identified as phosphate solubilizing bacteria or fluorescent *pseudomonas* on Pikovaskya media. 50 strains were isolated on the basis of morphological and biochemical traits i.e. *P.aeuroginosa, P.putida, P.struezti, P.fluoresence and Bacillus thrungeneis.* Biofertilizers refers to products consisting of selected and beneficial living microbes, which are added to soil as microbial inoculants. Phosphate Solubilizing microorganisms are presently being used as biofertilizers. Slowly emerging Phosphate Solubilizing Bacteria are especially as important organisms used to improve soil health the studies revealed that they bring about dissolution of rock phosphate thereby reducing phosphate deficiency in soil. In the present study phosphate Solubilizing bacteria were isolated from soil and their effect on germination of *Cicer arietinum* seeds as well as seedling growth was studied with an

objective to develop a biofertilizers to use these bacteria in improving soil quality which in turn, will lead to increased productivity. Molecular approach provide the diversity evaluated and compared with RFLP,RAPD,REP- PCR biotyping techniques and the phenotypic characteristics to form biofertilizers and assessing the diversity to alter.

II. MATERIAL AND METHODS

2. Sampling sites

Samples were collected from rhizospheric region of *Cicer arietinum,* of different District Betul, Hoshangabad, Raisen, Rajgarh, Chhindwara, shajapur, shahdol, and Bhopal of Madhya Pradesh, India. These districts lie in central part of Madhya Pradesh India. Soil samples were collected at depth of 0-30cm. Surface litter was scrapped away and soil samples were stored in a presterlized high-density polythene (HDPE) bags (Jitendra Malviya *et al*., 2012).

2.1 GIS (Geoinformatic Science)

The procedure adopted in this includes (i) delineation of cropped area using remote sensing (SPOT-Vegetation Index) derived products, (ii) assessment of crop biomass from crop statistics, (iii) estimation of soil carbon stock from point measurement using GIS, (iv) understanding the relationship of crop biomass C with cropping intensity, net irrigated area and nitrogen consumption using regression analysis (Nisha Wani *et al*., 2010).

2.2 Isolation and Identification of phosphate Solubilizing bacteria

Phosphate Solubilizing bacteria were isolated from soil sample by serial dilution technique (K Sharma, 2005). Bacterial suspensions ranging from 10^{-1} to 10^{-6} dilutions were cultured on Pikovaskaya Medium (Hi-media) (R.I. Pikovskaya, 1948)

2.3 Siderophore production

Production of siderophores was estimated qualitatively on Chrome Azurol S (CAS) agar medium (Hi-media) (B Schwyn and JB Neiland, 1987). For preparation, 1 L of blue agar 60.5 mg CAS was dissolved into 50 ml water mixed with 10 ml Fe III solution (1 mM FeCl3.6H2O, 10 ml HCl) (Ranbaxy),while stirring this solution add slowly 72.9 mg and hexadecyltrimethylammonium (HDTMA) dissolved in 40 ml distilled water. The resultant dark blue liquid was autoclaved. Also, sterilize mixture of 750 ml water 100 ml MM9 salts, 15 g agar, 30.24 g pipes (Piperazine-1,4-bis(2- ethanesulfonic acid) was added into it, later, NaOH solution (6 M) was added to raise the pH up to 6.8 and the solution was poured into Petri plates (90 diameters, Borosil). The active culture of the *Pseudomonas* isolates were spotted on the dark blue agar plates and these plates were kept into incubator for 24 - 48 h at 32 ± 1°C.

2.4 PGPR and cell wall degrading enzymes of fungal

2.4.1 Indole-3-acetic acid (IAA)

Production of IAA overnight grown single colony was Cultured on LB medium agar (Hi-media), which contain (per liter): 10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar amended with 5 mM L-tryptophan, 0.06% sodium dodecyl sulphate and 1% glycerol (Rankem). Plates were overlaid with sterile Whatman no. 1 filter paper (82 mm diameter) and bacterial strain was allowed to grow for 3 days at 28°C. After incubation, the paper was removed and treated with Salkowski's reagent having the formulation of 2% of 0.5 M ferric chloride in 35% per chloric acid at room temperature for 60 min. In a Petri dish, the filter papers were saturated by soaking in Salkowski's reagent (Merck) and the production of IAA (Hi-media) was identified by the formation of a red halo on the paper immediately surrounding the colony.

2.4.2 Aminocyclopropane-1-carboxylate (ACC) deaminase

The ACC deaminase activity was determined as described earlier on Dworkin and Foster (DF) minimal salts medium, (Fermentos) which contains (per liter): 4 g KH_2PO_4, 6 g Na_2HPO_4, 0.2 g $MgSO_4.7H_2O$, 2 g glucose, 0.2 g; 2 g gluconic acid and 2 mg citric acid with trace element solution (1 mg $FeSO_4.7H_2O$, 10 µg H_3BO_3, 11.19 µg $MnSO_4.H_2O$, 124.6 µg $ZnSO_4.7H_2O$, 78.22 µg $CuSO_4.5H_2O$ and 10 µg MoO_3 (Merck) . Filter sterilized ACC solution (3 mM) was spread over the agar plates, allowed to dry for 10 min and inoculated with bacterial strains. Observe the growth after 2 days of incubation at 28°C.

2.4.3 Hydrogen Cyanide production (HCN)

Hydrocyanic acid (HCN Hi-media) production was tested on 35-mm petri dish containing Kings B agar medium amended with 4.4 g glycine/l with filter paper dipped in picric acid in the upper lid and sealed with parafilm. After inoculation, plates were incubated at 30±1°C for 24 to 144 h as required based on the trait under study. Rating the bacterial isolates positive for plant growth-promoting trait was recorded based on growth and/or zone size accordingly. Screening was done thrice with two replications each.

2.4.4 Protease Activity

The protease activity was determined using skim milk agar medium, (Hi-media) which contains (per liter): 5 g pancreatic digest of casein, 2.5 g yeast extract, 1 g glucose, 7% skim milk solution and 15 g of agar. Bacterial cells were spot inoculated and after 2 days incubation at 28°C proteolytic activity was identified by clear zone around the cells.

2.4.5 Chitinase

The chitinase activity of strains was tested on chitin agar medium, (Hi-media) which contains (per liter): 1.62 g nutrient broth, 0.5 g NaCl, 6 g M9 salts, 8 g colloidal chitin and 15 g agar. Bacterial cells were spot inoculated and after 5 days incubation at 30°C, chitinase activity was identified by clear zone around the cells.

2.4.6 Cellulase Production

Strains were screened for cellulase production by plating on M9 medium agar (Hi-media) amended with 10 g of cellulose and 1.2 g of yeast extract per liter. After 8 days of incubation at 28°C, colonies surrounded by clear halos were considered positive for cellulase production.

2.4.7 Pectinase Production

Pectinase production was determined using M9 medium amended with 4.8 g of pectin per liter. After 2 days of incubation at 28°C, plates were flooded with 2 mol l^{-1} HCl and strains surrounded by clear halos were considered positive for pectinase production.

Test of antagonism

Total isolates were screened for in vitro antagonism against *Sclerotium rolfsii, Macrophomina phaseolina, Fusarium solani, and Fusarium oxysporum* on potato dextrose agar (Hi-media) plates using dual culture technique. An agar plug (5 mm in diam) was cut from an actively growing (96 h) fungal culture and placed at the center of the agar plate. Simultaneously, the bacterium (24 h grown) to be tested was streaked 2 cm away from the agar plug. Plates with only fungus without bacterial culture were used as controls. Plates were incubated at 30±1°C until fungal mycelia covered the agar surface of the control plate.

2.4.8 Test for Seed germination

Effect of isolated phosphate solubilizing bacteria on seed germination was studied by inoculating seeds of *cicer arietinum with* bacteria. Fifty seeds were dipped for 5 hrs in bacterial suspension ranging from 10-2 to 10-6 dilutions. Seeds dipped in distilled water served as control. Both treated and untreated seeds were incubated at 37 C in a moist chamber for 3 days. After 3 days of incubation radical and plumule length of germinated seeds was measured. The experiment was performed in triplicates and was repeated thrice.

2.5 DNA isolation

Isolation of DNA was done using Pseudomonas inoculums of 2.0 O.D., inoculated in 50 ml Pikovaskya broth at 28o ±20C for 24 hours in a shaking incubator. Cell pellet was then obtained by centrifugation at 10,000 rpm (Remi India). Cells were suspended in 25 ml of saline EDTA solution in Erlenmeyer flasks. Lysis of cells were done by addition of 1ml of lysozyme solution at 37°C for 30 min. followed by 25% of 2ml SDS treatment at 600 c for 10 min. It was then allowed to cool at room temperature and 5 ml of 3M sodium acetate was gently mixed to 50ml of 24:1 chloroform. Isoamyl alcohol was added and again centrifuged at 10,000 rpm for 30 min. Out of three layers obtained after centrifugation, the uppermost layer bearing was pipetted out. Two volumes of chilled ethanol was added to it, the white fibrous precipitate at the interface was gently spooled out with the help of glass rod. Isolated DNA was then preserved in tris-EDTA buffer for further study (T Maniatis *et al*., 1982).

2.5.1 The determination of %G+C:

The %G+C content (**Rapley, 1998**) was calculated from the following formula i.e. %G+C =2.44 (Tm – 69.4) .28 Where, Tm = Temperature of melting calculated from the graph.

2.5.2 16S r DNA- RFLP PCR:

Amplification of 16S rDNA was carried out by polymerase chain reaction using a thermo-cycler (Mini Cycler, TM MJ Research PTC-148 BIO-RAD). The PCR were carried out with 50-90 ng of pure genomic DNA. The primers forward and reverse, located respectively, at the extreme 5'and 3' ends of the ribosomal rDNA sequence, enable the amplification of nearly the entire gene. The amplification reactions were performed in a 100 µl volume reaction. The amplified product was run on a 0.8% Agarose gel along with 1Kb MW marker, at a constant voltage and visualized under UV light.

2.5.3 16Sr DNA-RFLP APMLIFICATION

The amplified 16S rDNA is then digested separately with 05 different restriction enzymes by incubating it over night at 370C.26 Enzyme activity is stopped by providing a low temperature of 4 0C and by adding 2 µl 6x loading buffer to it. Further the enzyme digested PCR product along with 1Kb DNA ladder (Bangalore genie INDIA) in a separate work was estimated by electrophoresis at 55 mV of 2% Agarose gel for four hours.

2.5.4 RAPD-PCR

Minor modification was used for caring out PCR reaction to produce RAPD profiles. Amplification of DNA fragments was carried out by the Polymerase Chain reaction (JGK Williams, 1990) using 30 cycles.

2.5.5 REP –PCR fingerprinting

The 18 nucleotides in the REP primer make it specific primer for genomic DNA analysis. Both reverse primer: REP 1R (5'-IIIICgICgICATCIggC-3') 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 master mix was prepared for all the 50 samples as mentioned in the table (table 2.1).The completed PCR reaction is stored at 40C and whole cell REP-PCR products preferably at –200C,to prevent breakdown of the amplified product.

2.6 Genetic Diversity and phylogenic 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 Jacard Coefficient. Construction of phylogenetic tree was based on the data produced from the clustering and similarity output constructed with the help of 0-1 of the agarose gel with separated DNA bands obtain from various bacterial cultures.

III. RESULTS AND DISCUSSION

Many rhizobacteria have been reported to stimulate plant growth under different conditions. Phosphate solubilizing microbes have been routinely isolated from rhizospheric soil isolates formed colourless colonies which did not produce pigment, cells were gram negative, rod shaped and on the basis of biochemical reactions it was found to be *Pseudomonas fluorescens*. Solubilization efficiency (SE) of *Pseudomonas fluorescens was* calculated as 200.00 (Jitendra Malviya *et al*., 2012) (Table 1.) *Pseudomonas* often predominates among plant rhizosphere associated bacteria. Soil microorganisms transform the insoluble forms of phosphorus into soluble forms and thus influence the subsequent availability of phosphate to plant roots. Stress induced solubilization of tri-calcium phosphate was observed in Pikovskya's medium (Miguel Sulbarán *et al*., 2009) All the isolates produced phosphate solubilizing on Pikovskay's agar medium by inducing clear zones (Fig 2). Phosphorus is considered as an essential macronutrient and a great portion of phosphorus from chemical fertilizers becomes insoluble by its conversion into calcium or magnesium salts in soils.

3.1 GIS (Geoinformatic Science)

Agricultural area map of Madhya Pradesh includes total geographical area of the state is 30.8 M ha, of which total net sown area in 2005-06 was 14.9 M ha (48.5%), net irrigated area is 5.5 M ha (36.9% of agricultural area) and total forest area is 8.68 M ha (**DES, 2006**). District-wise agriculture area distribution was delineated from the base map Guna and Umaria districts had the highest (6337 km2) and lowest net sown area (1109 km2), respectively which was attributed to extent of geographical area and forest cover. Among all other districts of Madhya Pradesh, Hoshangabad had the maximum net irrigated area (79.2% of net sown area) due to the availability of an irrigation canal and Dindori had the minimum net irrigated area (0.40%) as the district was mostly covered with forest and mountainous terrain. The majority of the crops in Madhya Pradesh were rainfed and only 36.9% of the net sown area was under irrigation shown in (Fig.1.) (Nisha Wani, 2010).

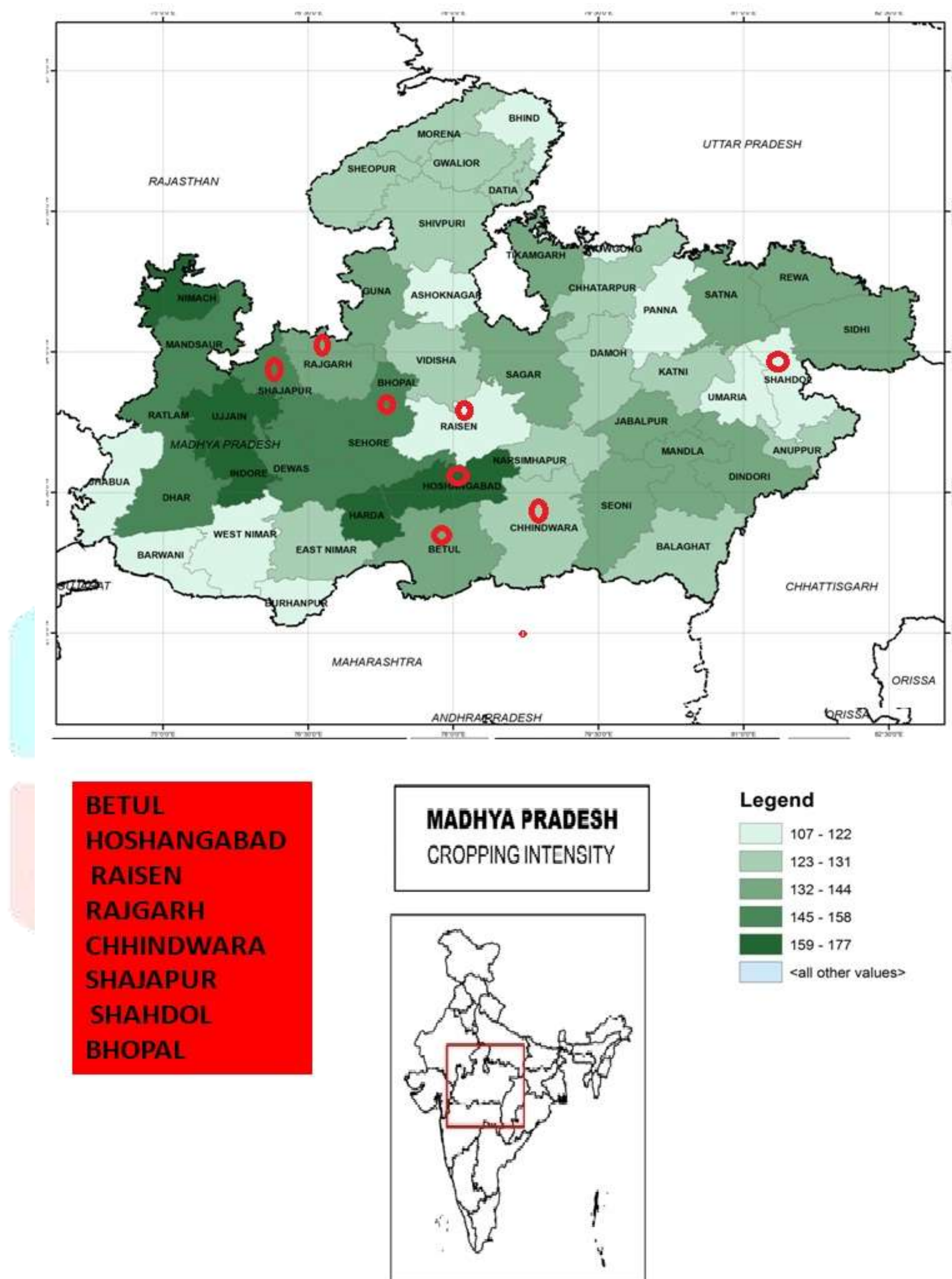

Fig.1. Map of Madhya Pradesh Showing the cropping intensity and soil sample area

Table 1. Morphology of Isolated phosphate Solubilizing Bacteria

S.No.	Characteristics	Bacterial morphology
1	Colony Size	Pinpoint
2	Surface	Shiny
3	Margin	Smooth
4	Elevation	Raised
5	Optical features Pigment Production	Yellowish green
6	Growth in liquid medium	Turbid
7	Microscopic Examination	Rod
8	Gram staining	Negative
9	Motality	Polar flagella
10	Endospore Formation	Negative
11	Morphology	Rod Shaped

Table 2. Biochemical Characterization of Phosphate Solubilizing Bacteria

S. No	Strain No.	Indole Test	Methyl Red	Vogaus proskeur	Oxi-dase Test	Glucose Fermen-tation	Fluorescent diffusible yellow pigment on F Pseudo agar	Non Fluorescent diffusible Blue pigment on P pseudo agar	Motility test	Gelatin	HCN	IAA
1	JM-1	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
2	JM-2	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
3	JM-3	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
4	JM-4	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
5	JM-5	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
6	JM-6	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
7	JM-7	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
8	JM-8	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
9	JM-9	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
10	JM-10	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
11	JM-11	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
12	JM-12	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
13	JM-13	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
14	JM-14	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
15	JM-15	-ve	-ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve	+ve	+ve
16	JM-16	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
17	JM-17	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
18	JM-18	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
19	JM-19	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
20	JM-20	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
21	JM-	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve

	21											
22	JM-22	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
23	JM-23	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
24	JM-24	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
25	JM-25	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
26	JM-26	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
27	JM-27	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
28	JM-28	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
29	JM-29	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
30	JM-30	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
31	JM-31	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
32	JM-32	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
33	JM-33	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
34	JM-34	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
35	JM-35	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
36	JM-36	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
37	JM-37	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
38	JM-38	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
39	JM-39	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
40	JM-40	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
41	JM-41	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
42	JM-42	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
43	JM-43	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
44	JM-44	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
45	JM-45	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
46	JM-46	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
47	JM-47	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
48	JM-48	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
49	JM-49	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve
50	JM-50	-ve	-ve	-ve	+ve	-ve	+ve	-ve	+ve	+ve	+ve	+ve

Fig 2 Colony Morphology of Phosphate Solublizing Bacteria on Nutrient Agar and King's B Plate after incubation at 30^0 C Showed Phosphate solubilization formed by *P. putida*

The PSMs are known to solubilize Ca-P complexes mainly by lowering the pH of the media by secreting organic acids and studies with mineral phosphates have shown that the nature of the organic acids was more important than the amount and it is strongly influenced by pH, solubility increases with pH when Ph. P –solubilization was accompanied due to acidification which in turn, reduces the ability of Fe-P chelating. The decrease in pH indicates the production of organic acids considered responsible for P-Solubilization (Miguel Sulbarán *et al*., 2009) Ability to solubilize various insoluble phosphates is always desirable to be a competent PGPR. These isolated strains were found highly efficient solubilizers of three common insoluble phosphates. Solubilization of Ca3 (PO4)2 was found in similar range as reported by (K. Sharma, 2005). All the strains were tested positive for the production of siderophore on the basis of change in colour of the CAS medium from blue to orange; the strains are showed positive for the product of plant growth promoting hormones IAA (Jitendra Malviya *et al*., 2017) These antagonistic strains also showed production of IAA, fungal cell wall degrading enzymes, such as cellulases, proteases and chitinases which are known to be involved in antagonistic activity against phytopathogenic fungi and insects (Park *et al*., 2013; Cho *et al*., 2015; Parray *et al*., 2015; Jitendra Malviya *et al* ; 2011). Aminocyclopropane-1 carboxylate (ACC) deaminase was observed less number of strains by their growth on Dworking and Foster minimal medium containing ACC. ACC deaminase producers were present in all sources studied and these groups of bacteria are known to promote root elongation and plant growth by hydrolyzing ACC and decreasing ethylene biosynthesis in roots. Some of the strains showed proteolytic activity by inducing clear zones around the cells on skim milk agar medium i.e. of all strains (Fig.2.). There are many reports on improved legume-rhizobia symbiotic performance by PGPR e.g., soybean (*G. max* L.) (Egamberdieva *et al*., 2016), thal tree (*A. gerrardii*) (Hashem *et al*., 2016), chickpea (*C. arietinum* L.) (Panjebashi *et al*., 2012; Yadav and Verma, 2014), and peanut (*Arachis hypogaea*) (Badawi *et al*., 2011).

Out of 50 only 30 strains showed chitanase activity inducing clear zones around the cells on chitin agar medium. Pseudomonas had cellulose degrading bacteria as a dominant class constituting 90% population of P- solubilizers, 30 strains were chitinase producers out of 50. Specific strains of fluorescent pseudomonad bacteria indirectly influence the plant health by preventing the deleterious effects of phytopathogenic microorganisms through the production of antibiotics, cell wall degrading enzymes, HCN metabolite and siderophores. Production of HCN by *P. fluorescens* was recognized as a biocontrol factor, against plant pathogenic fungi. Hydrogen cyanide is a secondary metabolite implicated in plant protection, quality of plant growth promoting rhizobacteria, inhibit plant disease development thus strengthening the host's disease resistance mechanism (Hashem *et al*., 2016). Phosphate solubilizing bacteria isolated from *Cicer arietinum* rhizosphere can play a critical role by making P available to all crops (A.A. Khan *et al*., 2009).All 50 isolates strains showed positive test for HCN and pectinase producers. More than 6 strains were selectively present in the source used in the study and comparatively lower than other group of bacteria. Currently, studies on the rhizosphere microbial flora are accumulating and most of these studies are concentrated in the crops (Jitendra Malviya *et al.,* 2012). Although studies on the bacteria of the rhizosphere were initiated late, they have become one of the research focuses. (Table 2.)

These results suggest that treatment with phosphate solubilizing bacteria is beneficial as a general increase in seed germination as well as radicle and plumule length as compared to control was observed in all cases. Enhancement in growth is concentration dependent as barring a few exceptions. Best results were observed due to treatment with 10-4 dilution and as compared to inoculation by individual bacteria, combined inoculation proved better. Inoculation with *P. fluorescens* resulted in 27.9%, 43.6% and 58.6% increase in radicle length at 10-2 to 10-4 dilutions respectively while 40.9%, 47.6% and 63.1% decrease in radicle length was observed at 10-2, 10-5 and 10-6 dilutions, respectively as compared to control. Similarly 19.0%, 22.7%, 29.1% and 32.0% increase in plumule length was observed at 10-2 to 10-5 dilutions due to treatment with *P. fluorescens* whereas 54.5% and 70% decrease was seen at 10-5 and 10-6 dilutions respectively as compared to control (Eljounaidi *et al*., 2016;Jitendra Malviya *et al*., 2012).

3.2 DNA isolation and PCR amplification

In order to identify *Pseudomonas* sp isolated from different samples of soil genomic analysis have been performed. Microbial diversity is based upon variation in organization of genomic content. Study of microbial diversity is a need of microbial ecological research .The production of accurate variation in genomic content is dependent upon external factors. DNA sample of

50 isolated strains were taken using Helms method (Helms et. al., 1985; Tracy, 1981; Mainatis *et. al*., 1982).Gel was performed at 25^{0}C with 0.8% agaorse. Each of the 50 species included in *Pseudomonas* genus showed ordinal profile of DNA content. Microorganisms have the ability to solubilize the insoluble phosphates and maintain the soil health and quality (Richardson *et al.,* 2001). Bacteria use several direct and indirect mechanisms of action to improve plant growth and health. Mechanisms such as phosphate solubilization (K.Y.Kim, 1998) aminocyclopropane- 1-carboxylate (ACC) deaminase (Penrose *et al.,* 2002), nitrogen cycle (Ahn T. *et al.,* 2007) and phytohormone production (O'Sullivan D.J. *et al.,* 1992) are considered as popular mechanisms. Beneficial effects of phosphate Solubilizing bacteria to crops have been documented well (Pal *et al*., 1998; Richardson *et al*., 2001). It is believed that microbial solubilization of phosphate in soil was correlated with the ability of microbes in producing selected organic acids and or extracellular polysaccharides (Halvorson *et al.,* 1990). This hypothesis has been corroborated by cloning pyrroloquinoline (PQQ) synthase (Goldstein A.H. *et al.,* 1987; Rodriguez H., 2000) and *gab*Y genes involved in gluconic acid production. Gluconic acid is the principal organic acid produced due to direct oxidation of glucose by *Pseudomonas* which was found to be involved in phosphate solubilization (Goldstein A.H. *et al.,* 1993).

Among phosphate solubilizing bacteria, fluorescent *Pseudomonads* that colonize aggressively the plant roots have been considered as an important group of bacteria due to their biofertilizing and biocontrol properties. In many occasions, plant growth promoting rhizobacteria often exhibit the production of antimicrobial metabolites, which take part in suppression of diseases caused by soil borne phytopathogens (Bano N. *et al.,* 2004 ; Ravindra Naik P. *et al.,* 2010) as well as involve in the induction of systemic resistance against insects and nematodes (Ramamoorthy V. *et al.,* 2001, Zahnder *et al.,* 2001). Selective strains of *fluorescent Pseudomonad* bacteria have also been reported for biodegradation of agricultural pollutants (Bano N. *et al*., 2003) as well as for weed control in agricultural fields (Chrudattan R *et a*l., 1991; Kremer RJ, 1990). These bacteria have been considered as an important bioinoculants due to their innate potential to produce plant growth promoting hormones and enzymes (Cattelan A.J. *et al.,* 1999, Sunish Kumar *et al.,* 2005). Though the bacteria belonging to *Mesorhizobium, Rhizobium, Klebsiella, Acinetobacter, Enterobacter, Erwinia, Achrobacter, Micrococcus, Aerobacter* and *Bacillus* have been reported as phosphate solubilizers, strains belong to *pseudomonads* are considered as efficient phosphate solubilizers (Villeges J. *et al.,* 2001). Soyabean and other Symbiotic leguminous plants are most important food crops of the world and are widely grown in developing countries. Fungal pathogens are important production constraints of rice, banana and other crops .Indiscriminate use of fungicides in agriculture is known to be hazardous to the environment and lethal toward other beneficial organisms; it can lead to the development of resistance against target organisms. Microbial solubilization of phosphorus, biodegradation of chemical pollutants and biocontrol of plant pathogens are cost-effective, novel biological restoration and ecofriendly techniques. Considering the multiple applications of phosphate Solublizing *fluorescent Pseudomonads* it is essential to study their diversity, which will be useful in designing strategies to use these native strains as bioinoculants for sustainable and organic agriculture without causing harm to the environment and farmers.

The objective of the present investigation was to study the genetic and functional diversity of phosphate Solubilizing *fluorescent Pseudomonads* associated with rhizospheric soils of soyabean and other Symbiotic and free-living microorganism by an array of *in vitro* assays, gene amplification techniques, fermentation methods and chromatographic analyses. Taxonomic affiliation of bacteria was done on the basis of 16S rDNA gene similarity and molecular phylogenetic analyses (Fig.3.) . Phosphorus Solubilizing bacteria are known as fluorescent *Pseudomonads* are often associated with rhizosphere .These bacteria are known to synthesize phytohormone and on the other hand they are known to suppress the growth of phytopathogens. Plant growth promoting rhizobacterial types of fluorescent *Pseudomonad*s use one or more mechanisms of direct or indirect in improving plant growth .these group of bacteria exhibit multiple functional traits such as solubilizing of inorganic phosphate and iron production, production of vitamin, phytohormone and antimicrobial metabolites. In the present investigation out of 50 isolates most of the *Pseudomonas* species have been identified as phosphate solubilizer. 16SrDNA of 50 isolated strains of pseudomonas were PCR amplified with Universal Primer *P. aeuroginosa, P. fluorescence, P.Putida, P. Streuzi.* The sequence of ribosomal DNA is known to determine phylogeny of microbes as well as higher organism the rate of mutation frequency of these conserved sequence is extremely low. Therefore, most of the species reflects, common pattern as they are conserved in nature. Restriction enzyme known for PCR analysis of 16SrDNA segments varies, from species to species although for present study *Hae III* restriction enzyme was preferred in case of 16SrDNA segment of *Pseudomonas* species.

The bands resulting after PCR reaction were seen on agarose gel. Use of *Hind III, Mbo I, Taq I,* results variation in pattern of appearance of bands when DNA is subjected to digestion. Bands appearance on acryl amide gel after digestion falls within 200-800bp .The DNA band patterns appearing on gel were further subjected to computational analysis using UPGMA profile the similarity index were subjected to analysis on NTSYS software using unweighted pair method. The results appearing using restriction enzymes treatment is illustrated (Fig. 3.).

Dendogram were prepared using coefficient scale shows point of divergent at 68% of similarity. Second measure growth of *pseudomonas* preferred with genetic relationship at 80% similarity (Fig. 3.) strains JM-6,JM-15,JM-16,JM-19 and JM-20 shows 20% dissimilarity among each other. However, a smaller group shows divergent at 87% similarity and shows only one species of *Pseudomonas* i.e. JM-3.Strains of *Pseudomonas* JM-5 and JM-14 shows dissimilarity of 7% i.e. they are much similar among each other. strain JM-36 is similar to JM-3,JM-33,JM-34,JM-45,JM-35,JM-48,JM-46 and shows similarity of 87% however, strain S-3, Shows only 4% dissimilarity to rest of the group. Minor deviation in similarity among all isolates of *Pseudomonas* shows there mutational competency and resistance towards strains .The bands appearing during 16SrDNA restriction enzyme treatment the level of divergent in base substitution during course of evolution. The phylogenetic distance between species i.e. JM-1 to S-50 of *Pseudomonas* the presence variation and arrangement of nucleotide sequence as supported by appearance of dendogram (Fig 2). The dissimilarity among base pair also affects the reading frame of organism *Pseudomona*s species isolated were subjected to random amplification to observe polymorphic pattern of DNA. Polymorphism is a natural phenomena which helps to study similarity amongst species using defined primers appearance of repeated sequence of a bases in pseudomonas

genus results banding pattern after performing PCR reaction following denaturation, annealing and extension of fragment of DNA .The reaction mixture were examine for band profile on 2% agarose gel (Fig 3).

The bands appeared expressed pattern of banding between 200 to 900 bp observations of RAPD were subjected to NTSYS program for reproduction of phylogeny (Fig .4). RAPD PCR of isolated strains of *Pseudomonas* represented high degree of variability. Strain JM-4, JM-10 and strains JM-42, JM-44, showed dissimilarity of 20% with a narrow range of bifurcation, strain S-43 an S-44 shows 100% similarity. The similarity index with the help of RAPD PCR of *Pseudomonas* species isolates divergent only with 15% of similarity index maximum similarity of appeared at 80%. Heterogeneity and variation with in species JM-27 and JM-38 shows 67% similarity strains JM-26 shows 77% dissimilarity with rest of the strain *Pseudomonas* isolated polymorphism represented repeated appearance of common sequence and in the present of observation occurrence of such bands for more prevalent . The site of action of enzyme most preferably more available thus, Thus, by implementing Jaccard's similarity coefficient and UPGMA cluster method minute degree of deviation resultant hieracy of polymorphism.Furthermore, standard PCR technique of REP-PCR was implemented and reproduced to show banding pattern.

The size of DNA fragment using REP-PCR primer range between 200 to 1000 bp all the 50 isolates of *Pseudomonas* from different soil of Madhya Pradesh although having approximately similar G+C % content and Tm value represented variation were subjected to REP-PCR fingerprinting tools . Purified DNA was subjected to digestion using forward and reversed primer REP 1R (5'-IIIICgICgICATCIggC-3') and REP 2I (5'-ICgICTTATCIggCCTAC-3') and illustrate in (Fig .5). Three distinct clusters were found with 55%, 60%, and 65 % respectively JM-13,JM-14,JM-15,JM-15,S-38,JM-16, JM-38,JM-39 JM-40,JM-41, and JM-46 falls with 65% similarity. However, strains JM-5, JM-8, JM-21, JM-24, JM-30 and JM-33 shows 60 % similarity amongst each other. Strain S-50 JM-2 JM-3 JM-26 JM-27 JM-28 differs with these 5 % to JM-25. The Present study get microbial isolation of *Pseudomonas species* from Raisen, Hoshangabad, Betul, Shajapur, Bhopal, Shahdol, Chhindwara, Rajgarh district of Madhya Pradesh helps to conclude bacterial species similar biochemical property having approximately common genetic content exhibit molecular diversity. Genomic observation used as tools to study differential .Appearance and relationship amongst *Pseudomonads* species. Molecular techniques offer advantage over biochemical parameters as genome resultants any metabolic process, thus, maintaining continuity of inheritance .All the 50 strains of pseudomonas sp such as *Pseudomonas aeruginosa*, *P.putida, P. fluroscence*, and *P. streuzi* were shown to have genetic similarity with variation in arrangement of nucleotides in genome. Expression of species with in the genus in regulated but reading frame of organism , therefore , with common concentration of DNA most of the species shows architectural motive once NTSYS prediction were made using UPGMA. Ornamentation of genome represent base substitution thus defining evolutionary distance along with constantly of character within species. All the *Pseudomonas* isolated looking into their biochemical profile can be used as PGPR and biocontrol agents (Walitang *et al.,* 2017)

This study results suggest that strains of bacteria isolated from soil are phosphate Solubilizing and use of bacteria as seed inoculant will improve seed germination and seedling growth as well as result in increased productivity. Amongst the strain, use of *P. fluorescens* will be more effective in increasing seed germination as well as seedling growth. Hence use of phosphate-solubilizing bacteria, as biofertilizer should be promoted (Jitendra Malviya *et al* ., 2011). Further studies on the rhizo competence of these strains are wants a molecular techniques which may suggested, gene amplification, fermentation and chromatographic analyses as well as taxonomic affiliation of bacteria on the basis of *16SrDNA*. The management of bacteria, soil and plant interactions has emerged as a powerful tool in view of the biotechnological potential of these interactions, evidenced by increased crop productivity, reduction of production costs by reducing the volume of fertilizers applied and a better conservation of environmental resources(Hungria *et al*., 2013). Moreover, inoculants are composed of beneficial bacteria that can help the plant meet its demands for nutrients. As previously discussed, these bacteria increase plant growth, accelerate seed germination, improve seedling emergence in response to external stress factors, protect plants from disease, and promote root growth using different strategies. Whether gram-negative or gram-positive, these bacteria require isolation in culture media and analysis of various genotypic and phenotypic aspects, as well as analysis regarding their beneficial interaction with the host plant in experimental and natural conditions (Rocheli de Souza *et al*., 2015).

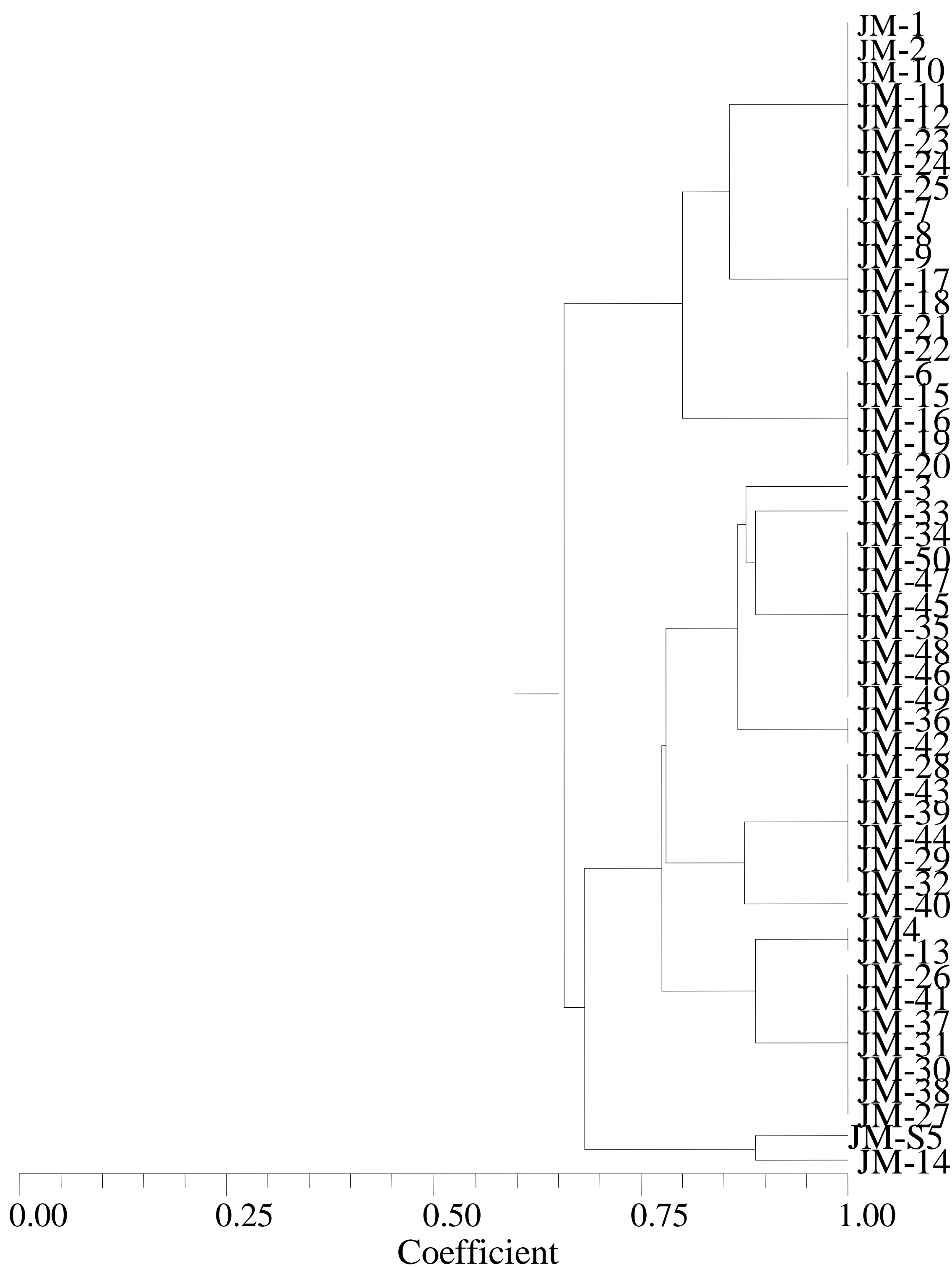

Fig.3. Dendogram based on *Hae III* treated 16S rDNA segment of *Pseudomonas* isolates showing genetic relatedness using Jaccord's similarity coefficient and UPGMA cluster method.

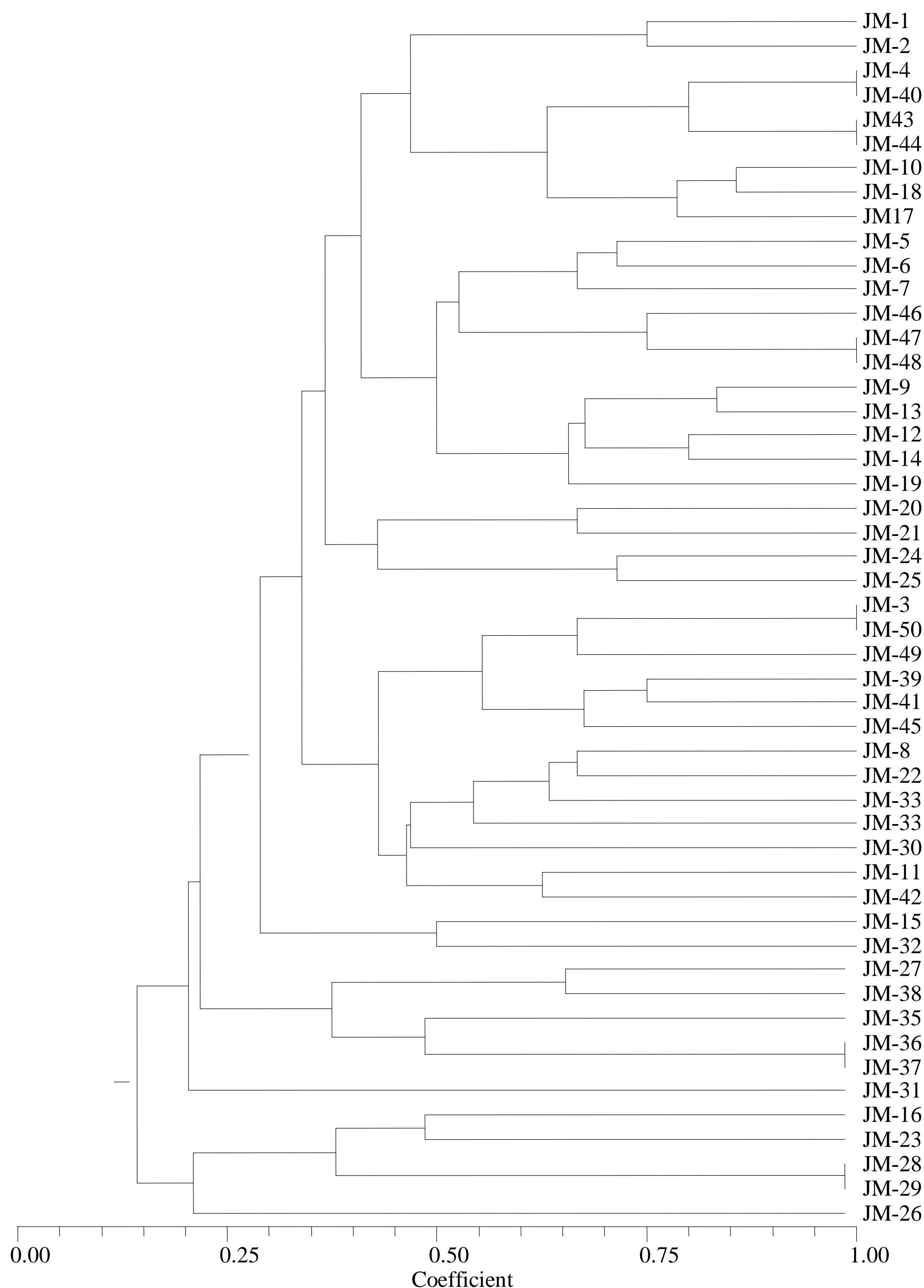

Fig.4. Dendogram based on RAPD-PCR of *Pseudomonas* isolates showing genetic relatedness using Jaccord's similarity coefficient and UPGMA cluster method.

Fig.5. Dendogram based on REP-PCR of *Pseudomonas isolates* showing genetic relatedness using Jaccord's similarity coefficient and UPGMA cluster method.

CONCLUSION

Phosphate solubilizing fluorescent pseudomonad bacterial strains with their multifunctional properties will attract more attention in the field of biofertilization and biological control Characterization of phosphate solubilizing fluorescent pseudomonas bacteria is required to study their ecological role in soil. Fluorescent pseudomonas reported in this study with phosphate solubilizing Bacteria potential and ability to excrete phytohormones and antimicrobial metabolites may be used as plant growth promoting bacteria and biocontrol agents in sustainable agriculture. Siderophore produced by different *Pseudomonas* species have been widely studied as biological agents and it is an alternative to take into account the control of phytopathogenic microorganism in agriculture as they bind to available form of iron in the rhizosphere, thus making it unavailable to the phytopathogens and protecting the plants health. The increase and eventual commercialization of fluorescent pseudomonas as biocontrol agents depends on in part to understanding the mechanism involved in the antagonist interaction between bacteria, pathogen and host plant. In these study we revealed the microbial diversity of fluorescent Pseudomonads with innate potential of mineralizing phosphate, plant growth promoting traits and biocontrol properties.

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