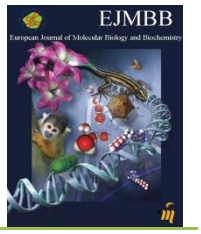
e - ISSN - 2348-2206
Print ISSN - 2348-2192

European Journal of Molecular Biology and Biochemistry

Journal homepage: www.mcmed.us/journal/ejmbb



INFLUENCE OF BIOFERTILIZERS IN INDUCING GENETIC VARIABILITY AMONG RELATED VARIETIES OF Oryza sativa LINN. USING RAPD ANALYSIS

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Article Info
Received 23/07/2014
Revised 16/08/2014
Accepted 19/09/2014

Key words:- *Oryza sativa*, RAPD, Biofertilizers, Cluster and Dendrogram.

ABSTRACT
Two varieties of *Oryza sativa* was treated with different Biofertilizers and the genetic diversity identified originally designed 10 Random Amplified Polymorphic DNA (RAPD) primers. Polymorphism among the rice varieties revealed by two major clusers in dendrogram of both varieties indicate the effect of biofertilizer of genetic makeup. The presence of branches within the same group indicates the mobilization of certain signals which act upon the DNA and have to ascertain further.

INTRODUCTION

Agriculture, plant germplasm and crop varieties were treated differently from the industrial products in the past but not anymore. There are reportedly 75,000 species of edible plants worldwide, but only 7,000 of them have been used for food and out of these less than 700 are actually commercialized currently. Origin and speciation of most crops is millions of years old while domestication is relatively recent. It is a notable fact that the currently exported food crops from the developed countries did not originate there. These crops were introduced in those "cash rich" countries, almost for free, from the "gene-rich" countries during the last 10,000 years, mostly during last 500 years. The agro-biodiversity created and fostered with human intervention across 12,000 years history of agriculture has stood the test of time and synergy with societies. Human civilizations exploited the germplasm for food, fiber, fuel and other purposes. The central dogma was the welfare of human beings with simple rules but not with individualization and profit motive.

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Scenes have changed now and the technology rich are exploiting the germplasm rich countries. Biofertilizers are preparations containing agriculturally useful microorganisms, which help in mobilizing plant nutrients through their biological activity. They are defined a preparation containing live or latent cells of efficient strains of N- fixing, P- solublizing or cellulolytic microorganisms used for the application to seed or soil [1]. Biofertilizers form an important part of Integral Plant Nutrient Supply System (IPNS) and organic farming, which constitutes the present as well as future mandate of Indian agriculture. Biofertilizers manufactured in India at present are carrier based, in general and suffer from short shelf life, poor quality, and high contamination and low unpredictable field performances. Rice (*Oryza sativa* L.) is the staple food for half of the world's population especially in oriental countries. In India, about 2500 varieties of rice are being cultivated, from which more than 1500 varieties are in southern India which are preferred over others, owing to their yield, good quality and quantity of grain, short duration of growth and resistance against pest and diseases. A large number of experiments have been conducted in several countries to investigate the effect of inoculation on various grasses [2,3]. The aim of

their application is to get fast growth, better health of the plant and higher yield. *Azotobacter*, *Nitrosomonas* and *Azospirillum* are known to increase yield under controlled conditions. Consequently, ensuring genetic diversity requires that rice land races are cultivated continuously and not simply stored in seed banks. Genetic diversity is known to substantially decrease a crop's vulnerability to nutrient availability of different biofertilizers. A perfect system to identify different races is the most fundamental requirement to enforce the propriety over plant varieties and genetic pool. Variability in morphological, biochemical and molecular markers are the tools available for characterizing variability. The presence of sufficient variability, the knowledge of nature of association among different characters and relative contribution of different characters to yield is a pre-requisite to any breeding programme.

MATERIALS AND METHODS

Biofertilizer Treatment

The genetic variability between plants of *Oryza sativa* varieties (ADT-36 & IR - 36) was studied by treatment with biofertilizers like Mycorrhiza, Phosphobacterium, Azospirillum and all mixed. The coding used were I_o Control for ADT - 36; I_a Mycorrhiza for ADT - 36; I_b Phasphobacterium for ADT - 36; I_c – Azosprillum for ADT - 36; I_d mixed for ADT – 36. Similarly II was used with the respective subscripts for IR-36.Commercially available preparations were used at 10^9 CFU/ml inoculum density as seed soaking before sowing. Triplicates of the study were maintained to study internal variance.

EXTRACTION OF GENOMIC DNA

The Leaves of each *Oryza sativa* variety was taken (I_a – Mycorrhiza, I_b- Phosphobaterium, I_c- Azospirillum and I_d- combined above fertilizers) and were frozen in liquid nitrogen and ground to a fine powder with the help of a pestle and mortar. This powder was then transferred to 20ml of CTAB extraction buffer maintained at 60℃ in water bath and incubated for 1 hr. with intermittent mixing. Then equal volume of chloroform: isoamyl alcohol (24:1) was added and was mixed gently by inverting for 5 min. It was then spinned at 17000 rpm for 10 min. The aqueous phase was transferred to a fresh centrifuge tube and equal amount of isopropanol was added. DNA was allowed to settle down for 20 min. and later on spooled out.

DNA pellets were washed twice with 70% ethanol and then it was vacuum dried. It was then dissolved in 10:1 TE buffer (10mM Tris, 1mM EDTA, pH 8.0). The dissolved DNA was treated with RNAse and pronase K, each for 1 hr. The RNA free DNA was treated with equal volume of phenol: Chloroform (1:1) twice. After this treatment DNA supernatant was further purified by giving two washings with chloroform : isoamyl alcohol. DNA was precipitated by adding 1/10 volume of 3M NaOAc (Sodium acetate) and chilled ethanol. Extract salts were removed and vacuum dried in a lyophilizer (Savant Refrigerated speed Vac.Se 110). Dried pellets were dissolved in TE (10:1) buffer at room temperature. Quantity of DNA (3µl) was checked by using 0.8% agarose gel.

RAPD Assay

RAPD assay was carried out in 25 µl reaction mixture 2.5 µl 10X amplification buffer (500mM KCl, 100Tris HCl, 1.0% Triton X- 100 and 15mM $MgCl_2$), 200µM each of dATP, dGTP, dCTP and dCTP, 1.0 U of *Taq* DNA polymerase (Merck, Mumbai) 25pM of 10mer in separate reactions (Operon Technologies Inc, Merck) and 50ng of genomic DNA. Amplification was performed in Eppendorf Research Thermal Cycler. The sequential steps were 1 cycle of 2 min at 93ºC, 2 min 35ºC and 2 min at 72ºC followed by 38 cycles of 1 min at 93ºC, 1 min at 36ºC and 2 min at 72ºC. The last cycle was followed by 10 min extension at 72ºC.

PCR products were mixed with 2.5µl of gel loading buffer (6x buffer: 0.25% bromophenol blue, 0.25%xylene cyanol FF, 30% glycerol in water) and spun briefly in a microfuge before loading [4]. The amplification products were electrophoresed in 1.2% agarose gel at 100 volts in 1xTAE buffer. A 100bp/1kb DNA ladder was used as a molecular standard. The gels were stained with ethidium bromide and gels were documented under the gel documentation system (Genei, Bangalore, India). Individual bands were scored as one of two discrete character status (0 and 1 for absence and presence, respectively, of RAPD bands). Molecular weight of bands was determined by comparison with standard 1-Kb molecular weight DNA ladder (Hi media, Pvt., Mumbai, India). The experiment was repeated twice with all the strain and random primers tested. Jaccard's similarity coefficient values for each pair-wise comparison between accessions were calculated [5] and a similarity coefficient matrix was constructed. This matrix was subjected to unweighted pair-group method of arithmetic averages analysis (UPGMA) to generate a dendrogram.

RESULTS

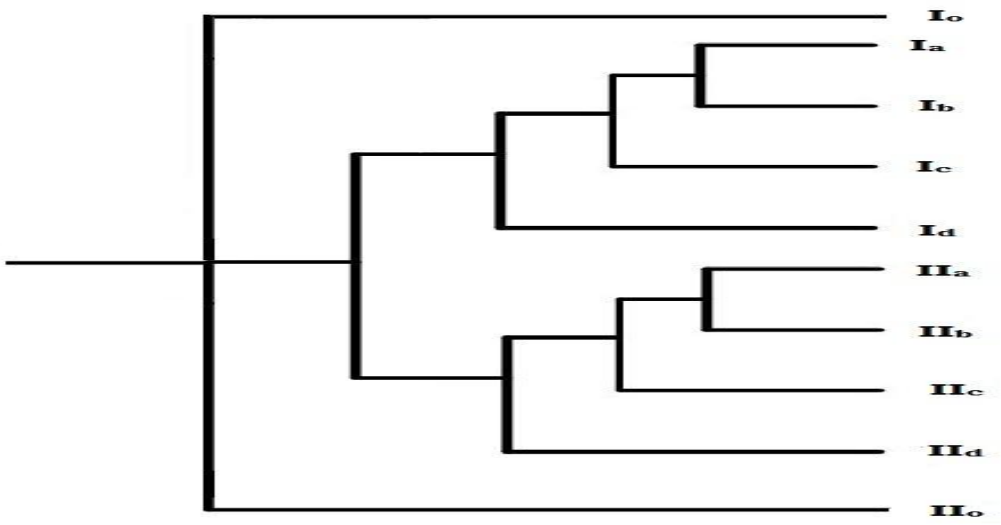
Genetic variability

Genomic DNA was isolated by CTAB method. The quantity of DNA was checked by using 0.8% agrose gel and the image was presented in plate No. 1. RAPD profiles of randomly selected *Oryza sativa* varieties were compared. All ambiguous RAPD bands were excluded from scoring in order to avoid fragments that could be artifact. The 10 RAPD primers used in this study yielded a total of 133 clear and reproducible DNA bands. Several polymorphic bands were observed for each primer in the range between 200 and 1900bp (Table 1). Out of all the bands scored for 10 RAPD primers, 49 bands were

polymorphic. OPN 11 primer gave the fewest bands (11 bands). The number of bands per primer ranged between 11 to 17 with a mean of 13.3. The percentage of polymorphic bands for each primer observed ranged from 25 to 36.23%. The pair wise Jaccard's coefficient genetic similarity matrix was prepared on the basis of RAPD data. The genetic similarity coefficient among all 10 populations varied from 0.614 to 0.963 (Table 2).

To understand overall genetic relationships among *Oryza sativa* varieties treated with various biofertilizrs, cluster analysis was carried out based on similarity Coefficients generated from 142 RAPD bands using UPGMA. This was further used for developing a dendrogram (Figure 1). Though all accessions could be grouped into one cluster at 76%, two major clusters were detected at 82% similarity level. The first cluster contained *Oryza sativa* variety (ADT 36) treated with biofertilizers; second set of cluster was variety (IR 36) of *Oryza sativa.* Individual two branches also been observed by means of control plants of *Oryza sativa* for both varieties. The UPGMA based cluster analysis is a good indication of the genetic relationship existing among the biofertilizers treated populations of *Oryza sativa* varieties. The result indicated that there was a common tendency towards availability of nutrients in respective biofertilizers and divergences with biofertilizers treated plants of distance between the populations.

Figure 1. Dendrogram based on co-efficient similarity



I_o – Variety (I) Control; I_a - Variety (I) Mycorrhiza; I_b - Variety (I) Phasphobacterium; I_c - Variety (I) Azosprillum; I_d - Variety (I) Mixed

II_o – Variety (II) Control; II_a - Variety (II) Mycorrhiza; II_b - Variety (II) Phasphobacterium; II_c - Variety (II) Azosprillum; II_d - Variety (II) Mixed

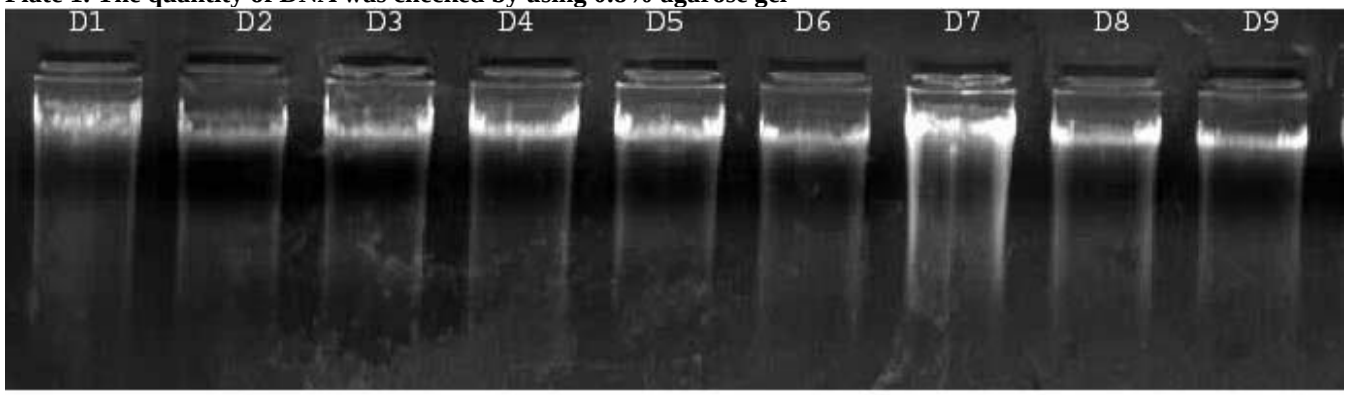
Table 1. Scoring of amplified DNA bands generated by RAPD markers

S.No	Name of the Primer	Sequence (5' – 3')	Total No. of bands amplified	No. of poly-morphic bands	No. of mono-morphic bands	Percentage of polymorphic bands
1.	OPN 11	TCGCGCCAAA	11	7	4	36.36
2.	OPP 01	GTAGCACTCC	13	8	5	38.46
3.	OPQ 01	GGGACGATGG	14	9	5	35.71
4.	OPQ 20	TCGCCCAGTC	13	8	5	38.46
5.	OPR 03	ACACAGAGGG	12	9	3	25.00
6.	OPT 03	TCCACTCCTG	16	9	7	43.75
7.	OPT 07	GGCAGGCTGT	11	8	3	27.27
8.	OPT 08	AACGGCGACA	17	10	7	41.17
9.	RPi 07	ACATCGCCCA	12	8	4	33.33
10.	RPi 10	ACGATGAGCG	14	8	6	42.85
		Total	**133**	**84**	**49**	**362.36**
		Mean	**13.3**	**8.4**	**4.9**	**36.23**

Table 2. Similarity matrix of *Oryza sativa* from different treatments

	Io	Ia	Ib	Ic	Id	IIo	IIa	IIb	IIc	IId
Io	1.000									
Ia	0.730	1.000								
Ib	0.714	0.760	1.000							
Ic	0.635	0.780	0.796	1.000						
Id	0.614	0.840	0.861	0.823	1.000					
IIo	0.963	0.739	0.745	0.684	0.615	1.000				
IIa	0.724	0.756	0.789	0.695	0.654	0.724	1.000			
IIb	0.739	0.725	0.765	0.625	0.698	0.734	0.784	1.000		
IIc	0.654	0.685	0.714	0.689	0.651	0.645	0.794	0.791	1.000	
IId	0.625	0.651	0.754	0.685	0.654	0.671	0.860	0.877	0.843	1.000

Plate 1. The quantity of DNA was checked by using 0.8% agarose gel



D1 to D9- Isolated DNA samples from one to nine respectively

Plate 1a. Electrophorotic bands of DNA (*Oryza sativa* Var*)* at 0.8% agarose gel

1.**Primer : OPN 11**

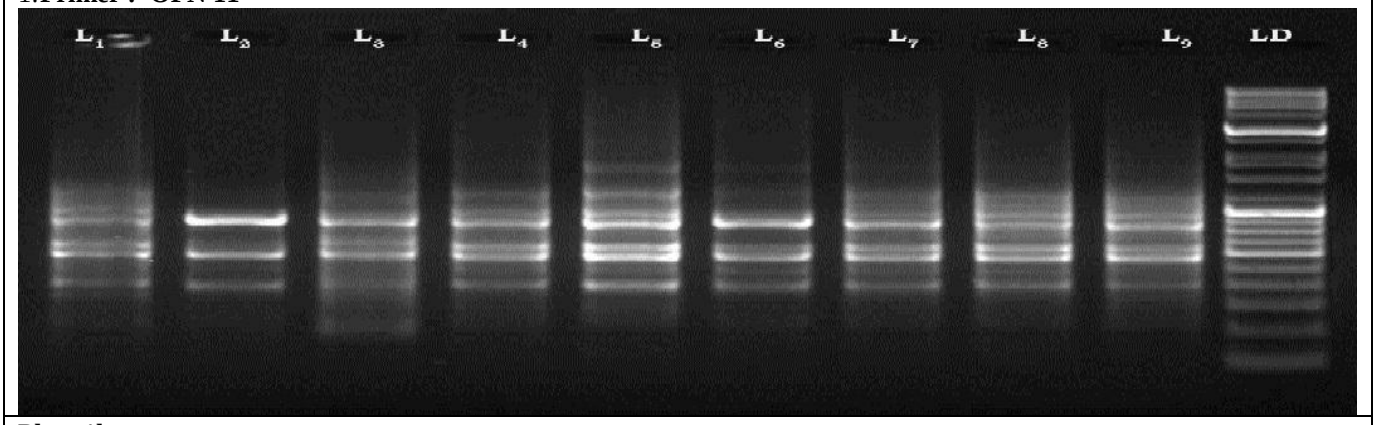


Plate 1b.

2. Primer : OPP 01

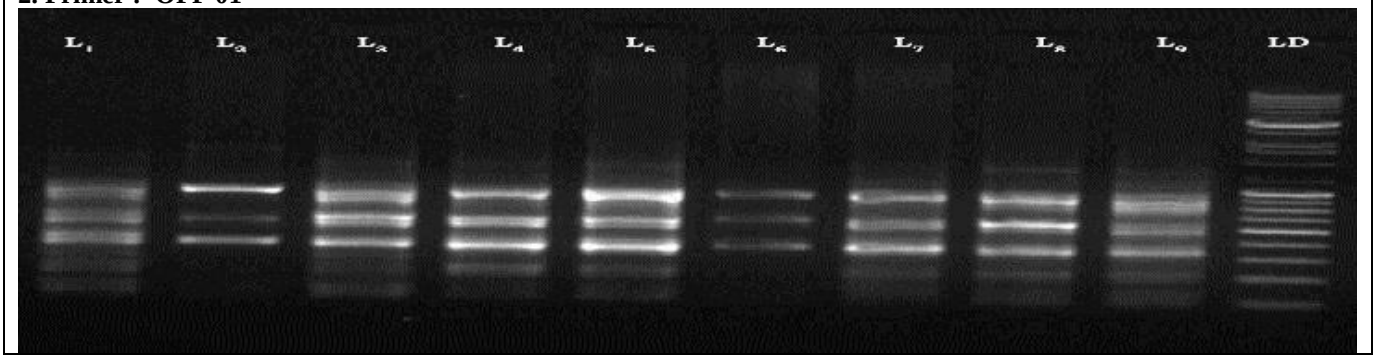


Plate 1c.
3. Primer : OPQ 01

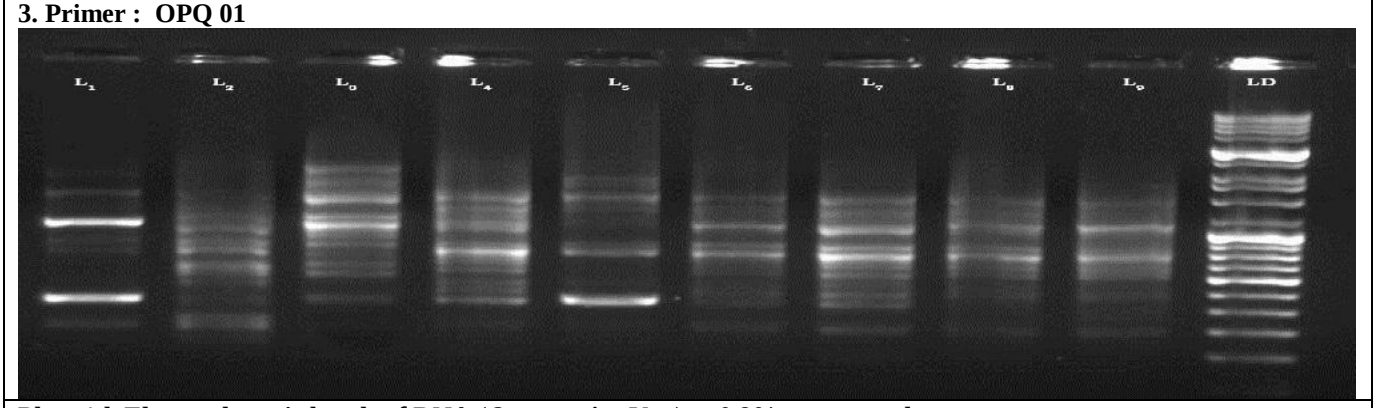
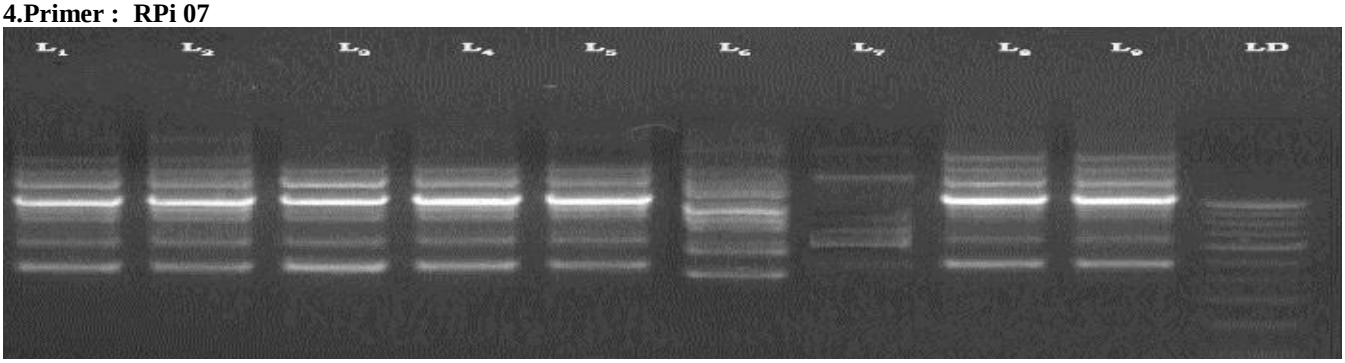


Plate 1d. Electrophorotic bands of DNA (*Oryza sativa* Var*)* at 0.8% agarose gel
4.Primer : RPi 07



LD- Ladder, L_1- Control, L_2- Variety I- Mycorrhiza, L_3- Variety I- Phasphobacteria, L_4- Variety I- Azosprillum, L_5- Variety I-Mixed, L_6- Variety II- Mycorrhiza, L_7- Variety II- Phasphobacteria, L_8- Variety II- Azosprillum, L_9- Variety I-Mixed

DISCUSSION

The ability of a species to adapt to environmental changes (treating various Biofertilizers) depends greatly on the genetic diversity in species [6]. Narrowing of gene pool and reduced genetic diversity pose challenges in the selection pressure brought in by addition of various inoculums [7]. The RAPD technique to detect genetic variation at the level of DNA was found to be sensitive and powerful tool in *Oryza sativa* varieties. The dendrogram based on co-efficient of similarity derived from RAPD analysis using 10 primers showed two main branches. Biofertilizers treated with both Oryza sativa varieties occupied two groups of clusters and can be grouped separately. Both varieties' control falls in individual branch.. The variation may be due to the traces isolation and their nutrient traces availability between these main groups. This report is supported by the observations made in same species by Amanda *et al* [8]. Therefore, high genetic diversity further supports greater differentiation [9,10]. Our study pronounced that genetic differentiation among varieties influence the biofertilizers treatment and this can be attributed to low or absent gene flow between the populations.

SUMMARY

In rice genetic diversity studied, highly significant and positive correlation was observed in every treatment of biofertilizers by individual varieties. RAPD was used to generate amplifications. The extent of polymorphism percentage was observed in RAPD with markers revealed that bioavailability of nutrient traces in fertilizers and combined fertilizers grouped into two major distinct clusters.

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