

# GENETIC DIVERSITY STUDY OF *ORYZA SATIVA* L. VARIETY TPS-3 AND AMBAI-16 USING DIFFERENT BIOFERTILIZERS

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**Abstract :** Biofertilizers are preparations containing agriculturally useful microorganisms which help in mobilizing plant nutrients through their biological activity. Rice (*Oryza sativa* L.) is the staple food for half of the world's population. The aim of application of biofertilizers is to get fast growth, better health and yield. Successful crop improvement programme depends on availability of sufficient genetic variability that arises from genetic diversity DNA markers, being independent of environment interference are indispensable tools in the study of genetic variability. RAPD markers have been used to characterize variation at the DNA level. This study clearly indicated that the combined effect of biofertilizers showed variation in the genetic diversity of *oryza sativa* variety TPS-3 and Ambi-16.

**Keywords -** RAPD, DNA marker, PCR.

## INTRODUCTION

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 present are carrier based, in general and suffer from short shelf life, poor quality, and high contamination and low and unpredictable field performances. Biofertilizers are preparations containing agriculturally useful microorganisms which help in mobilizing plant nutrients through their biological activity. Biofertilizers generally are defined a preparation containing live or latent cells of efficient strains of N-fixing, P-solubilizing or cellulolytic microorganisms used for the application to seed or soil (Motsara *et al.*, 1995).

Rice (*Oryza sativa* L.) is the staple food for half of the world's population. In India, about 2500 varieties of rice are being cultivated, from which more than 1500 varieties are in southern India. A large number of experiments have been conducted in several countries to investigate the effect of inoculation of various grasses (Smith *et al.*, 1976; Watanabe *et al.*, 1981). The aim of the application of *Azospirillum* is to get fast growth, better health of the plant and higher yield. It is known to be very active nitrogen fixing bacteria viz., *Azotobacter*, *Nitrosomonas* and *Azospirillum* to increase yield under controlled conditions.

DNA markers, being independent of environmental interference are indispensable tools in the study of genetic variability. RAPD markers have been used to characterize variation at the DNA level, both within species and among closely related taxa (Welsh and McClelland, 1990). These are, therefore, suitable for gene mapping, population genetics, phylogenetic studies and genotype identification (Martin and Sánchez-Yélamo, 2000).

Successful crop improvement programme depends on availability of sufficient genetic variability that arises from genetic diversity (Rana and Bhat, 2004). There is a wide genetic variability in rice among and between wild relatives and varieties which has great scope for trait improvement in breeding program. Approximately 100,000 unique rice germplasm accessions have been collected worldwide (Perret, 1991).

## MATERIALS AND METHODS

### Random amplified polymorphic DNA (RAPD) analysis

#### Extraction of genomic DNA

The Leaves of each *Oryza sativa* (treated with biofertilizers and control in both species) was collected separately. Each leaves was frozen in liquid nitrogen and ground to a fine powder with the help of a pestle and mortar. This powder was then transferred to 20 ml of CTAB extraction buffer maintained at 60°C in water bath and incubated for 1 hr. it was mixed intermittently. 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 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 phenol:chloroform treatment, DNA supernatant was further purified by giving two washings with chloroform:isoamyl alcohol. DNA was precipitated by adding 1/10 volume of 3 M Na O Ac (Sodium acetate) and chilled ethanol. Extract salts were removed and vacuum dried in a lyophilizer (Savent 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<sub>2</sub>), 200µM each of dATP, dGTP, dCTP and dTTP, 1.0 U of *Taq* DNA polymerase (Merck, Mumbai) 25pM of 10mer (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 93°C, 2 min 35°C and 2 min at 72°C followed by 38 cycles of 1 min at 93°C, 1 min at 36°C and 2 min at 72°C. The last cycle was followed by 10 min extension at 72°C.

PCR products were mixed with 2.5µl of gel loading buffer (6x I buffer: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) and spun briefly in a microfuge before loading (Sunagawa *et al.*, 1989). 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, Banaglore, 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 kilo base molecular weight DNA ladder (Hi media, Pvt., Mumbai, India).

### Agarose gel electrophoresis

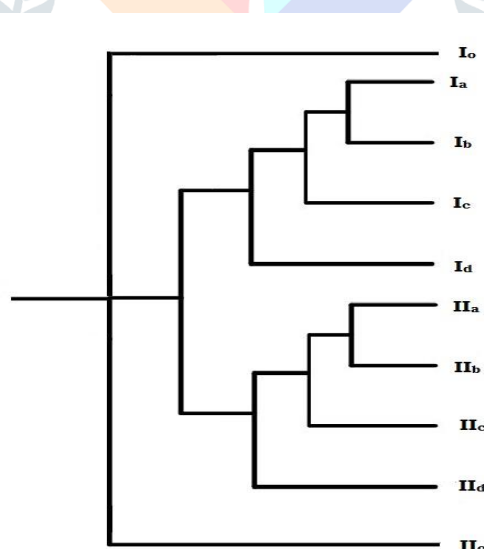
The amplified products were visualized in a 2% agarose gel containing 0.5 mg ml/L of ethidium bromide and documented by a gel documentation system (UVTEC). Amplified products that were reproducible, re-bandable, re-scorable and consistent in performance were chosen for data analysis. The bands were scored based on the molecular weight marker (1kb DNA ladder, Merk, Bangalore). 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 (Rohlf, 2004) 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.

## RESULT

### Molecular Analysis

To study the genetic variability between plants of *Oryza sativa* varieties (TPS-3 and Ambai-16) treated with biofertilizers like, *Mycorrhizae*, *Phosphobacterium*, *Azospirillum* and combined fertilizers were collected and stored in liquid nitrogen during the transportation. Genomic DNA was isolated by CTAB method. The quantity of DNA was checked by using 0.8% agarose gel RAPD profiles of randomly selected *Oryza sativa* varieties was compared. PCR amplification product obtained from the following RAPD primers (OPN-11 to Rpi-10) and they were separated using 1.2% agarose gel. 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 band. Several polymorphic bands were observed for each primer in the range between 200 and 1900bp. 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.

Figure 1 : Dendrogram based on co-efficient similarity



Variety (I) – TPS-3

I<sub>o</sub> – Variety (I) Control;

I<sub>a</sub> - Variety (I) Mycorrhizae;

I<sub>b</sub> - Variety (I) Phosphobacterium;

I<sub>c</sub> – Variety (I) Azospirillum;

I<sub>d</sub> - Variety (I) combined fertilizers - Variety (II) – Ambai-16

II<sub>o</sub> – Variety (II) Control;

II<sub>a</sub> - Variety (II) Mycorrhizae;

II<sub>b</sub>– Variety (II) Phosphobacterium;

II<sub>c</sub> - Variety (II) Azospirillum;

II<sub>d</sub> - Variety (II) combined fertilizers

To understand overall genetic relationships among *Oryza sativa* varieties treated with various biofertilizers, cluster analysis was carried out based on similarity Coefficients generated from 142 RAPD bands using UPGMA. This was further used for developing a

dendrogram. (Fig.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 (I) treated with biofertilizers; second set of cluster was variety (II) of *Oryza sativa* individual two branches also been observed by the means of control plants of *Oryza sativa* 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 tendency towards availability of nutrients in respective biofertilizers and divergences with biofertilizers treated plants of distance between the populations.

## DISCUSSION

*Oryza sativa* L. variety (TPS-3 and Ambai-16), using PCR with originally designed RAPD (Random Amplified Polymorphic DNA) primers and many sequence-specific primers, used for amplifying four specific DNA fragments. Use of these primers revealed polymorphisms among varieties in rice and facilitates DNA fingerprinting. Dendrograms of both species based on polymorphisms were constructed and genetical relationships were established (Toshiya Yamamoto *et al.*, 1994). In rice, half the numbers of amplified bands were polymorphic and almost all varieties differentiated. Some of the PCR fragments were variety or type specific, which could be used for indicators for type-selection. The dendrogram obtained showed differentiated clusters of crisp head, leaf and butter head type, findings in good accord with the classification based on the genetic background (Toshiya Yamamoto *et al.*, 1994).

The ability of a species to adapt to environmental changes (treating various Biofertilizers) depends greatly on the genetic diversity in species (Zheng, *et al.*, 1991). Narrowing of gene pool and reduced genetic diversity pose challenges in the selection pressure brought in by addition of various inoculums (Zheng, *et al.*, 1991; Moola Ram, *et al.*, 2011). 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 three main branches. Biofertilizers treated with both *Oryza sativa* varieties were occupied in separate two group of cluster and can be grouped separately. The both varieties of non- biofertilizers treated *Oryza sativa* falls in individual branch. These three branches are based in their genetic DNA band divers in each varieties distribution. 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.*, 2005. Therefore, high genetic diversity further supports greater differentiation (Paran and Michelmore, 1993; Anulakh *et al.*, 2001; Amanda *et al.*, 2005). Our study pronounced that genetic differentiation among the biofertilizers treatment was found in every treatment of each species and this was attributed to low or absent gene flow between the populations.

## CONCLUSION

This study clearly indicated that the combined effect of biofertilizers showed variation not only in the morphology but also in the genetic diversity of *Oryza sativa* variety TPS-3 and Ambai -16.

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