ABSTRACT

Soil microorganisms play an important role in soil formation, fertility and so also productivity. Nitrogen is an important element to support plant growth. Plants depend on soil microorganisms for fixed nitrogen. Among the nitrogen fixing microorganisms, the role of Rhizobium is quite significant. The aim of this study was to study diversity of Rhizobium spp. in agricultural lands of Madhya Pradesh. Physicochemical properties of soil was studied using standard methods while molecular methods used to study diversity within Rhizobium species. Further, population analysis of Rhizobium species in relation to genetic diversity was carried out using 16S rDNA-RFLP PCR. Rhizobium were identified and genetically by determining the %Guanine plus Cytosine content of the whole genome, followed by Restriction enzyme (Mbo I, Hap II, Taq I) treatment of Polymerase Chain Reaction (PCR) amplified product of 16S rDNA segment was performed. The sequences recognized by the restriction enzymes are distributed at variable intervals in the genome of an organism and also vary in number. The separation carried out by electrophoresis (1.6-2% agarose gel) resulted in specific banding pattern differing within as well as among different species. The technique used was helpful in characterizing Rhizobium isolates to be used as inoculants for improving agricultural land quality of Madhya Pradesh (India).

METHODS AND MATERIALS

Soil samples (0-30cm depth) were collected from 10 agricultural sites of Neemuch, Hoshangabad, Betul-Multaib, Sehore, Bhopal, Tikamgarh, Chhindwara, Raisen Vidhisha- Sanchi and Ujjain districts of Madhya Pradesh. These districts fall in central part of the province. Surface litter was scrapped away and soil samples stored in pre-sterilized high density polythene (HDPE) bags (Forster, J., 1995). Samples were passed through 2 mm sieve to have homogenous particles for further analysis. The Rhizobium species confirmed from soil samples were named as R1- R20.

The dilutions (10^1 to 10^6) were inoculated on YEMA (Yeast Extract Mannitol Agar) plates and incubated at 280 ± 2°C for 24 to 72 h. Fast growing Rhizobium species appeared...
within 24 hours and the slow growing needed cells further incubation of 72-96 h. The glistening white Rhizobium, like colonies were picked up and purified by continuous streaking on YEMA and CRYEMA plates (Subba Rao, N.S., 1984). The composition of media was mannotol-10g, K HPO₄-0.5g, MgSO₄-7H₂O-0.2g, NaCl-0.1g, yeast extracts-0.4g, agar-15.0g, distilled water-1 L and Congo red solution (10.0ml), pH of the medium was adjusted to 6.8 and sterilized at 15 psi (15 min). Yeast extract mannotol agar had the following ingredient: mannotol-10 g, K HPO₄-0.5g, MgSO₄-7H₂O-0.2g, NaCl-0.1g, yeast extracts-0.4g, agar-15.0g, distilled water-1 L, pH of the medium was adjusted to 6.8 and sterilized at 15 psi (15 min).

DNA isolation
Isolation of DNA was done by Marmur’s method (1961), with slight modifications. The Rhizobium inoculums (2.0 O.D₅) was added to 50ml YEM broth and put at 280 ± 2°C (24 h) in a shaking incubator, the cell pellet obtained by centrifugation (10,000 rpm). Cells were suspended in 25ml saline EDTA solution in Erlenmeyer flasks. Lysis of cells as done by addition of 1ml of lysozyme (37°C, 30 min) followed by 25% of 2ml SDS at 60°C (10min). The suspension was allowed to cool at room temperature, and 5ml of 3M sodium acetate was gently mixed in 50ml of 2:1 Chloroform-isomyl alcohol followed by centrifugation (10,000 rpm, 30min). Out of the three layers obtained following centrifugation, the uppermost one bearing nucleic acid was pipetted out in 100 ml beaker and two volumes of chilled ethanol added (Heims, C., 1985; Tracy, S., 1981). The white fibrous precipitate at the interface was gently pooled out with the help of glass rod.

Determination of Tm value
The Tm of each DNA sample was determined as described by Mandel and Marmur (Mandel, et al., 1968; Marmur, J., 1961). The % G+C content of the samples was determined by using the equation %G+C = 2.44 (Tm – 69.4) as suggested by De Ley, J. (1970).

PCR-RFLP of amplicon
Polymerase chain reaction (PCR) is most useful widely used genetic tool in study of molecular biology of organisms. It is widely applied on cloning, sequencing and phylogenetic study. The efficiency of PCR technique is based on “master mix” preparation consisting buffer, dNTP’s mix (2mM), Primer 1, Primer 2, Taq polymerase, sterile water except the DNA template. The reproducibility and reliability of results depended upon proper pipetting of all the components of “master mix” and their further distribution. After addition of template DNA, it was exposed to temperature cycles in a thermal cycler (PTC-1148, MJ Mini Thermal cyler, BIORAD, USA). The conserved sequence in DNA i.e., the 16S rDNA was amplified using the reverse primer (5’ ACGGCTACCTTCTTAGCATT3′) and the forward primer (5’ AGAGTTGATCCTGCGTCAAG3′) at 55°C (annealing) for 30 cycles in PCR unit (PTC-1148, MJ Mini Thermal cycler, BIO-RAD, USA). Amplified DNA was subjected to RFLP analysis using restriction enzymes (Taq I, Hpa II, Mbo I) after amplification. The amplified 16S rDNA was then digested separately with 3 different restriction enzymes, by incubating overnight at 37°C. Enzyme activities was stopped by low temperature (4°C) and by adding 2µl of 6x loading buffer. Further, the enzyme digested PCR product along with 1kb DNA ladder (Bangalore Genie, India) in a separate well was estimated by electrophoresis (Walker, et al., 1998) at 55mV on 2% agarose, 2x). The Bio-Rad Gel DocTM XR and ChemiDocTM XRS gel documentation system are easy-to-use, high-performance systems. They use a CCD camera to capture image in real time, which allows you to more accurately position and focus the image (Molecular Imager Gel Doc XR System 170-8170, 170-8171, BIO-RAD, USA).

Data analysis:
All restriction patterns were coded in binary form and analysed using NTSYS software (Rohlf, 1990). A simple matching coefficient was calculated to construct a similarity matrix and the UPGMA algorithm was used to perform hierarchical cluster analysis and to construct a dendrogram.

Result and Discussion
Present study, however, concentrated on the nitrogen fixing strains. Hofer’s alkaline broth test conducted is because Agrobacterium grows at higher pH levels and not rhizobia. The isolates strains failed to utilize peptone when were grown on glucose peptone agar medium. Rhizobium respond negatively to ketalactose test. Microscopic observations on pure culture cells confirmed the gram-negative nature. In addition, gelatin was not liquefied by cells grown on gelatin medium. Bacterial cells once inoculated on pre-sterilized yeast extract mannotol agar (YEMA) produced white, translucent glistening colonies with entire margin soil samples from Neemuch, Hoşangabad, Betul-Multai, Ujjain, Sehore, Bhopal, Tikamgarh, Chhindwara, Sanchi-Visdisha, Raisen etc were subjected to the above mentioned biochemical parameters.

The composition of DNA in bacterial genome is similar as it shows presence of all the four defined bases. The helix of DNA with double stranded structure shows pairing between A+T and G+C, thus (A+T)/ (G+C) ratio or (G+C) content reflects compatibility of microbial strain in relation to evolutionary strategy. The G+C content is examined in the form of temperature of melting (Tm). The bases are joined by hydrogen bonds and show regular pairing. It is obvious that the DNA with higher G+C content will stand higher melting temperature as more energy is needed to separate the double stranded DNA. The melting temperature thus is calculated by observing midpoint of the rising curve. The optical density of DNA shows further use in the presence of greater amount of G+C content. In all the organisms ranging between eukaryotes to prokaryotes, the highest degree of variation is observed in case of microbes (between 25-85%). The composition of G+C content with slight variation shows similar base sequences thus giving emphasis on relatedness among species in contrast to dissimilarity as observed in PCR-based observations. Samples analyzed presently, amongst 20 strains i.e., R1 to R20, the Tm ranged between 94.3 to 95.6°C. However, % G+C content of isolated strains ranges between 60.7 to 63.9%. All the bacteria tested and examined for Tm values and G+C content, were similar with narrow range of difference with respect to % G+C. Thus present observation as listed in provides most similarity amongst microbial isolates in relation to DNA as a parameter.

Furthermore, the pattern of genetic diversity was studied using known and established molecular biotyping methods. Genotyping of the isolates was done by using molecular methods. Taq I restriction enzyme when used to have 16S rRNA digestion the group of strains showed variation in pattern on DNA profiling studies with 2.0 % agarose (Fig. 1& 2). In the presence of Taq I the digestion of 16S rDNA showed bands between 200 bp and 500bp. DNA profile once put on computation data on NTYSUS using unweight pair group method with arithmetic averaging (UPGMA) over Taq I digested rDNA, Rhizobium isolates. The dendrogram shows divergence at 100% similarity into two broader groups and S16, S15, S12 and S2 strain shows 65% dissimilarity with all the strains isolated from agricultural soil of Madhya Pradesh.

Figure 1: Restriction enzyme Taq I treated 16S rDNA segments of isolated strains of Rhizobium spp. on 2% agarose gel.
Conclusions

On the basis of different physiological and biochemical parameter studied, it seems all the strain of Rhizobium sp. are same. Findings on the isolated Rhizobium strains from agricultural soils of M.P. with regard to the level of gene sequences will help establish the improved strains as biofertilizers. The utility of present observation falls with relative similarity between patterns of genome i.e., alignment of bases of DNA, although preference was given to the pattern of base-sequences in NTSYS based genomic analysis. Thus, the present observations give an insight on molecular orientation of Rhizobium species occurring naturally in the agricultural soils of Madhya Pradesh.

Acknowledgements

This investigation was supported by a National Bureau of agriculturally Important Microorganisms (NBAIM)/Indian Council of Agricultural Research (ICAR), grant to Prof. Kiran Singh.