Journal of Advances in Microbiology Research

E-ISSN: 2709-944X
P-ISSN: 2709-9431
JRM 2021; 2(2): 33-45

www.microbiojournal.com
Received: 18-05-2021
Accepted: 29-06-2021

Archana Dhole
B.A. College of Agriculture, Anand agricultural university, Anand, Gujarat, India

Harsha Shelat
B.A. College of Agriculture, Anand agricultural university, Anand, Gujarat, India

Correspondence
Archana Dhole
B.A. College of Agriculture, Anand agricultural university, Anand, Gujarat, India

Genetic diversity of plant growth promoting rhizobial and non-rhizobial isolates from green gram (*Vigna radiata* L.) based on Amplified ribosomal DNA restriction analysis

Archana Dhole and Harsha Shelat

Abstract
The increasing awareness about organic farming in farming community of India has stimulated the isolation and identification of large number of rhizobia strains from the root nodules and directed to studies on their diversity in the nodule niche. The bio-fertilizer inoculants containing diverse strains of rhizobia have been developed to improve soil fertility and increase symbiotic nitrogen fixation in the legumes. In spite of this success, there is paucity of data on diversity and genetic variation of rhizobia and associated genera present in root nodules as well as interaction between them. So this study aims to isolate and find out the genetic diversity of rhizobial and non rhizobial strains from root nodules of green gram. Comparisons of communities and variations in their DNA sequences existing in PCR-amplified 16s *rRNA* genes were studied by Amplified ribosomal DNA restriction analysis (ARDRA). The amplified PCR product from the 15 Rhizobial isolates and 38 non rhizobial isolates obtained from green gram root nodules were digested by tetra cutter restriction endonucleases (*Hae III* and *Hinf I*). ARDRA profile specified that the structure of community of rhizobial type and non rhizobial isolates was diverse and variable since four different clusters were observed separately by rhizobial type and non rhizobial isolates. Furthermore, 17 isolates showed phosphate solubilization ability within 5 days and production of IAA was varied greatly among both rhizobial type and NRE isolates ranging from 116. 50 µg ml^{-1} to 10.50 µg ml^{-1}. Therefore, the results of this study certainly suggests that the rhizobial and NRE isolates have great diversity and variability inside the nodule environment which help the plant growth, development and proliferation due to phosphate solubilization and IAA production. Thus, these isolates as a consortium may be explored as bio-inoculants for sustainable agriculture.

Keywords: Amplified ribosomal DNA restriction analysis, Microbial diversity, 16s *rRNA* gene, Root nodule

Introduction
Green gram (*Vigna radiata* L.) is an important pulse crop belongs to the family *Leguminosae* and subfamily *papilionaceae*. Due to the cheaper source of protein and essential amino acids green gram has been assigned in the higher order of nutritive merit. Besides its nutritional value, as a legume its root nodules established to be habitat for plant growth promoting rhizobacteria (PGPR). PGPR enhance plant growth and yield through variety of mechanisms like production of plant growth stimulating hormones, suppression of plant pathogens, N_2 fixation, increased availability of macro and micro nutrients, improvement in soil structure and thereby positively influencing root growth as well as promote other beneficial plant-microbe symbiosis. Earlier, it was believed that the root nodules of leguminous plants harbor only endophytic bacteria of genus *Rhizobium*, *Mesorhizobium, Bradyrhizobium, Ensifer* and *Azorhizobium* collectively called *Rhizobials* and fix atmospheric nitrogen symbiotically with plant. Bacteria of genus *Rhizobium* well perceived for symbiotic association with legumes that reside in legume nodules, mainly involved in biological nitrogen fixation (BNF) wherein atmospheric nitrogen is converted to ammonia by the enzyme nitrogenase complex. However, root nodules also accommodate various non-rhizobial endophytes (NRE) having definite influence on the survival, nodulation and yield of the crop. In recent years' nitrogen fixing root nodule bacteria have also been described in other Alpha-proteobacterial genera, including *Ochrobactrum* (Trujillo *et al*., 2005), *Methylobacterium* (Sy *et al*., 2001), *Microvirga* (Ardley *et al*., 2012; Radl *et al*., 2014) [1, 20], *Devosia* (Rivas *et al*., 2003) [22] and *Phyllobacterium* (Zakhia *et al*., 2006) [34].

Furthermore, so-called Beta-rhizobia have in the last ten years been described in the Betaproteobacterial genera *Burkholderia* and *Cupriavidus* (Chen *et al*., 2001; Moulin *et al*., 2001; De Meyer *et al*., 2014) [3, 16, 4]. So there is crucial need to focus on the diversity and interaction of rhizobial and non rhizobial population dynamics in the root nodules of green gram.

Different genetic fingerprinting techniques such as ARDRA, SSCP (Single-Strand Conformation Polymorphism), T-RFLP (Terminal Restriction Fragment Length Polymorphism), DGGE (denaturing-gradient gel electrophoresis), RISA (Ribosomal Intergenic Spacer Analysis), LH-PCR (Length heterogeneity PCR) and RAPD (Random Amplified Polymorphic DNA) for the partial community analysis of bacteria were widespread in current era. ARDRA is the extension of the technique of RFLP for the gene encoding the small (16s) ribosomal subunit of bacteria. The technique involves an enzymatic amplification using primers directed at the conserved regions at the ends of the 16s gene, followed by digestion using tetracutter Restriction enzymes. Patterns obtained from several restriction enzymes can be used to phylogenetically characterize cultured isolates. 16S-ARDRA is a simple method that can be routinely used in laboratories because it does not require specialized equipment. It is also less expensive than 16S rDNA sequencing (costs of identification depend primarily on the price of reference strains and restriction enzymes). The power of discrimination of ARDRA depends on the restriction enzymes used, which can be selected on the basis of *in silico* analysis using 16S *rDNA* sequences accumulated in public databases. Although ARDRA provides little or no information about the type of microorganisms present in the sample, the method is still useful for rapid monitoring of microbial communities over time, or to compare microbial diversity in response to changing environmental conditions. ARDRA is also used for identifying the unique clones and estimating OTUs in environmental clone libraries based on restriction profiles of clones (Smit *et al.*, 1997) [26].

Material and Methods

Isolation of rhizobial and NRE

In order to isolate rhizobial and NRE, total 5 samples were collected from different locations *viz*. Agronomy farm Anand, Dahod, Medicinal farm Anand, Vadodara, Jabugaon. Mud and soil particles were thoroughly removed by proper washing of root nodules under tap water. Healthy and pink nodules were safely cut from the root and were surface sterilized for 30 sec in 70% ethanol solution. Then treated with 0.1% $HgCl_2$ for 2 min and successively washed three times with sterile distilled water under aseptic condition for 1 min each. Then the nodules were crushed and the 100 µl contents were spread on Yeast Extract Mannitol Agar (Yeast Extract-1.0 g, Mannitol-10.0 g, K_2HPO_4- 0.50 g, $MgSO_4.7H_2O$- 0.20 g, Nacl- 0.1 g, Congo red-2.5 ml, Agar- 15 g, Distilled water-1000 ml, pH-7.0), Nutrient agar (Peptone-10.0 g, Meat or Beef extract- 03.0 g, Sodium chloride-05.0 g, Agar- 15 g, Distilled water-1000 ml, pH-7.4) and King's B medium (Peptone- 16 g, $MgSO_4$-1.6 g, K_2HPO_4-1.6 g, Glycerol-10 ml, Distilled water-1000 ml, Agar 15 g, pH-7.0). The petri plates were incubated at 28 ± 2 ˚C for 24 to 36 hrs. In order to verify surface sterilization, water from final rinse was spreaded onto the R_2A medium (Peptone- 0.5 g, Starch-0.5 g, Glucose-0.5 g, Yeast Extract-0.5 g, Casein Hydrolysate- 0.5 g, Dipotassium phosphate-0.3 g, Sodium pyruvate- 0.3 g, Magnesium sulphate anhydrous- 0.024 g, Distilled Water- 1000 ml, Agar- 15 g, pH-7.2) and incubated at 28 ± 2˚C for 24 to 36 hrs. Isolated colonies from root nodules were sub cultured and maintained at 4 ^{0}C.

DNA isolation and ARDRA profiling

All the isolates were grown in Luria broth for 24 h, and genomic DNA was extracted using the cetyltrimethyl ammonium bromide (CTAB) method (Sambrook *et al*. 1989; Jhala *et al*. 2014) [24, 12]. The integrity and concentration of the purified DNA was determined by agarose gel electrophoresis. The total genomic DNA extracted was dissolved in Tris-EDTA buffer and stored at 4 °C. 16S rDNA amplification was performed in a thermal cycler (Eppendorf Mastercycler; Eppendorf AG, Hamburg, Germany) with a 25-µl reaction mixture containing 50 ng of genomic DNA, 0.2 mM of each dNTPs, 1 µM of each primer (Lee *et al*. 2005), 2.5 mM of MgCl2, and 1 U of Taq DNA polymerase (Bangalore Genei, India) and the buffer supplied with the enzyme.

16S *rRNA* PCR product (15 µl) of all isolates was used to carry out the restriction digestion with two different restriction enzymes with 20 µl reaction mixture containing 2 µl 10X assay buffer, 5 U Restriction enzyme (*Hae III* or *Hinf I*), 10 µl PCR product and 6.25 µl nuclease free water. All the reaction mixtures were incubated at 37˚C for 3 hrs and enzymes were deactivated at 65˚C for 10 min after incubation. Restriction digested product (5 µl) together with 100 bp DNA ladder was separated on 3% agarose gel in 1 X TAE buffer, containing 0.5 µg ml^{-1} ethidium bromide.

The relationship between isolates was established using data from restriction enzymes that adequately differentiated isolates. A binary scoring system (1 for presence of band and 0 for the absence) was used to generate input matrix, which was analyzed using un weighted pair group method using average (UPGMA) algorithm (Sneath and Sokal, 1973) [27]; a dendrogram was generated from the matrix using NTSYS pc software (Rohlf, 1997) [23].

Phosphate Solubilization

Phosphate solubilization activity of all the isolates was determined by method described by Taurian *et al*. (2010). Bacterial isolates were inoculated by using toothpick on respective Sperber agar plates (Glucose-10.0 g, $CaCO_3$-05.0 g, Yeast Extract- 05.0 g, 10% K_2HPO_4- 20 ml, $MgSO_4.7H_2O$- 0.25 g, $CaCl_2$- 0.1 g, Agar- 15 g, Distilled water- 1000 ml, pH-7.0) under aseptic condition and were incubated at 30 ± 2 ^{0}C for five days with observation on colony diameter and solubilization of TCP every 24 h. Clear zone formation around the growing colony indicated the phosphate solubilization activity.

Growth Hormones Production

All the isolates were grown in glucose phosphate broth (Glucose- 05.0 g, K_2HPO_4- 05.0 g, Peptone- 05.0 g, Distilled water- 1000 ml, pH-7.0) containing L-tryptophan (0.005M) for 3 days at 30+2 ^{0}C on shaker at 100 rpm and then centrifuged at 3000 rpm for 20 min. One ml (1:2) supernatant was mixed with 2 ml of Salkowaski's reagent. Un-inoculated control was kept for comparison. The intensity of pink color developed within 30 min was measured at 535 nm in UV/VIS spectrophotometer

(Glickmann and Dessaux, 1995) [9]. The quantity of IAA was determined by comparison with an IAA standard curve.

Result and Discussion

Isolation of bacteria

Total 53 bacteria were isolated from five different samples on different media like CRYMA, NA and King's B agar. All the isolates were divided in to two categories based on medium as rhizobial (15) and Non rhizobial (38) as mentioned in Table 1 and 2. Out of which five isolates from Agronomy Farm, Anand; three from Muvaliya farm, Dahod; one from medicinal farm, Anand; three from Model Farm Vadodara and three from Jabugam Farm.

Table 1: Isolates from green gram root nodules on YEMA medium

Sr. No.	Isolate Name	Source of mung bean nodule samples from AAU farms
1.	A1	Agronomy Farm, Anand
2.	A2	
3.	A3	
4.	A4	
5.	A5	
6.	D1	Hill Millet Research Station, Muvaliya farm, Dahod
7.	D2	
8.	D3	
9.	M1	ICAR- Directorate of Medicinal and Aromatic Plants Research
10.	B1	Pulse Research Station, Model Farm, Vadodara
11.	B2	
12.	B3	
13.	J1	College of Agriculture, Jabugam
14.	J2	
15.	J3	

Table 2: NRE isolates from green gram root nodules on different media

Sr. No.	Isolate Name	Media	Source of mung bean nodule samples from AAU farms
1	A6	NA	Agronomy Farm, Anand
2	A7	NA	
3	A8	NA	
4	A9	King's B	
5	A10	King's B	
6	D4	NA	Hill Millet Research Station, Muvaliya Farm, Dahod
7	D5	NA	
8	D6	NA	
9	D7	NA	
10	D8	NA	
11	D9	NA	
12	D10	King's B	
13	D11	King's B	
14	D12	King's B	
15	D13	King's B	
16	M2	NA	ICAR- Directorate of Medicinal and Aromatic Plants Research
17	M3	NA	
18	M4	NA	
19	M5	King's B	
20	M6	King's B	
21	M7	King's B	
22	M8	King's B	
23	B4	NA	Pulse Research station, Model Farm, Vadodara
24	B5	NA	
25	B6	NA	
26	B7	NA	
27	B8	NA	
28	B9	NA	
29	B10	King's B	
30	B11	King's B	
31	B12	King's B	
32	B13	King's B	
33	B14	King's B	
34	B15	King's B	
35	B16	King's B	
36	J4	NA	College of Agriculture, Jabugam
37	J5	NA	
38	J6	King's B	

Diversity study by Amplified Ribosomal DNA Restriction Analysis (ARDRA) of promising isolates

ARDRA is the extension of the technique of RFLP (restriction fragment length polymorphism) to the gene encoding the small (16s) ribosomal subunit of bacteria. It is proved to be useful for relatedness of bacterial strains at different taxonomic levels, depending on selection of conserved or variable regions in the ribosomal genes for the analysis (Heyndrickx *et al*., 1996 and Koeleman *et al*., 1998). It is widely used to check the clonal diversity of the isolates.

The results of the virtual restriction, chosen two tetra cutter restriction enzymes namely *Hinf*I and *Hae*III were employed for the characterization of fifty three isolates. The amplified products obtained from all the isolates were subjected to restriction analysis yielding total of 200 and 203 fragments by *Hinf*I and *Hae*III restriction enzymes, respectively ranging from 126 bp to 1494 bp.

ARDRA analysis for rhizobial type isolates

In order to assess the existence of species, specific restriction patterns were performed for each of the enzymes utilized and have showed different and distinguished pattern of all isolates. Enzyme *Hae*III restriction pattern obtained 66 DNA restriction fragments (Table 3) ranging from 126 bp to 1060 bp (Fig. 1). Restriction analysis of rhizobial type isolates allowed three species specific profile. While, enzyme *Hinf*I showed 71 DNA restrictions fragments and allowed four specific restriction profiles (Fig. 2) ranging in size from 135 bp to 1494 bp.

Digestion with *Hae*III restriction enzyme revealed, three different clusters showing great phylogenetic diversity among the rhizobial type isolates (Fig. 3). However, isolates A1, A3, A4, J3, A5, D1, D2, and J2 demonstrated 100% similarity with each other in first cluster but more than 80% dissimilarity with the isolate B1. In second cluster two isolates were found with more than 80% dissimilarity from each other. Third cluster contains three isolates from which two (D3, M1) were identical but 80% diverse from third isolate (B2). Moreover, gram negative isolate B3 were found to be completely distinct from the all other isolates.

Digestion with *Hinf* I restriction enzyme revealed, four different clusters as shown in Fig. 4. Isolates A1, A5, D3, J3 and J1 demonstrated 100% similarity with each other which were more than 60% diverse from three isolates (D1, M1 & B2). Similarly isolates A3, A4, J2 were 100% similar and had 75% similarities with D2 as well as only 50% similarity with isolate B1. While, gram negative isolates B3 and A2 are more distinct and separated from all other rhizobial type isolates found in separate clusters.

Table 3: ARDRA pattern obtained for rhizobial type with enzyme *Hae*III and *Hinf*I

Sr. No.	Isolate	Enzyme *Hae*III		Enzyme *Hinf*I	
		No. of bands	RE fragment size (bp)	No. of bands	RE fragment size (bp)
1.	A1	5	126, 15,5 176, 197,303	7	323, 677, 874, 1072, 1218, 1349, 1494
2.	A2	1	544	1	1494
3.	A3	5	127, 155, 171, 200, 298	3	135, 321, 673
4.	A4	5	127, 155, 169, 194, 298	3	135, 311, 654
5.	A5	5	133, 167, 183, 213, 327	7	331, 660, 920, 1045, 1250, 1384, 1573
6.	D1	5	136, 167, 187, 217, 321	6	331, 695, 897, 1019, 1218, 1384
7.	D2	5	129, 155, 169, 197, 298	3	139, 321, 654
8.	D3	4	144,333, 502, 667	7	331, 644, 789, 968, 1158, 1384, 1613
9.	M1	4	141, 333, 502, 667	5	323, 611, 789, 993, 1218
10.	B1	7	134, 159, 171, 203, 298, 444, 599	6	144, 172, 321, 383, 616, 673
11.	B2	4	141, 247, 502, 690	5	340, 1072, 1250, 1420, 1573
12.	B3	1	1060	1	810
13.	J1	5	141, 173, 190, 225, 333	7	331, 677, 874, 1045, 1218, 1384, 1573
14.	J2	5	130, 159, 173, 203, 307	3	148, 330, 694
15.	J3	5	141, 170, 194, 221, 339	7	323, 677, 920, 1045, 1218, 1384, 1573

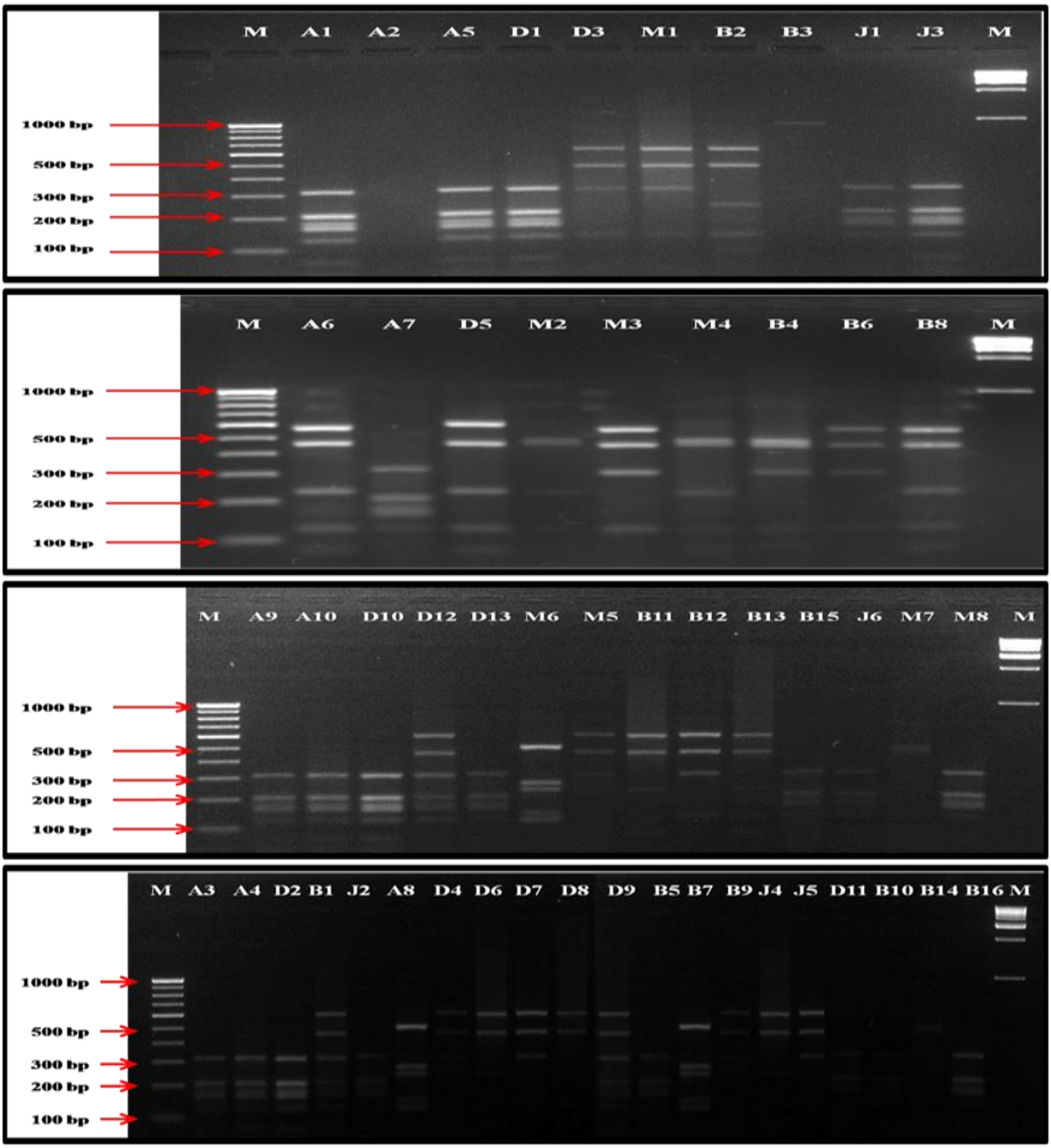

Fig 1: ARDRA pattern of all the rhizobial and NRE isolates with *Hae* III

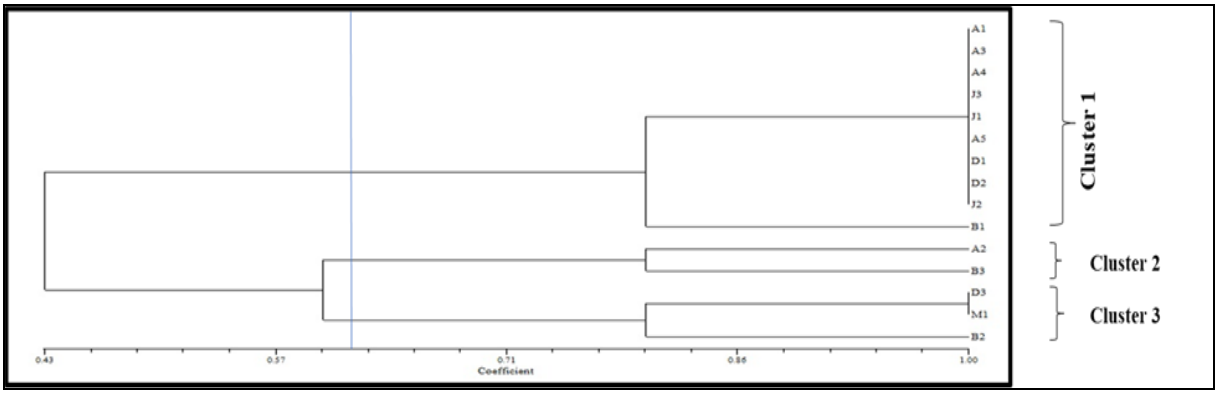

Fig 2: Dendrogram of Rhizobial isolates based on ARDRA profile of *Hae* III

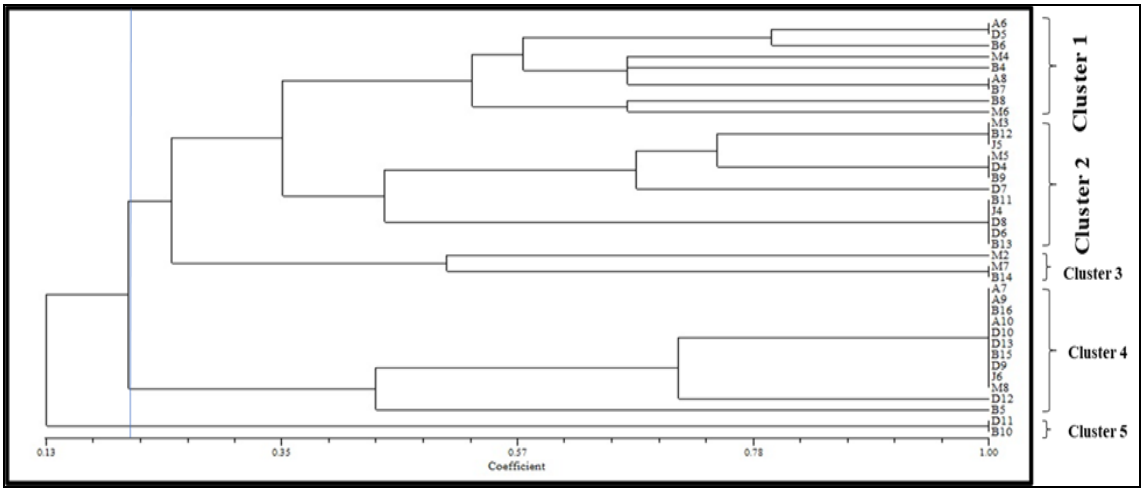

Fig 3: Dendrogram of Non rhizobial isolates based on ARDRA profile of *Hae*III

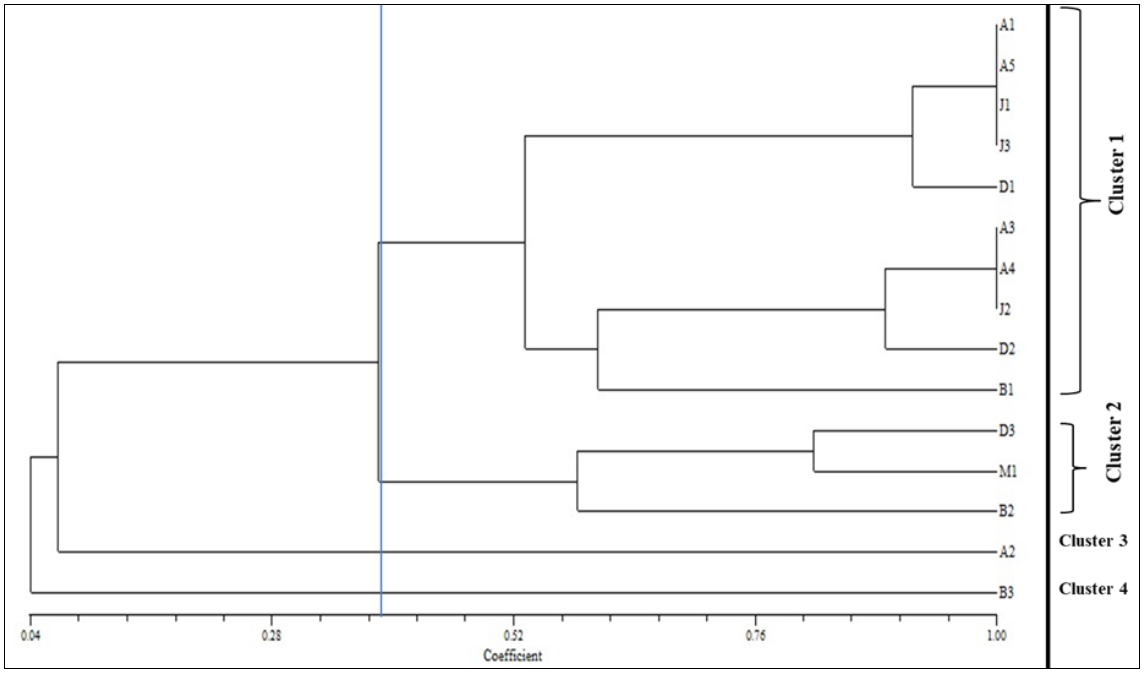

Fig 4: Dendrogram of Rhizobial isolates based on pooled ARDRA profile

Table 4: ARDRA pattern of NRE isolates obtain with enzyme *Hae*III and *Hinf*I

Sr. No.	Isolate	Enzyme *Hae*III		Enzyme *Hinf*I	
		No. of bands	RE fragment size (bp)	No. of bands	RE fragment size (bp)
1.	A6	5	92, 128, 221, 451,572	4	175, 333, 373, 552
2.	A7	5	122, 151, 170, 196,308	5	148, 175, 219, 333, 690
3.	A8	5	132, 153, 227, 259, 491	4	167, 330, 361, 616
4.	A9	5	128, 155, 166, 199, 296	3	141, 317, 678
5.	A10	5	120, 155, 166, 195, 296	3	141, 302, 661
6.	D4	3	302, 450, 616	3	157, 302, 781
7.	D5	5	87, 128, 216, 451, 600	3	185, 343, 1050
8.	D6	3	231, 450, 616	3	157, 311, 933
9.	D7	4	150, 311, 457, 616	4	157, 302, 340, 580
10.	D8	3	237, 450, 616	3	157, 311, 933
11.	D9	7	130, 159, 176, 200, 298, 450	3	124, 148, 302, 654
12.	D10	5	131, 158, 170, 199, 289	3	138, 310, 645
13.	D11	5	134, 169, 186, 221, 316	3	100, 253, 580
14.	D12	7	128, 155, 174, 199, 296, 438	6	145, 177, 317, 370, 613
15.	D13	5	128, 162, 174, 195, 302	3	149, 326, 695
16.	M2	2	221, 451	3	185, 353, 1111
17.	M3	4	125, 287, 451, 559	3	190, 363, 1050
18.	M4	5	94, 119, 221, 441, 473	3	196, 373, 1111
19.	M5	3	302, 448, 620	3	164, 317, 769
20.	M6	5	128, 148, 229, 257, 492	4	169, 317, 370, 613

21.	M7	1	459	1	942
22.	M8	5	138, 166, 182, 214, 230	3	138, 326, 695
23.	B4	5	96, 128, 294, 430, 473	3	201, 384, 1021
24.	B5	5	130, 164, 178, 206, 307	3	148, 321, 673
25.	B6	4	125, 294, 441, 559	3	196, 384, 1174
26.	B7	5	132, 150, 227, 262, 498	2	340, 990
27.	B8	5	99, 131, 232, 451, 559	4	201, 384, 429, 617
28.	B9	3	302, 457, 625	4	128, 148, 321, 694
29.	B10	5	136, 171, 189, 212, 316	3	98, 238, 531
30.	B11	3	234, 438, 605	3	156, 310, 918
31.	B12	4	141, 310, 459, 620	4	160, 302, 343, 568
32.	B13	3	229, 459, 620	3	152, 302, 918
33.	B14	1	470	3	120, 145, 781
34.	B15	5	144, 170, 190, 214, 324	3	131, 294, 628
35.	B16	5	140, 166, 178, 206, 302	4	124, 245, 276, 486
36.	J4	3	227, 450, 607	4	107, 120, 268, 598
37.	J5	4	146, 307, 463, 616	2	268, 854
38.	J6	5	141, 162, 190, 214, 324	3	302, 334, 678

Pooled analysis

Pooled analysis of ARDRA for rhizobial type isolates are represented by dendrogram (Fig 5). Pooled analysis represented four different clusters. Isolate A1, A5, J1, J3 were 100% similar and 92% similar with D1. Similarly, A3, A4 and J2 were 100% similar as well as all the three isolates showed 89% similarity with D2 and 39% with isolate B1. Isolate D3 were 83% similar with M1 and 50% with B2 in second cluster. Cluster three and four having single isolate each A2 and B3, respectively.

Overall ARDRA results indicated that there was great diversity among the rhizobial type isolates from the root nodules of green gram. All isolates were of different type and non-identical to each other, indicating good phylogenetic diversity in native isolates.

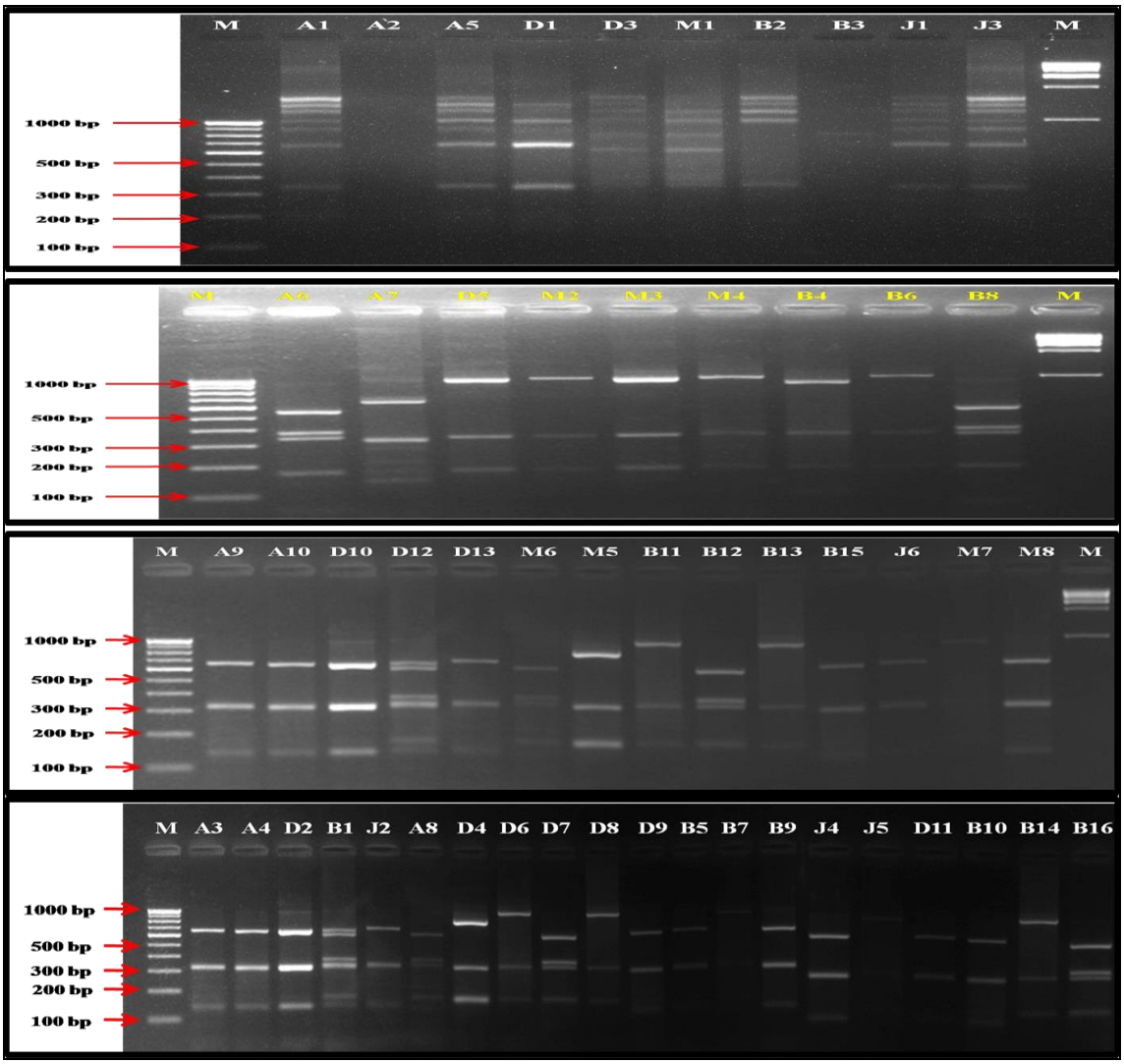

Fig 5: ARDRA pattern of all the rhizobial and NRE isolates with *Hinf*I

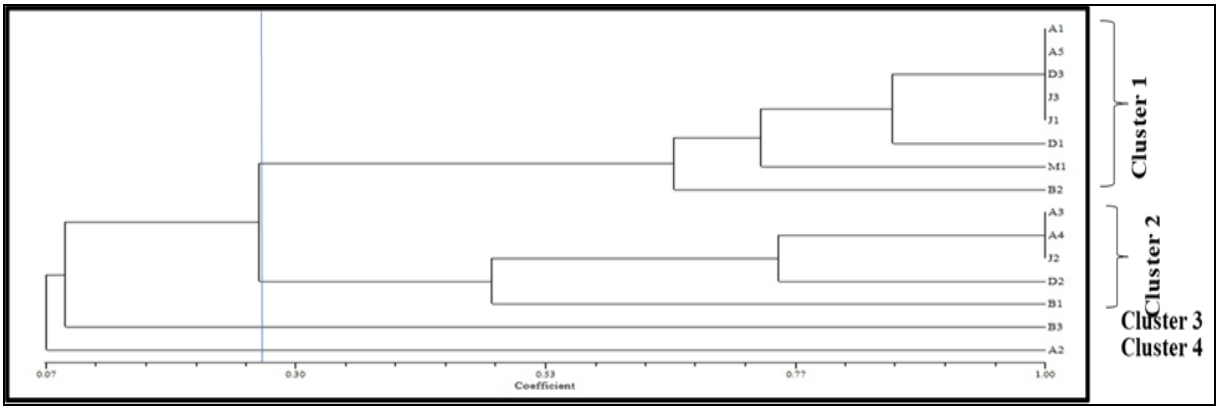

Fig 6: Dendrogram of Rhizobial isolates based on ARDRA profile of *Hinf*I

ARDRA analysis of non rhizobial isolates

Great diversity was found among the non rhizobial isolates after digestion of 16s *rDNA* with restriction enzymes as shown in Fig. 6. Enzyme *Hae*III restriction pattern obtained 162 DNA restriction fragments (Table 4) ranging in size 92 bp to 620 bp. Restriction analysis of non rhizobial isolates allowed five species specific profile (Fig. 1). While enzyme *Hinf*I showed 124 restriction fragments and found four specific restriction profiles (Fig. 2) ranging from 98 bp to 918 bp.

Digestion of non rhizobial isolates with *Hae*III restriction enzyme obtained five different clusters as shown in Fig. 6. In first cluster all the isolates were diverse having less than 80% similarity. The isolate A6 and D5 were found to have 100% similarity and all the other isolates namely B6, M4, B4, A8, B7, B8 and B6 were have less than 80% similarity. M3, B12 and J5 were found 100% with each other as well as M5, D4 and B6 were 100% similar with each other and less than 70% similar with above three isolates showing diversity within cluster. Another five isolates from second cluster were 100% similar namely B11, J4, D8, D6 and B13. The isolate M2 was found in separate branch showing less than 60% similarity with all the isolate found in second cluster. Third cluster includes only three isolates i.e. M2, M7 and B14. While, the two isolates (M7 and B14) were 100% similar with each other and 40% dissimilar with the isolate M2. The fourth cluster contains twelve isolates out of which ten isolates i.e. A7, A9, B16, A10, D10, D13, B15, D9, J6 and M8 were found 100% similar. While, isolate D12 and B5 were found in separate branches showing 30% dissimilarity with all remaining isolates.

*Hinf*I restriction enzymes digestion found total four clusters as shown in Fig. 7. Cluster one had twelve isolates, namely A6, M6, B12, B16, A8, D7, B8, D12, B15, D9 and B10. However, eleven isolates with more than 80% similarity were found in second cluster which includes A7, D10, A9, A10, D13, J6, D11, B5, M8, J4 and B9. Remaining fifteen isolates were found in third cluster namely D5, M2, M3, B14, M4, B4, B6, D8, D6, B11, B13, B7, J7, M5 and D4. Only single isolate M7 was found in separate fourth cluster.

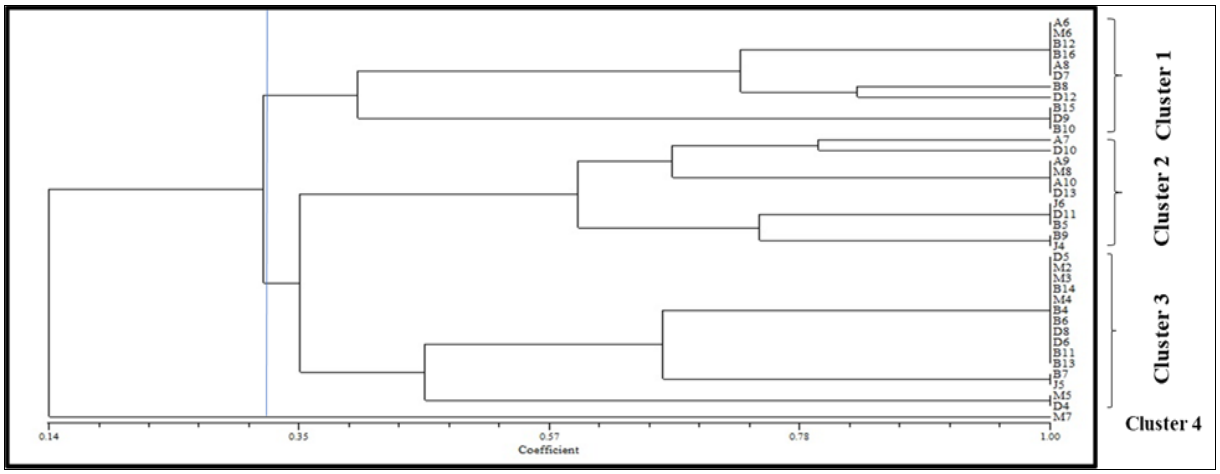

Fig 7: Dendrogram of Rhizobial isolates based on ARDRA profile of *Hinf*I

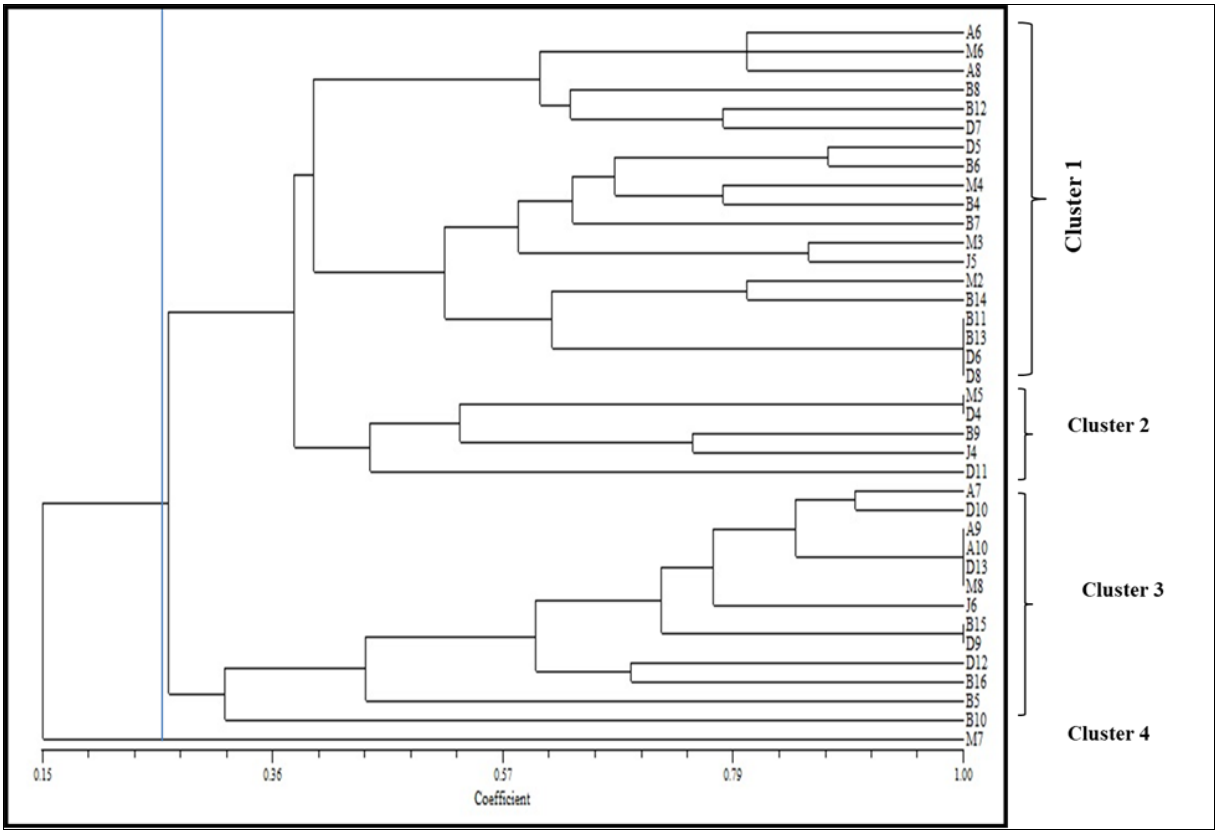

Fig 8: Dendrogram of Non-rhizobial isolates based on pooled ARDRA profile

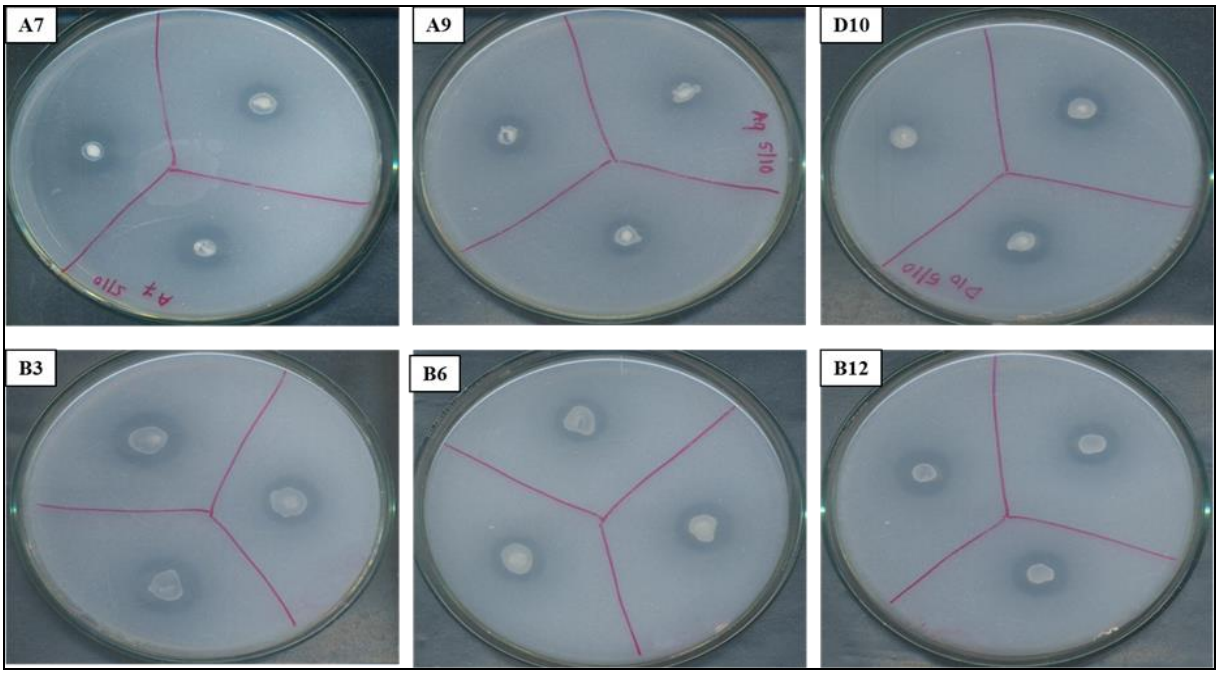

Fig 9: *In vitro* screening of rhizobial (B3) and NRE (A7, A9, D10, B6 & B12) isolates for phosphate solubilization after 5 DAI on Sperber agar

Pooled analysis

Pooled analysis for the two enzymes *Hae*III and *Hinf*I was represented by Fig. 8. Dendrogram showed that all isolates were dispersed in four different clades. First clade represents nineteen different isolates having less than 50% similarity. Out of which A6, M6 and A8 are 80% similar with each other and 45% similar with other three isolates namely B8, B12 and D7. While, four isolates were 100% similar with each other namely B11, B13, D6 and D8. Five isolates were found in second cluster out of which two were 100% similar (M5 & D4). Remaining fourteen isolates were found in fourth cluster in which isolate A9, A10, D13 and M8 were completely similar exhibiting 100% similarity. In addition isolate B15 & D9 were 100% similar with each other. Isolate M7 was completely distinct found in fourth cluster.

Phosphate solubilization

All the isolates were screened for phosphate solubilization on sperber agar. Clear zone formed around the growing colonies indicating the phosphate solubilization activity. Total 15 rhizobial type and 38 NRE isolates were screened for phosphate solubilization on sperber agar containing Tri-Calcium Phosphate as insoluble source. Wherein, the 17 isolates showed clear zone around their colony exhibiting phosphate solubilization ability within 5 days (Table 5). However, isolates A7, A9, D10, B3, B6 and B12 shown strong phosphate solubilization activity exhibiting clear zone around their colony (Fig. 9).

IAA production

Glucose phosphate broth containing L-tryptophan (0.005M) was used to screen IAA production and intensity of pink color developed within 30 min was measured at 535 nm in UV/VIS spectrophotometer. The quantity of IAA was determined by comparison with an IAA standard curve.

Production of IAA was varied greatly among both rhizobial type and NRE isolates with the highest by isolate A5 (116. 50 µg ml^{-1}) and the lowest by isolate D11 (10.50 µg ml^{-1}) as shown in Table 6. Isolate A5 (116.50µg ml^{-1}) and B6 (116.33µg ml^{-1}) exhibited the highest IAA production followed by J3 (115.33 µg ml^{-1}), A2 (114.5µg ml^{-1}) and B12 (107.17 µg ml^{-1}). IAA production was reported as common in plant associated bacteria as part of colonization strategy that involves phytostimulation and circumvention of plant defense mechanisms (Tariq *et al.,* 2012) [31].

Table 5: Phosphate solubilization (PS)

Isolate Name	Isolates	PS	Isolate Name	Isolates	PS	Isolate Name	Isolates	PS
A1	R	-	D9	NRE	-	B6	NRE	++
A2		+	D10		++	B7		-
A3		-	D11		-	B8		-
A4		-	D12		+	B9		-
A5		+	D13		+	B10		-
A6	NRE	-	M1	R	-	B11		-
A7		++	M2	NRE	-	B12		++
A8		-	M3		-	B13		-
A9		++	M4		-	B14		-
A10		-	M5		-	B15		+
D1	R	+	M6		-	B16		-
D2		-	M7		-	J1	R	-
D3		-	M8		-	J2		-
D4	NRE	+	B1	R	-	J3		-
D5		-	B2		-	J4	NRE	+
D6		-	B3		++	J5		+
D7		-	B4	NRE	-	J6		+
D8		+	B5		-			

Note: ++ Strongly Positive + Positive, - Negative R- Rhizobial type isolates
NRE- Non Rhizobial Endophytes

Table 6: Indole Acetic Acid production

Isolate	IAA (µg ml^{-1})	Isolate	IAA (µg ml^{-1})	Isolate	IAA (µg ml^{-1})
A1	87.33	D9	32.33	B6	116.33
A2	114.50	D10	12.83	B7	23.67
A3	36.50	D11	10.50	B8	18.17
A4	57.00	D12	30.67	B9	20.83
A5	116.50	D13	48.83	B10	53.00
A6	48.17	M1	35.83	B11	80.50
A7	27.17	M2	22.50	B12	107.17
A8	56.83	M3	52.33	B13	22.50
A9	105.17	M4	11.33	B14	27.67
A10	22.33	M5	29.17	B15	58.83
D1	47.17	M6	102.17	B16	21.33
D2	90.17	M7	15.50	J1	95.17
D3	16.33	M8	28.00	J2	12.00
D4	40.00	B1	15.83	J3	115.33
D5	29.17	B2	38.67	J4	25.17
D6	46.17	B3	98.83	J5	18.17
D7	41.33	B4	13.83	J6	66.33
D8	69.33	B5	20.17		

Discussion

In nitrogen limiting condition, association between legumes and symbiotic associates belonging to α and β Proteobacteria results in development of specialized structure for N_2 fixation called root/stem nodules. Regardless of the benefits of symbiotic association to both partners, entry of rhizobia into host plant roots occurs by a series of well coordinated events from both symbionts. An

increasing number of α, β and γ Proteobacteria have been isolated from root nodules of a wide range of legumes regardless of symbiosis specificity at multiple phases of the interaction between both the partners and are reported as nodule associated bacteria or nodule endophytes (Zakia *et al*., 2006; Kan *et al*., 2007) [13]. Even though there is very high specificity between legume crops and *Rhizobium*, the non-rhizobial endophytes such as *K. pneumoniae*, *P. fluorescence*, *Bacillus* sp., *Enterobacter* sp., *Klebsiella* sp. and *Paenibacillus* sp. were frequently isolated from root nodules by different scientists (Pandya *et al*. 2013; Zhang *et al*. 1996; De Meyer *et al*. 2015; Selvakumar *et al*. 2008; Ibanez *et al*. 2009; Tariq *et al*. 2012; Rajendran *et al*. 2011; Egamberdieva *et al*. 2010) [19, 35, 4, 37, 11, 31, 21, 7].

In present investigation total 53 rhizobial (15) and non rhizobial (38) endophytes were isolated and studied for their diversity, phosphate solubilization and IAA production. The molecular technique always offers huge potential for studying the microbial ecosystems (Smit, 1997) [26]. Although various other *rRNA* gene analysis-based techniques were available to carry out more rapid analysis (e.g., denaturing or temperature gradient gel electrophoresis), the ARDRA approach used in this investigation provided elevated level of resolution and the generation of additive and retrievable data which might also be used to generate other taxonomical probes and primers (e.g., to target smaller groups of microorganisms) for use in further studies (Filion *et al*., 2004) [8]. ARDRA analysis has emerged as useful tool which provide a very useful data regarding the microbial diversity and timely changes in community structure within specified niche. The 16S-ARDRA technique proved to be an alternative to more laborious and expensive methods for the identification of eubacteria, as it is simple, relatively fast and highly repetitive (Dec *et al*., 2016) [5]. Our study clearly supports the hypothesis that the structure of community of rhizobial type and non rhizobial isolates was diverse and variable inside the root nodules since four different clusters were observed separately. For this study in present investigation universal primers (27f and 1492r) were used to amplify the entire 16S *rDNA* to obtain a highest discriminatory power. However, care must be taken since the discriminatory power will decrease if primers are chosen in such a way that less restriction sites are present in the amplification products (Stakenborg *et al*., 2005) [29]. The combined application of two restriction enzymes (HaeIII and HinfI) for digestion of 16S rDNA allowed dividing the total 53 isolates into several phylogenetic groups.

Afterwards, 17 out of 53 isolates were exhibited the phosphate solubilization ability and it is well established that after nitrogen, phosphorus (P) is the most limiting nutrient for plant growth. It exists in both inorganic (bound, fixed or labile) and organic (bound) forms; its concentration depends on the parental material. Although the parent material has a strong control over the soil P status, the availability of P to plants is influenced by pH, compaction, aeration, moisture, temperature, texture and organic matter of soils, crop residues, extent of plant root systems and root exudates secretions and available soil microbes. The phosphate-solubilizing activity characterizes the microorganisms with ability to produce and release metabolites such as organic acids that chelate the cations bound to phosphate, converting them into soluble forms. Hence, rhizobia and NRE isolates helps in P release to the plants that absorb only the soluble P like monobasic ($H_2PO_4^-$) and dibasic ($H_2PO_4^{2-}$) forms (Hajjam *et al*., 2016; Dinic *et al*., 2014; Kumar and Ram, 2014; Palaniappan *et al*., 2010; Sridevi and Mallaiah, 2007) [10, 14, 17, 28].

Furthermore, all the 53 isolates showed variable pattern of IAA production. IAA is the foremost phytohormone that accelerates plant growth and development by improving root/shoot growth and seedling vigor (Tariq *et al*., 2012; Rajendran *et al*., 2011) [31, 21]. IAA is involved in cell division, differentiation and vascular bundle formation and an essential hormone for nodule formation. IAA also plays significant role in plant signaling pathways to coordinate the physiological and morphological responses. IAA-mediated induction of primary and adventitious root development in young seedlings will augment their establishment in soil and facilitate the uptake of nutrients and water. In addition, IAA plays an important role in the regulation of plant abiotic stresses induced by ethylene (Sekar *et al*., 2018; Bhutani *et al*., 2018; Pandey *et al*., 2015) [2].

Conclusions

A unique feature of the plants of the legume family (*Leguminosae* or *Fabaceae*) is establishment of some nitrogen-fixing symbiotic bacterial genera in root or stem nodules, collectively called rhizobia. *Rh*izobia are included in the well-known genera *Rhizobium*, *Bradyrhizobium*, *Sinorhizobium*, *Mesorhizobium*, *Azorhizobium* and *Allorhizobium* of *Alphaproteobacteria*. The rhizobium-legume symbiosis has been studied intensively, because of their economic and ecological importance. However, root nodules also accommodate various non-rhizobial endophytes (NRE) having definite influence on the survival, nodulation and yield of the crop. Therefore, in present investigation 53 rhizobial and non rhizobial endophytes were isolated and their diversity was studied. Based on results obtained it is concluded that the great diversity of rhizobial and non rhizobial endophytes present inside the root nodule environment. This result opens up the door for study and development of consortium containing rhizobial and non rhizobial isolates for the application in leguminous plants.

References

1. Ardley J, Parker MA, De Meyer SE, O'Hara G, Reeve W, Yates RJ, Dilworth M, *et al*. *Microvirga lupini* sp. nov., *Microvirga lotononidis* sp. nov. and *Microvirga zambiensis* sp. nov. are alphaproteobacterial root nodule bacteria that specifically nodulate and fix nitrogen with geographically and taxonomically separate legume hosts. Int J Syst Evol Microbiol. 2012;62:2579-2588.
2. Bhutani N, Maheshwari R, Negi M, Suneja P. Optimization of IAA production by endophytic *Bacillus* spp. from *Vigna radiata* for their potential use as plant growth promoters. Israel J Plant Sci. 2018;65:1-2. Doi:10.1163/22238980-00001025.
3. Chen WM, Laevens S, Lee TM, Coenye T, DeVos P, Mergeay M, Vandamme P. *Ralstonia taiwanensis* sp. nov. isolated from root nodules of Mimosa species and sputum of acystic fibrosis patient. Int J Syst Evol Micro. 2001;51:1729–1735.
4. De Meyer SE, Cnockaert M, Ardley JK, Van Wyk BE, Vandamme PA, Howieson JG. *Burkholderia dilworthii* sp. nov., isolated from *Lebeckia ambigua* root nodules. Int. J. Syst. Evol. Microbiol. 2014;64:1090-1095.

5. Dec M, Puchalski A, Urban-Chmiel R, Wernicki A. 16S-ARDRA and MALDI-TOF mass spectrometry as tools for identification of Lactobacillus bacteria isolated from poultry. BMC Microbiology. 2016;16:105. DOI 10.1186/s12866-016-0732-5
6. Dinić Z, Ugrinović M, Bosnić P, Mijatović M, Zdravković J, Miladinović M, *et al*. Solubilization of Inorganic Phosphate by Endophytic *Pseudomonas* sp. from French Bean Nodules. Ratar Povrt. 2014;51(2):100-105.
7. Egamberdieva D, Berg G, Lindstrom K, Rasanen LA. Coinoculation of *Pseudomonas* spp. with *Rhizobium* improves growth and symbiotic performance of fodder galega (*Galega orientalis* Lam.). Eur J Soil Biol. 2010;46:269–272.
8. Filion M, Hamelin RC, Bernier L, St-Arnaud M. Molecular Profiling of Rhizosphere Microbial Communities Associated with Healthy and Diseased Black Spruce (*Picea mariana*) Seedlings Grown in a Nursery. Appl Environ Microbiol. 2004;70(6):63541–3551.
9. Glickmann E, Dessaux Y. A critical examination of the specificity of the Salkowski reagent for indolic compounds produced by phytopathogenic bacteria. Appl Environ Microbiol. 1995;61(2):793.
10. Hajjam Y, Alami IT, Udupa SM, Cherkaoui S. Isolation and evaluation of phosphate solubilizing rhizobia from root nodules of faba bean *(Vicia faba L.)* in Morocco. J Mater Environ Sci. 2016;7(11):4000-4010.
11. Ibanez F, Jorge A, Tania T, Marıa LT, Adriana F. Endophytic occupation of peanut root nodules by opportunistic Gammaproteobacteria. Sys Appl Micro. 2009;32:49–55.
12. Jhala YK, Vyas RV, Shelat HN, Patel HK, Patel HK, Patel KT. Isolation and characterization of methane utilizing bacteria from wetland paddy ecosystem. World J Microbiol Biotechnol. 2014;30(6):1845–60.
13. Kan FL, Chen ZY, Wang ET, Tian CF, Sui XH, Chen WX. Characterization of symbiotic and endophytic bacteria isolated from root nodules of herbaceous legumes grown in Qinghai–Tibet plateau and in other zones of China. Archives of Microbiol. 2007**;**188(2):103-115.
14. Kumar GK, Ram MR. Phosphate Solubilizing Rhizobia Isolated from *Vigna trilobata.* Am J Microbiol Res. 2014;2(3):105-109.
15. Lee KD, Bai Y, Smith D, Han HS, Supanjani. Isolation of plant growth-promoting endophytic bacteria from bean nodules. Res J Agric Biol Sci. 2005;1(3):232-236.
16. Moulin L, Munive A, Dreyfus B, Boivin-Masson C. Nodulation of legumes by members of the beta-subclass of Proteobacteria. Nature. 2001;411:948-950.
17. Palaniappan P, Chauhan PS, Saravanan VS, Anandham R, Sa T. Isolation and characterization of plant growth promoting endophytic bacterial isolates from root nodule of *Lespedeza* sp. Biol Fertil Soils. 2010;46:807–816.
18. Pandya M, Rajput M, Rajkumar S. Exploring plant growth promoting potential of non rhizobial root nodules endophytes of *V. radiata.* Microbiol. 2015**;**84(1):110–119.
19. Pandya M, Shalini R, Kumar GN. Invasion of rhizobial infection thread by non-rhizobia for colonization of *Vigna radiata* root nodules. FEMS Micro Lett, 2013, 1-8.
20. Radl V, Simoes-Araujo JL, Leite J, Passos SR, Martins LMV, Xavier GR, *et al*. *Microvirga vignae* sp. nov., a root nodule symbiotic bacterium isolated from cowpea grown in semi-arid Brazil. Int J Syst Evol Microbiol. 2014;64:725-730.
21. Rajendran G, Patel MH, Joshi SJ. Isolation and characterization of nodule-associated *Exiguobacterium* sp. from the root nodules of Fenugreek (*Trigonella foenum-graecum*) and their possible role in plant growth promotion. Inter J Microbiol. 2011;2012:1-8.
22. Rivas R, Willems A, Subba-Rao NS, Mateos PF, Dazzo FB, Kroppenstedt RM, Martínez-Molina E, *et al*. Description of *Devosia neptuniae* sp. nov. that nodulates and fixes nitrogen in symbiosis with *Neptunia natans*, an aquatic legume from India. Syst Appl Microbiol. 2003;26:47-53.
23. Rohlf F, NTSYS-Pc. Numerical taxonomy and multivariate analysis system version 2.02e. Exeter Software. New York, 1997.
24. Sambrook J, Fritsch EF, Maniatis T. Analysis and cloning of eukaryotic genomic DNA in Molecular cloning. Cold Spring Harbor Laboratory Press, 1989, 9-19.
25. Gupta P, Sharma VK. Organic farming in Himachal Himalaya: Learning lessons from past and present. Int. J Agric. Nutr. 2021;3(1):14-18. DOI: 10.33545/26646064.2021.v3.i1a.40
26. Smit E, Leeflang P, Wernars K. Detection of shifts in microbial community structure and diversity in soil caused by copper contamination using amplified ribosomal DNA restriction analysis. FEMS Microbiol Ecology. 1997;23:249-261.
27. Sneath IHA, Sokal RR. Numerical taxonomy. W. H. Freeman and Company, San Francisco, 1973.
28. Sridevi M, Mallaiah KV. Phosphate solubilization by *Rhizobium* strains. Indian J Microbiol. 2009;49:98-102.
29. Stakenborg T, Vicca J, Butay P, Maes D, Baere TD, Verhelst R, *et al*. Evaluation of amplified rDNA restriction analysis (ARDRA) for the identification of *Mycoplasma* species. BMC Infectious Diseases. 2005;5:46.
30. Sy A, Giraud E, Jourand P, Garcia N, Willems A, de Lajudie P, *et al*. *Methylotrophic Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J Bacterio. 2001;183:214-220.
31. Tariq M, Hameed S, Yasmeen T, Ali A. Non-rhizobial bacteria for improved nodulation and grain yield of mung bean [*Vigna radiata* L. Wilczek]. Afri J Biotech. 2012;11(84):15012-15019.
32. Taurian T, María SA, Jorge GA, María LT, Liliana L, Dayana P, *et al*. Phosphate-solubilizing peanut associated bacteria: screening for plant growth-promoting activities. Plant Soil. 2010;329:421–431.
33. Trujillo ME, Willems A, Abril A, Planchuelo AM, Rivas R, Ludena D, *et al*. Nodulation of *Lupinus albus* by strains of *Ochrobactrum lupini* sp. nov. Appl Environ Microbiol. 2005;71:1318-1327.
34. Zakhia F, Jeder H, Willems A, Gillis M, Dreyfus B, Lajudie P. Diverse bacteria associated with root nodules of spontaneous legumes in tunisia and first report for *nifH*-like Gene within the Genera *Microbacterium* and *Starkeya*. Microb Ecol. 2006;51(3):375-393.
35. Zhang F, Dashti N, Hynes RK, Smith DL. Plant growth

promoting rhizobacteria and soybean [*Glycine max* (L) Merr] nodulation and nitrogen fixation at suboptimal root zone temperatures. Ann Bot. 1996;77:453-459.
36. Zhao LF, Xu YJ, Ma ZQ, Deng ZS, Shan CJ, Wei GH. Colonizat ion and plant growth promoting characterization of endophytic *Pseudomonas chlororaphis* strain Zong1 isolated from *Sophora alopecuroides* root nodules. Brazilian J Microbiol. 2013;44(2):629-637.
37. Selvakumar G, Kundu S, Gupta AD, Shouche YS, Gupta HS. Isolation and characterization of non-rhizobial plant growth promoting bacteria from nodules of Kudzu (*Pueraria thunbergiana*) and their effect on wheat seedling growth. Curr Microbiol. 2008;56:134-139.