Journal of Pharmacognosy and Phytochemistry
Available online at www.phytojournal.com

E-ISSN: 2278-4136
P-ISSN: 2349-8234
JPP 2018; 7(1): 418-423
Received: 18-11-2017
Accepted: 20-12-2017

Rahul V Kadam
B.N.F. Schemeand Department of Agricultural microbiology, College of Agriculture, Pune, (MH) India

Dr. SW Jadhav
B.N.F. Schemeand Department of Agricultural microbiology, College of Agriculture, Pune, (MH) India

PB Abhang
Department of Plant pathology PGI, Dr. P.D.K.V. Akola (MH) India

Correspondence
Rahul V Kadam
B.N.F. Schemeand Department of Agricultural microbiology, College of Agriculture, Pune, (MH) India

Molecular characterization of MPKV biofertilizers

Rahul V Kadam, Dr. SW Jadhav and PB Abhang

Abstract
The present investigation entitled, **"**Molecular characterization of MPKV biofertilzer" was focused on molecular diversity among the bacterial spp. by employing DNA fingerprinting technique, Random Amplified Polymorphic DNA.
DNA fingerprinting at different bacterial spp. were carried out using RAPD analysis. To find out the relatedness and diversity based on polymorphism in RAPD profile. The total genomic DNA of bacterial strains was isolated by following the method given by Ivanova *et al.;* Ten RAPD primers of OPB series used for RAPD analysis, ten primers viz., RBa-01, RBa-02, RBa-03, RBa-04, RBa-05, RBa-06, RBa-07, RBa-08, RBa-09and RBa-10. RBa-01, RBa-04 and RBa-06 yielded good and different banding pattern. In the present study the size range of the bands amplified with selected 10 random primers was found in between 5500bp to 250bp when compared with DNA Molecular weight marker of 1kb. Thus, on the basis of variation of size number and intensity of bands, *Rhizobium* species were grouped into two groups: group I contains *Bradyrhizobium japonicum, Rhizobium loti* and *Rhizobium spp.* and group II contains *Rhizobium meliloti, Rhizobium leguminasorum* which depicts the diversity between them. This study demonstrates that the RAPD markers are useful in assessing diversity at genetic level rather than morphological markers which are subjected to environmental fluctuations.

Keywords: MPKV biofertilizers, DNA fingerprinting technique, *Rhizobium loti.*

1. Introduction
The use of biofertilizers is steadily increasing day by day. There are number of firms producing different types of biofertilizer, however the major question remaining unanswered regarding of quality. The quality depends largely on genetic makeup of the microbes used in production of that particular biofertilizer under such circumstance branding and intellectual property right (IPR) assumes paramount importance. Keeping these aspects in mind it is thought to be very essential to have DNA fingerprinting of different bacterial strains used for the production of biofertilizers under BNF scheme at College of Agriculture, Pune-5.
Fast and accurate identification of bacterial isolates is becoming increasingly important for epidemiology, taxonomy and ecological studies. Traditional identification method was involved slow and cumbersome and primarily based on phenotypic microbiological and biochemical characterization of traits such as antibiotic resistance, phase sensitivity or iso-enzymes comparison. Genotypic identification methods have also been used including restriction endonuclease fingerprinting, hybridization with strain specific DNA**.**
RAPD is the most popular method being used to differentiate among individuals (Williams *et al.* 1990) [10]. In this procedure, an arbitrary 10 base pair nucleotide sequence anneals to complementary template sequence throughout the genome and act as a primer for extension by a heat stable Taq DNA polymerase, the results are a series of DNA fragments that are amplified in each individual. Each individual has a specific amplification pattern that may differ as a result of mismatch between different primer and template sequence.

Material and methods
Experimental material
The experimental material of present study comprised of bacterial culture of *Rhiizobium species (Bradyrhizobium japonicum, R. loti, R. melioloti, R. leguminosarum, R. spp, R. phaseoli), Acetobacter (PAL-5), Azotobacter (strain-3), PSB (Bacillus megaterium), Azospirillum (Azospirillum brassilens)* and *BGA (Nostoc)* which are used for commercial production of different Biofertilizers.

Bacterial growth characteristics

Table 1: The pure culture of bacterial strains and used respective media.

Sr.no.	Name of Liquid medium	Name of bacterial stain
1	Yeast extract manitol agar medium	*Rhizobium spp*
2	Jensen's medium	*Azotobacter strain-3*
3	LGIP Medium	*PAL-5*
4	Pikovskya's Medium	*Bacillus megaterium*
5	Sodium malate medium	*Azospirillum brassilens*
6	Fogg's medium	*BGA*

Molecular analysis

DNA Extraction

The total genomic DNA of bacterial cultures was isolated by following method given by lvanova *et al.*, (2000) [4] with some modification.

Protocol for DNA extraction-

1. Take 10 ml of bacterial culture and cetrifuse it at 10000rpm for 10 minutes at 4 °C.
2. Resuspend the pellet in 10 mM Tris, 100mM Nacl and cetrifuse it at 10000 rpm for 10 minutes at 4 °C.
3. Resuspend the pellet in 2.5 ml of TE (Tris 50mM,EDTA 20mM) buffer containing 500 µlit of lysozyme (100mg/µl)
4. Incubate at 37 °C for 20 minutes containing 25 µlit of RNase A (10mg/µl).
5. Add 2.5 ml of 2% SDS.
6. Incubate at 50 °C for 45 minutes.
7. After incubation add 50 µl of protenase k (20mg/µl)
8. Again incubate at 55 °C for 10 minutes.
9. Add equal volume of CIA and cetifuge it at 10,000 rpm for 10 minutes at 4 °C.
10. After centrifugation an aqueous phase is obtained and in that add ice cold ethanol (double volume)
11. Centrifuge it at 10,000 rpm for 10 minutes at 4 °C.
12. After centrifugation pellet is obtained.
13. Air dry the pellet and dissolved in 50-100 µl of TE buffer

DNA quantification and purity analysis

Protocol for DNA Quantification

1 Confarmation of DNA in the sample was carried out on 0.8% agarose gel containing ethidium bromide @ 0.5 mg/ml.

2 5µl of sample was loaded and after 5 cm run, gel was observed under UV light and the DNA yield and quality was confirmed.

3 After confirmation of the DNA integrity it was quantified using Spectrophotometer. The concentration of purified DNA were measured by measuring absorbance at 260 and 280 nm.

4 Five µl of all DNA extracts were electrophoresed (Bio Rad sub cell model 96 USA) in 0.8% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide at 60 V/cm in TBE buffer.

5 After electrophoresis the band intensity of genomic DNA was visualized on gel documentation unit. And compared to standard unit λ DNA. This gels also provided a visual measure of the purity and integrity of the DNA.

Quality of DNA

Quality of DNA was observed by running 50ng DNA in 0.8 % agrose gel.And measuring the absorbance at 260 280 nm.

Dilution of DNA

Dilution were made of the samples to reach the value up to 25 ng/µl. The dilution were also checked by compairing them with the DNA quantification standards in agarose gel.

DNA amplification by RAPD-PCR technique:

RAPD amplification was performed as per protocol by Williams *et al.* (1990) [10]. However, modifications were made to enhance reproducibility and consistency in RAPD profiles. Amplifications were performed in a 0.2ml PCR tubes.

Result and discussion

Molecular characterization

Rapd analysis

The genomic DNA of 8 bacterial strains was analyzed using RAPD primer set obtained from RBa series. Ten random primers were selected for their ability to yield clear amplification patterns. The analysis showed variation in the profiles depending on the primer and genomic DNA used

In the similar way *Bacillus megatherium*, *Azatobacter strain-3* and *Azospirillum brassilens* strains shared different DNA banding pattern, but there was no DNA amplification in strain of *Acetobacter* and BGA. RAPD technique has been frequently used for identification and differentiation of bacterial stains such as *Rhizobium* **(**Hebb *et al.*; 1998.) [3] and *Azospirillum* (Fani *et al.*; 1993.) [2] According to Young and Cheng (1998) [11], RAPD is a potential tool for identification of the genetics and systematic of different populations.

Molecular characterization

The random and genomic wide nature of the RAPD technique is able to indicate over all genetic relatedness or dissimilarity than sequence analysis of a single region of the genome (Achenbach *et al.*; 1996) [1]. This assay is rapid independent of gene expression and proving to be beneficial for the grouping of bacterial strains. In DNA fingerprinting, the present challenge is to compile standardized pattern in a database for inter laboratory use and future reference. RAPD is applicable at different levels of taxonomic resolution (species, subspecies and strains etc).

In the present study, RAPD marker analysis was conducted to detect the genetic relatedness and variation between bacterial strains. The total genomic DNA of bacterial strains was isolated by following the method given by Ivanovo *et al.*; (2000) [4]. With some modifications giving higher yield and pure DNA.

Diversity is important for genetic characterization with different nitrogen fixing capacities, using diversity analysis; novelties of new *Rhizobium spp.* with high nitrogen fixing potential could be revealed which have not been identified ever before. Genomic DNA fingerprinting using random amplification of polymorphic DNA (RAPD) has been found

to be useful in differentiating between closely related bacteria. Randomly amplified polymorphic DNA (RAPD) profiles have provided new tools for investigating genetic polymorphism. This method was used by Van Rossum *et al.;* (1995) [9] for genetic analysis of *Bradyrhizobium* stains nodulating *Arachis hypogea* and nodule isolates of *Arachis spp*. (Khbaya *et al.*; 1998) [5]. Using diversity analysis, novelties of bacterial spp. with high nitrogen fixing potential could be revealed. Genomic DNA fingerprinting using Random amplification of polymorphic DNA (RAPD) has been found to be useful in differentiating between closely related bacteria. This result implies that *Rhizobium* strains belonging to group I and those from group II are significantly diverse.

The result indicates that RAPD is discriminative and efficient method for differentiating and studying genomic diversity of *Rhizobium* strains. RAPD fingerprinting was used for identification and assessment of genetic diversity within the field population of *B. japonicum* by Sikora *et al.*; 1997 [8]. From the analysis it is clear that most of the genetic diversity is found within the population.

Diversity among the bacterial strains was assessed on the basis of variation of size number and intensity of bands (Saleena *et al.*; 2001) [7]. DNA amplified by using RAPD – PCR revealed banding pattern depending on the number and size of amplified products which were observed in *Rhizobium* species, *Bacillus megatherium, Azotobacter strain-3, Azospirillum brassilens.* All the bacterial strains shared different DNA banding pattern.

RAPD technique has been frequently used for identification of bacterial strains such as *Rhizobium* (Hebb *et al.*; 1998) [3] and *Azospirillum* (Fani *et al.*; 1993) [2]. According to Young and Cheng (1998) [11], RAPD is the potential tool for the identification of the genetics and systematic of different populations.

In the present study, the size range of the bands amplified with selected 10 random primers was found to vary between 5500 bp to 250 bp when compared with DNA molecular weight marker of 1kb. P. Lognathan *et al.;* (1999) recorded overall size of amplification product ranging from 0.2 kb to 5 kb when a comparison was made between the types strain and the isolates, in the characterization of two genetically distant groups of *Acetobacter* diazotrophicus.

Thus, the present study indicated its usefulness in assessing genetic diversity of *Rhizobium* species by analyzing differences at DNA level rather than morphological markers which are subjected to environmental fluctuations.

Thus, the assessment of genetic diversity by using DNA polymorphism is a potent tool to obtain clear cut picture of genetic distances among the various bacterial strains which may have close relatedness.

Table 2: DNA number in bacterial strain on the basis of RAPD-PCR analysis using random primer of RBa sereies

Sr. no	Bacterial strain	No. of bands observed in a Primer									
		RBa1	RBa 2	RBa 3	RBa 4	RBa 5	RBa 6	RBa 7	RBa 8	RBa 9	RBa 10
	Bradyrhizobium japonicum	8	4	6	2	4	3	0	3	2	3
	Rhizobium loti	4	6	0	5	2	4	3	5	4	7
	Rhizobium meliloti	3	3	3	4	4	3	0	3	4	0
	Rhizobium leguminasorum	5	3	3	0	5	3	0	3	4	0
	Rhizobium spp.	4	7	2	4	4	2	6	5	3	1
	Bacillus megaterium	3	1	4	4	4	7	5	3	7	5
	Azotobacter chrococum	4	5	6	8	7	7	3	4	3	4
	Azospirillum brassilens	3	1	1	7	3	4	3	2	4	3

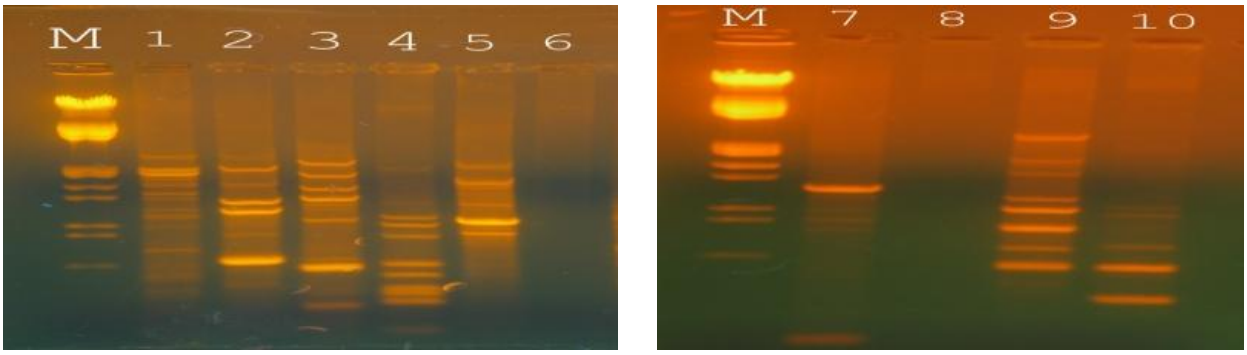

Plate 2: DNA Amplification profile of different bacterial species using primer – 1

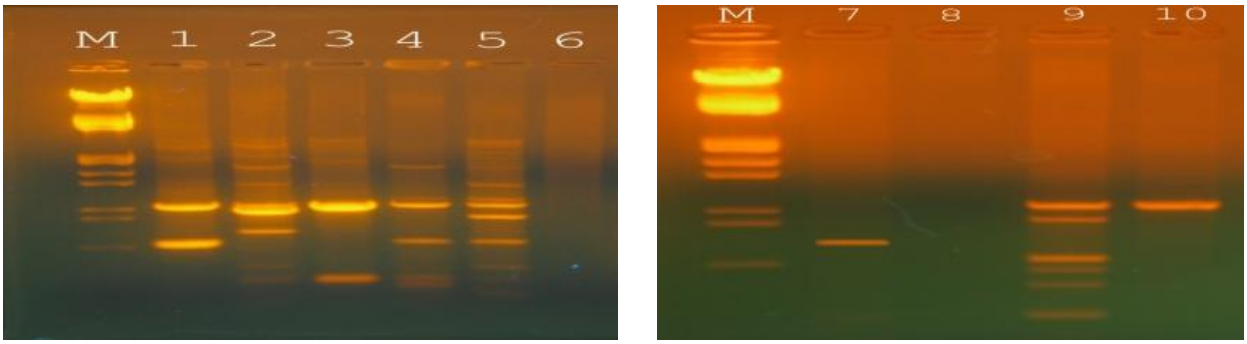

Plate 3: DNA Amplification profile of different bacterial species using primer – 2

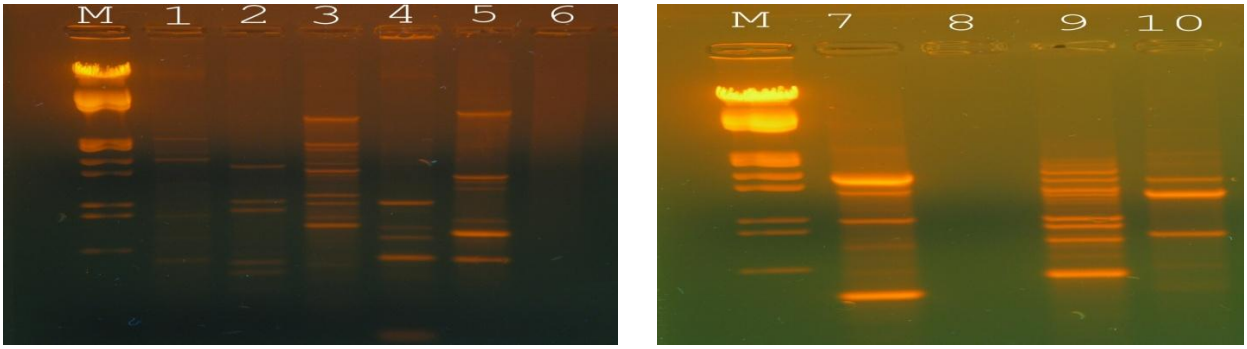

Plate 4: DNA Amplification profile of different bacterial species using primer – 3

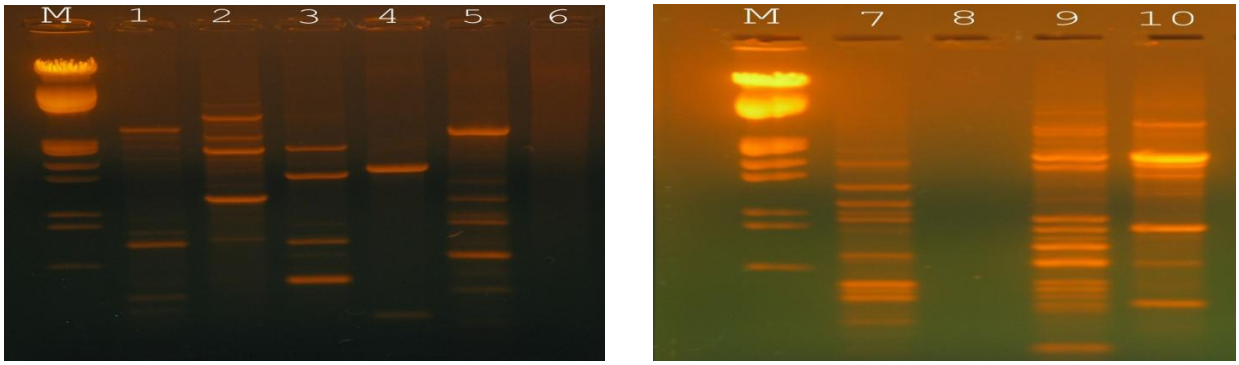

Plate 5: DNA Amplification profile of different bacterial species using primer – 4

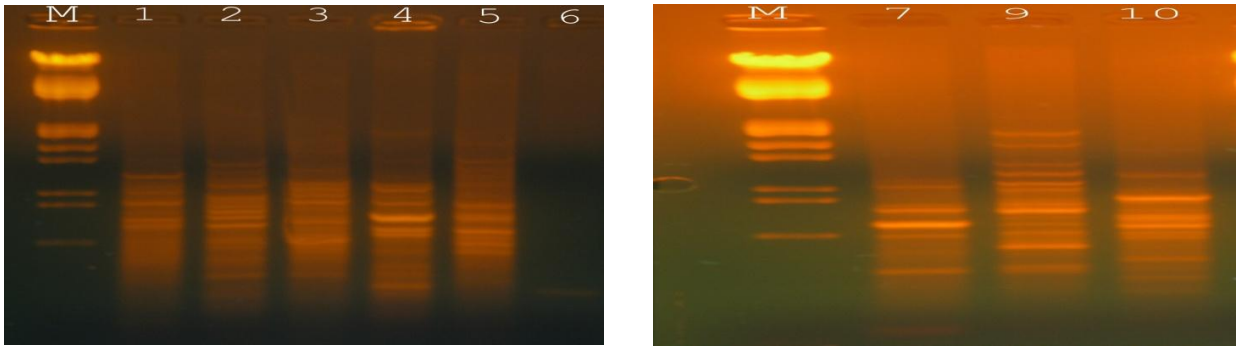

Plate 6: DNA Amplification profile of different bacterial species using primer – 5

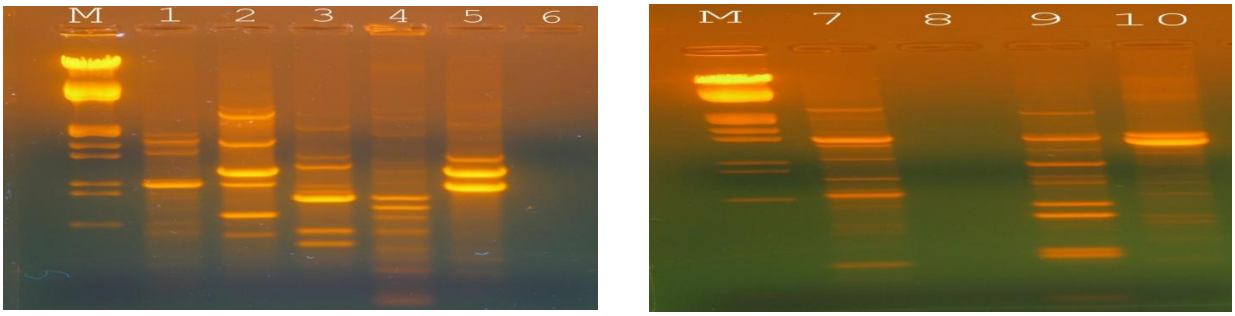

Plate 7: DNA Amplification profile of different bacterial species using primer – 6

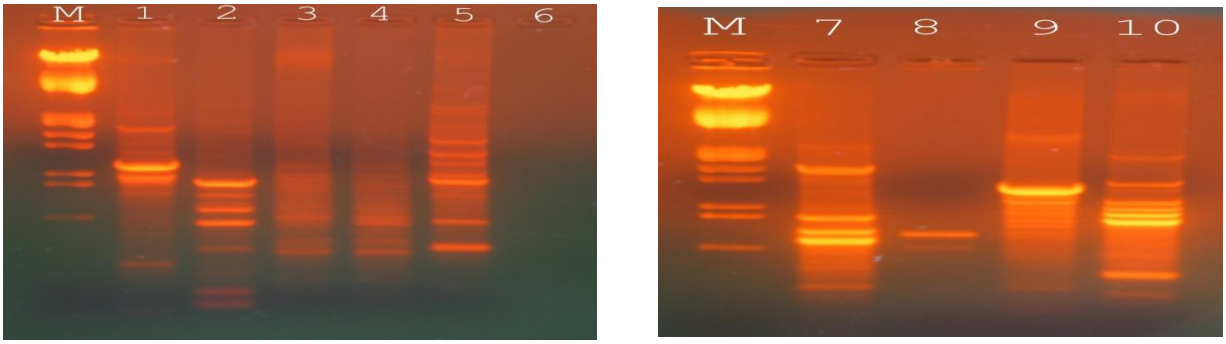

Plate 8: DNA Amplification profile of different bacterial species using primer – 7

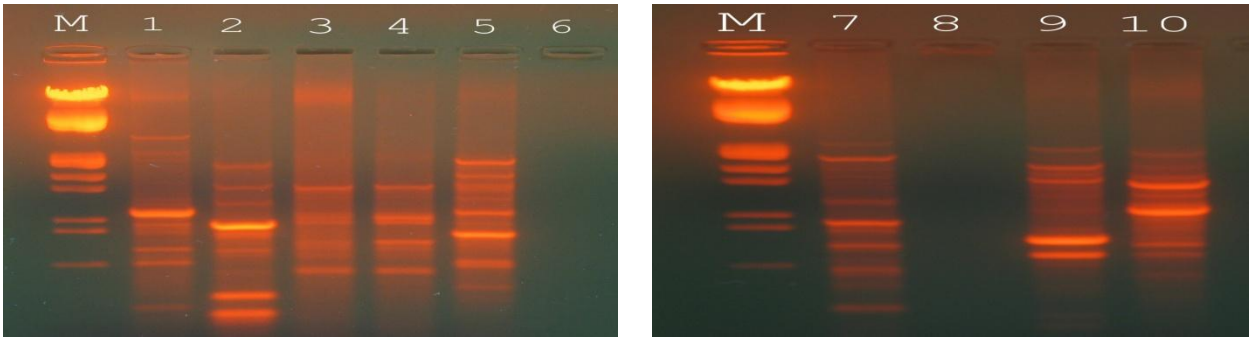

Plate 9: DNA Amplification profile of different bacterial species using primer – 8

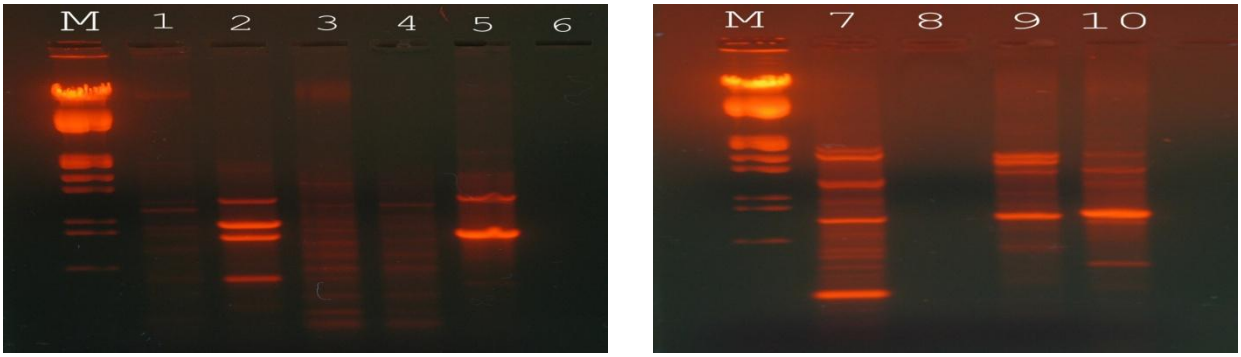

Plate 10: DNA Amplification profile of different bacterial species using primer – 9

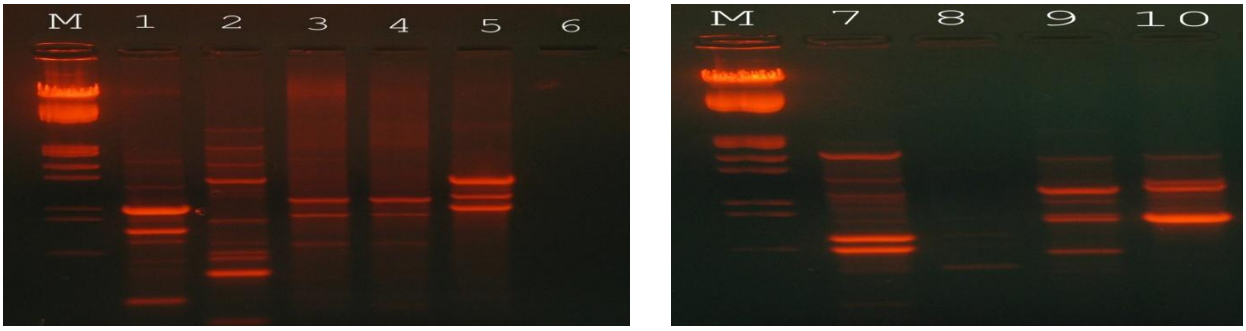

Plate 11: DNA Amplification profile of different bacterial species using primer – 10

M – DNA Marker (Ecor1/Hind III/Double digest)

1) *Bradyrhizobium japonicum*
2) *Rhizobium loti*
3) *Rhizobium meliloti*
4) *Rhizobium leguminasorum*
5) *Rhizobium spp.*
6) *Rhizobium phaseoli*
7) *Bacillus megaterium*
8) *PAL-5*
9) *Azotobacter chrococum*
10) *Azospirillum brassilens*

Conclusions

In the RAPD analysis 10 random primers were used viz; OPB-01, OPB-02, OPB-03, OPB-04, OPB-05, OPB-06, OPB-07, OPB-08, OPB-09 and OPB-10. Yielded the good and scorable amplified products. All 10 random primers yielded polymorphic amplified products average band size ranging from 5500 bp to 250 bp in all 10 random primers.

References

1. Achenbach LA, Patric J, Gray. Use of RAPD markers as a diagnostic tool for the identification of *Fusarium solani* isolates that cause Soybean sudden death syndrome. Plant dis. 1996; 80:1228-1232.
2. Fani R, Claudio Maria GB, Serigio C, Giuseppe D, Annamaria G, Marco B. RAPD fingerprinting is useful for identification of *Azospirillum* Strains. Microbe. releases, 1993; 1:217-21.
3. Hebb D, Richardson MAE, Reid R, Brockwell J. PCR has an ecological tool to determine the establishment and persistance of *Rhizobium* strains introduced in to the field as seed inoculant. Australian J. Agric. Res. 1998; 49:923-34.
4. Ivanova EG, Doronia NV, Trotsenko YA. Aerobic methelobacteria are capable of synthesizing auxins. Microbiol. 2000; 70:392-397.
5. Khbaya B, Neyra M, Normand P, Zerhari K, Filalimaltouf AK. Genetic diversity and Phylogeny of *rhizobia* that nodulate acasia spp. in Morocco accesed by analysis of rRNA genes. Appl. environ. Microbiol. 1998; 64:4919-4917.
6. Longanathan P, Sunita R, Parida AK, Nair S. Isolation and characterisatiion of two genetically distant groups of *Acetobacter* Diazotrophius from a new host plant *Elusine carcana* L. Journal of applied microbiology. 1999; 87:167-172.
7. Saleena LM, Lognathan P, Rangaran P, Nair S. Genetic

divercity of *bradyrhizobium* stains isolated from *Arachis hypogea*. Canadian J. Microbiol. 2001; 47:118-22.
8. Sikora S, Radzepovic S, Pejic I, Kozumpnik V. Genitic diversity of bradyrhizobium japonicum field population revealed by RAPD Finger Printing. J. Appl. Microbiol. 1997; 82:527-531.
9. Van Rossum DV, Schuarmans FP, Gills M, Muyakha A, Vanvenseverd HW, Stotuamer AH *et al*. Genetic and phenotypic analysis of *bradyrhizobium* strsains modulating peanut (*Arachis hypogea* L.) roots Appl. Environ. Microbiol. 1995; 61:1599-1609.
10. Williams JG, Kubelic AR, Livak KJ, Rafalaski JA, Tngey SV. DNA polymorphisms amplified by arbitrary primers are useful as genetic Markers. Nucleic acids Res. 1990; 18(22):6531-6535.
11. Young CC, Cheng KT. Genetic diversity of fast and slow growing Soybean *Rhizobia* determined by random amplified polymorphic DNA analysis. *Biol.Fertil.Soils*. 1998; 26:254-6.