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RESTRICTION FRAGMENT LENGTH POLYMORPHISM ANALYSIS OF *RHIZOBIUM* SP. ISOLATED FROM *DOLICHOUS LABLAB*

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ABSTRACT

RFLP analysis is a technique used to identify patterns that occur in DNA. No two organisms have identical DNA, so this procedure can be used to identify if a sample of DNA came from a particular individual. *Dolichous lablab* is known for its dietary protein source, medicinal properties and symbiotic nitrogen fixation by *Rhizobium* present in its root nodules. *Rhizobium* sp. was identified by biochemical methods from root nodules of *Dolichous lablab*. High molecular weight range of R1, R2 and R3 lies between 4.07 to 3.05 kbp. RFLP assay with restriction endonuclease *EcoRI* used to distinguish among the three species. This RFLP assay provides an inexpensive and simple means of identifying the *Rhizobium* sp. RFLP analysis of R1, R2 and R3 confirm the genetic variation of the species. Genetic diversity can be identified within breeding populations in plants and animals.

KEYWORDS: RFLP–Restriction Fragment Length Polymorphism, Genetic diversity, Restriction Endonuclease, *Rhizobium*, *Dolichous lablab*, SDS- Sodium Dodecyl Sulfate.

INTRODUCTION

Dolichous lablab is a twining vine (climbing or trailing plant) belongs to the family *Fabaceae. Rhizobia* are soil bacteria that fix nitrogen after becoming established inside root nodule (Wilson., 1970). These are the site of Nitrogen fixation and can be used as a biofertilizer, help in reducing the dosage of nitrogenous fertilizers like urea and increases the protein content of the seeds. It also provides nitrogen to plant and increases yield.

When the genomic DNA's of many individuals of one species are separately cleaved by restriction enzymes, passed through electrophoresis, blotted on nitrocellulose membrane and probed with a radio labeled DNA, there arises polymorphism in hybridization patterns of digested DNA's. This shows differences in sequences between the two individuals. These unique, common and repeated DNA sequences found in several individuals are called restriction fragment length polymorphs (RFLP) and are used as markers for gene mapping.

Analysis of RFLP variation in genomes was a vital tool in genome mapping and genetic disease analysis. If researchers were trying to determine the chromosomal location of a particular disease gene, they would analyze the DNA of members of a family afflicted by the disease, and look for RFLP alleles that show a similar pattern of inheritance as that of the disease. Once a disease gene was localized, RFLP analysis of other families could reveal who was at risk for the disease or who was likely to be carriers of the mutant genes. Hence the present study was chosen.

MATERIALS AND METHODS

Sample Collection

The Lablab bean root nodules were collected from Kattur, Trichy.

Rhizobium ISOLATION

YEMA medium was prepared; Congo red solution was separately sterilized and added to the medium at the time of pouring in Petri plates. Roots of the leguminous plants were uprooted and brought to the laboratory. The root system was washed with running tap water for removing the adhered soil particles. Healthy pink, unbroken and firm root nodules were selected and it was washed in water. The nodules were immersed in 0.1% Mercuric chloride for 5 mins for surface sterilization. Wash the nodules repeatedly in sterile water for 3-4 times to remove the sterilizing agent. The nodules were placed in 70% ethyl alcohol for 3 minutes. Nodules were washed repeatedly in sterile water. Nodules were crushed in 1 ml of water with a mortar and pestle. Uniform suspension of *Rhizobia* was made with water. Serial dilutions of the nodules extract were made. 1 ml of each of the suspension from various dilutions was spread on YEMA plates with Congo red dye. The plates were incubated at 37^0C for 4 – 7 days.

Cultural and biochemical characterization (Norris and Ribbons., 1972)

The motility test and Gram staining technique **(**Gram, H.C., 1884) and biochemical tests like Indole, Methyl Red, Voges Proskauer, Citrate Utilization, Triple Sugar Iron Agar Test, Starch Hydrolysis Test, Gelatin Hydrolysis Test, Catalase Test, Urease Test, Glucose Peptone Agar Test (GPA) and Lactose Test were performed by methods described by Norris and Ribbons, (1972) to identify the bacteria.

Isolation of Genomic DNA

The bacteria were grown on Luria Bertani (LB) broth. Two tubes were taken for each isolate and then it was inoculated and incubated for 24 hours in a rotary shaker. The fully grown culture was centrifuged on the second day at 6000 rpm for 10 minutes. The pellet was washed in sterile PBS (2ml),it was again centrifuged at 6000 rpm for 10 minutes. The PBS discarded and (0.05N NaOH) 100 µl of NaOH and 100 µl of SDS (25mg/100ml) was added into the tubes and it was mixed fully and the contents were transferred to the eppendorf carefully. The tubes were kept in a boiling water bath for 3 minutes. Immediately it was cooled on ice and centrifuged in a microfuge at 10,000 rpm for 10 minutes and it was stored at 4ºC and was used. 1% agarose gel was prepared for electrophoresis. 7 µl sample and 3µl of tracking dye was mixed and was loaded on the wells and the order of loading was noted down. The samples were electrophoreses at 50 -100 V for 1 – 2 hours.

RFLP Analysis

The vials containing restriction enzyme (ECoR1 - 20µl) was taken. 20µl of DNA was added to each of the enzyme vials. 25µl of 2x assay buffer was added to DNA mixture by tapping the tube. The vial was incubated at 37ºC for 1 hour. 1% agarose gel was prepared for electrophoresis. 1.5 ml of the vial was labeled and 10µl of marker and 1µl of gel loading buffer was added to it. The digested samples, substrate and marker were loaded and the order of loading was noted down. The samples were electrophoreses at 50 – 100 V for 1 – 2 hours.

RESULTS AND DISCUSSION

Nitrogen fixing bacteria (*Rhizobia)* was isolated from the healthy nodules of *Dolichous lablab*. Three different types of bacterial culture were isolated from the master plate. Then these cultures were subjected to biochemical test for identification and the results were given in Table 1.

Rhizobial cells were able to grown on the GPA media showing the utilization of glucose as the carbon source by the *Rhizobium* sp.(Plate 1,2,&3) It is the confirmatory test for

Rhizobium and they are able to utilize glucose as carbon source (Kucuk *et al.*, 2006). However, pure *Rhizobium* isolates are unable to grow on lactose. It was observed that Rhizobial cells do not produce gelatinase enzymes as medium containing gelatin solidified when kept at 4ºC for 30 minutes. Negative gelatinase activity is also a feature of *Rhizobium*. Yellow slants and red butt were obtained showing the utilization of glucose and sucrose in the triple sugar iron agar medium (Hajnaa, 1945).

RFLP Analysis

The genomic DNA was extracted from three *Rhizobium* sp. was subjected to agarose gel electrophoresis for confirmation (Fig.1). The strains were subjected to RFLP analysis and the genetic variation of the strains was confirmed based on the RFLP result. This technique has been used to differentiate the bacteria especially *Rhizobium* sp.

The efficient bean nodulating *Rhizobium* strains were characterized by RFLP. RFLP analysis of bacterial strain revealed the various changes in genotype of the *Rhizobium* sp. that taken for this study. There was totally 12 bands were observed. In R_1, the first band with high molecular weight range lies between 4.07 to 3.05 kbp, the second and third bands with low molecular weight range lies between 3.05 to 2.04 kbp and the fourth and fifth bands of very low molecular weight range lies between 2.04 to 1.02 kbp. RFLP analysis of R_1, R_2 and R_3 confirmed the genetic variation of the species. In R_2, the first two bands with high base pair lie between 4.07 to 3.05 kbp and next two bands low base pair lies between 3.05 to 2.04 kbp. In R_3, the first band with high molecular weight range lies between 4.07 to 3.05 kbp and the next two bands lie between 3.05 to 2.04 kbp(Fig.2).

Identification of species level of all isolates was characterized by RFLP method. RFLP patterns obtained with restriction endonuclease *EcoRI* revealed that all isolates differ significantly from R1, R2 and R3. *Rhizobium* strains which stimulated the growth of *Alfalfa* and red clover by about 10%. *Rhizobium sp*. are also able to colonize the Rhizosphere of cereals (Wilson, 1970) stimulating the nitrogenase activity and phytohormone production of native rhizosphere microorganisms and promoting the growth of wheat.

Genomic types of ADV (Aujeszky's Disease Virus) strains based on Restriction Fragment Length Polymorphism (RFLP) patterns with the endonuclease *Bam* HI were associated to viral geographic and chronological distribution in many countries (Christensen and, Soerensen., 1995). Using the *Bam*HI/ RFLP analysis, ADV strain confirmed its classification

as genomic type I (Hartmann *et al.,* 1982*).* Hartmann *et al.*, proposed a systematization of ADV genomic types based on the genomic *Bam*HI RFLP analysis of a large number of ADV isolates. This systematization has been adopted by different authors around the world (Banks, 1993).

RFLP analysis detects mutations or rearrangements in the genome which alter the distribution of specific restriction endonuclease recognition sites within defined regions of the genome. Increasingly, RFLP‘s are being used to study the genetic structure of higher organisms. Analysis of RFLP’s has been applied to human (Botstein *et al*., 1980) and plant (Helentjaris and King., 1985) genetics, but has been used in genetic studies of phytopathogenic bacteria (Denny *et al.,* 1988; Gabriel, *et al.,* 1988). RFLP analysis has demonstrated the diversity of symbiotic plasmid types within naturally occurring populations of *Rhizobium leguminosarum* (Gabriel, *et al.*, 1988; Demezas, *et al*., 1995).

Table 1: Biochemical and Cultural Characterization.

TESTS	R1	R2	R3
Motility	Motile	Motile	Motile
Gram’s Reaction	-	-	-
Shape	Rods	Rods	Rods
Indole	-	+	-
Methyl Red	-	-	+
Voges Proskauer	-	-	-
Citrate utilization	+	+	-
Triple sugar iron	Acid butt, alkaline slant	Acid butt, alkaline slant	Acid butt, alkaline slant
Urease	+	+	+
Gelatin hydrolysis	-	-	-
Nitrate reduction	+	+	+
Starch hydrolysis	-	-	-
Catalase	-	-	-

R1- *Rhizobium* sp.1, R2- *Rhizobium* sp.2, R3- *Rhizobium* sp.3

(-) – Negaive; (+)- Positive

Plate 1,2&3 **Fig: 1**

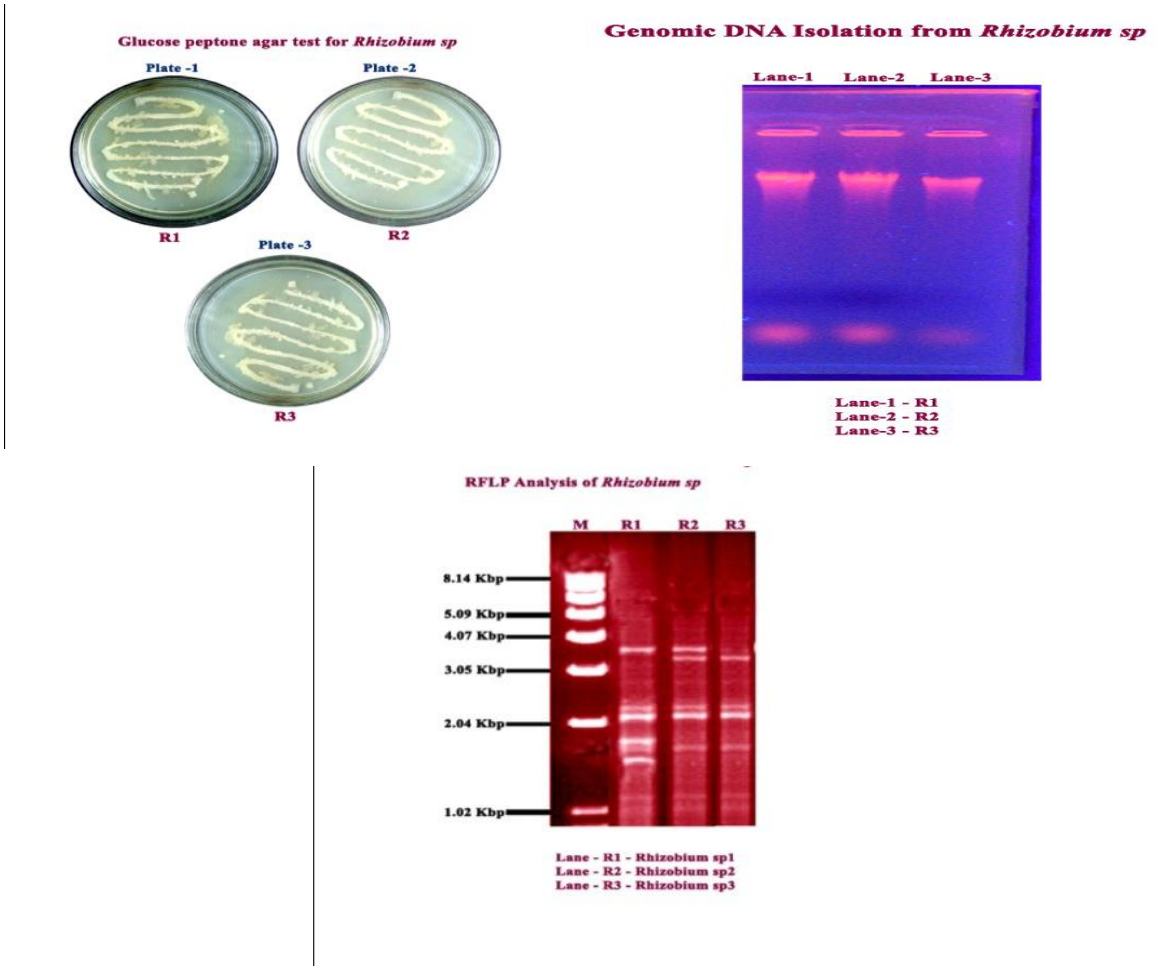

Fig: 2.

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