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MOLECULAR DIVERSITY ASSESSMENT OF PGPR USING ENTEROBACTERIAL REPETITIVE INTERGENERIC CONSENSUS (ERIC) PCR

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Abstract

In the present investigation, an attempt has been made to study the diversity of Plant Growth Promoting Rhizobacteria isolated from various rice fields of Indo-Nepal border region. It is reported here that diversity in PGPR of East Champaran, West Champaran and Varanasi district of India and Bara and Parsa district of Nepal showed 76 to 97% similarity among them based on ERIC-PCR. Serratia, Agrobacterium, Klebsiella, Pseudomonas etc are predominantly present in the rhizosphere of rice fields of these areas. Protein profile showed that all the isolates were 49-79% similar among each other. The three members consisting species of Klebsiella showed 72% similarity to each other and two species of Agrobacterium showed 80% similarity to each other.

Key words

ERIC-PCR; Molecular diversity; PGPR; Indo-Nepal border; Rhizobacteria.

Introduction

PGPR are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and/or indirectly. In last few decades a large array of bacteria including species of *Pseudomonas*, *Azospirillum*, *Azotobacter*, *Klebsiella*, *Agrobacterium*, *Enterobacter*, *Alcaligens*, *Arthobacter*, *Burkholderia*, *Bacillus* and *Serratia* have been isolated and reported to enhance plant growth (Okon and Labandera-Gonzalez, 1994; Glick, 1995; Shrivastava, 2012, 2015 & 2016).

Plant rhizosphere is known to be preferred ecological niche for various types of soil microorganisms due to rich nutrient availability. It has been documented that inoculation with diazotrophic bacteria like *Rhizobium*, *Azotobacter* and *Azospirillum* enhances the plant growth due to their ability of N_2 fixation and production of substances useful for plant growth promotion (Shrivastava, 2015 & 2018).

The genetic diversity of putative diazotrophic bacteria is evaluated using amplified ribosomal DNA restriction analysis (ARDRA), rep-PCR genomic fingerprinting and small subunit (SSU) ribosomal DNA (rDNA) sequencing etc. (Grange and Hungria, 2004). One of the most common PCR-based genomic fingerprinting methods is Repetitive (Rep) PCR i.e. Enterobacterial Repetitive Intergenic Consensus (ERIC) elements (Versalovic *et al.*, 1991; Hulton *et al.*, 1991). Although, PCR based fingerprinting methods are suitable for characterization of bacterial communities and diversity study, however, these techniques cannot be employed for the identification of bacteria (Ventura *et al.*, 2001). This research work was carried out with an attempt to characterize the diazotrophic PGPR present in the rhizosphere of rice plant and to elucidate the diazotrophic bacterial diversity present in the rice rhizosphere of Indo-Nepal border region.

Material and methods

Bacterial Isolates and Growth Media

Diazotrophic rhizobacteria isolated from different rice fields of border area of India and Nepal were taken for this study. The method of isolation and culture conditions previously documented (Shrivastava and Kumar, 2011; Shrivastava, 2013).

Procedure of Genomic DNA Isolation

5 mL of exponentially grown culture was harvested by centrifugation at 8000 rpm for 5 min. The pellet was washed with 1 mL STE (Sodium chloride-Tris-EDTA buffer) and resuspended in 567 µL TE buffer. 30 µL SDS (10%) and 3 µL proteinase K (20 mg/mL) were added and then the samples were properly mixed by vortexing and incubated for 1 h for complete lysis of cells. Thereafter, 100 µL 5M NaCl was added followed by the addition of 80 µL CTAB/NaCl and the tube was incubated at 65°C for 10 min. Equal volume of phenol/chloroform/isoamyl alcohol (25:24:1) was added to the suspension, mixed and spun at 12000 rpm for 10 min at 4°C. Upper aqueous phase was collected carefully in fresh centrifuge tube (1.5 mL) and again equal volume of chloroform/isoamyl alcohol (24:1) was added, mixed and spun as mentioned above. Again upper clear aqueous phase was collected in fresh centrifuge tube and then 2 µL of DNase inactivated RNase (US Biologicals, Massachusetts, USA) from 10 mg/mL stock was added followed by incubation at 37°C for 1 h. DNA was precipitated with 0.1 volume of 3 M sodium acetate (pH 5.2) and 0.6 volume of isopropyl alcohol. The precipitated DNA was washed twice with 70% ethanol, and the DNA pellet was air-dried. Finally, DNA was suspended in minimal volume of TE. Electrophoresis of genomic DNA was performed in a horizontal slab gel of 0.8 % agarose in a LKB 2012 Maxiphor Electrophoresis Unit attached with LKB 2301 Macro Drive Power Supply (Hoefer Scientific Instruments, San Fransisco, CA, USA). Ethidium bromide was present in the gel at a final concentration of 0.5µg/mL. 1 X TAE buffer was used for electrophoresis. DNA samples were prepared in 1 X gel loading buffer and the samples were run at 50 V for 3 h. The genomic DNA was monitored under long wave UV-light by a trans-illuminator (Bio-Rad Laboratories, USA).

Enterobacterial Repetitive Intergeneric Consensus (ERIC) PCR

The oligonucleotide sequence of the pair of primers used for ERIC-PCR was:

ERIC-1R: 5′-ATG TAA GCT CCT GGG GAT TCA C-3′

ERIC-2: 5′-AAG TAA GTG ACT GGG GTG AGC G-3′

Amplification was carried out in 20 µL reaction mix containing; 1 X *Taq* DNA polymerase assay buffer, 1.25 mM each of dNTPs, 10 pmol of each primer (ERIC-1R, ERIC-2), 1 U *Taq* DNA polymerase (Bangalore Genie, India), 10mM $MgCl_2$ and 50-60 ng of template DNA.

Amplification of inter-rep elements was obtained using thermal program of: initial denaturation at 95°C for 3 min, followed by 35 cycles of denaturation at 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 1 min 30 s and final extension at 72°C for 5 min and storage at 4°C. The amplified products were analyzed by agarose gel electrophoresis (1.5%).

Results and discussions

Enterobacterial Repetitive Intergeneric Consensus (ERIC) PCR assay of 33 isolates was employed to attain the proposed objective (Fig. 1). Eight isolates among these 33 isolates showed most efficient result in plant growth promoting characters (data not shown) were analysed by their protein profile. SDS PAGE was performed, and based on banding pattern. phylogenetic tree was constructed using UPGMA method (Fig. 2).

Enterobacterial Repetitive Intergeneric Consensus (ERIC) PCR and Construction of Dendrogram

ERIC-PCR of all the 33 isolates showed appearance of 1-6 bands following agarose gel electrophoresis. ERIC-PCR profile revealed that all the isolates shared 76 to 97% similarity to each other (Fig. 1). All the isolates from Bara district of Nepal except BN-7C were closer and showed 83% similarity. On the other hand there was distinct diversity among the isolates of Varanasi district of India, altogether 9 clusters were formed. However most of the isolates of East Champaran were clustered together with 92% similarity but, a few isolates of this district showed similarity with Varanasi district and Parsa district of Nepal (Fig. 1).

ERIC-PCR was preferred and used for rapid study of molecular polymorphism among bacterial isolates. In general the distribution of repetitive sequences (BOX and ERIC) has been employed in elucidating the genomic diversity in a number of bacteria (Selenska-Pobel *et al.*, 1995). In our study, the bacterial isolates were analyzed by ERIC-PCR where 2-7 bands were generated and 76-97% similarity was observed. Reproducible result of the ERIC profile was evident by the fact that identical banding pattern was obtained

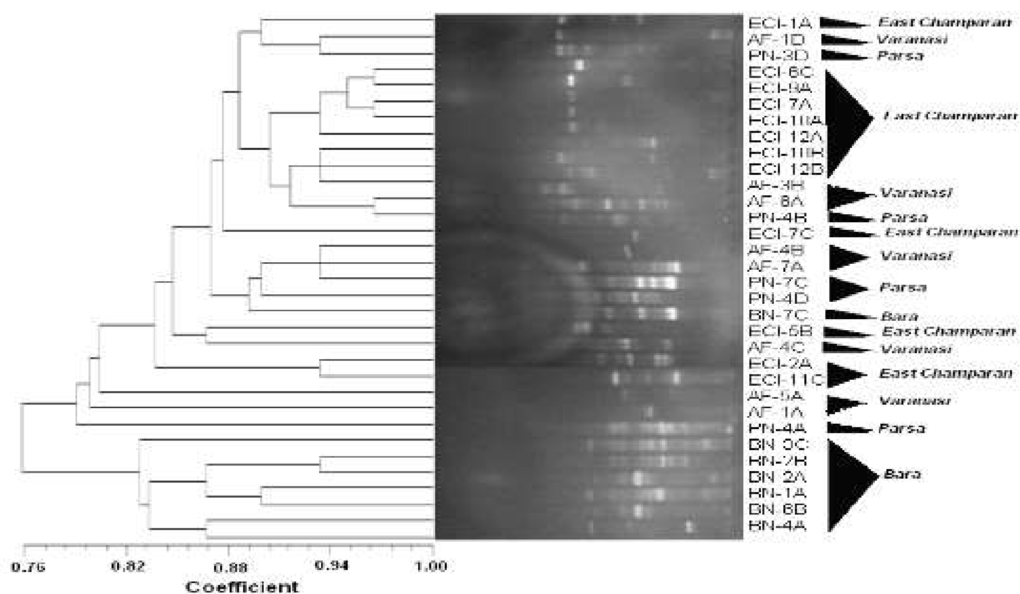

Fig. 1: *Dendrogram based on ERIC-PCR of 33 diazotrophic isolates.*

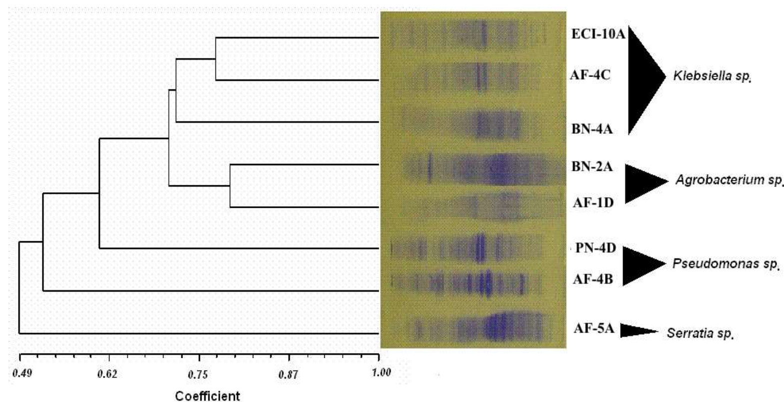

Fig. 2: *SDS- PAGE protein profile of eight isolates and dendrogram showing % similarity.*

when replicate samples were run together. ERIC-PCR- generated fingerprints, allowed us to grouping the isolates into nine clusters. Similar to our approach other workers have also applied ERIC- and BOX-PCR for studying the bacterial diversity of rice-associated diazotrophic bacteria (Mirza *et al.,* 2000) and putative endophytic bacteria isolated from seedlings of rice (Stoltzfus *et al.,* 1997).

In general genetic information on natural bacterial communities and correlation with their specific environmental conditions are limited and such study has not been made in Indo-Nepal border region till date. With the advent of molecular techniques, elucidation of microbial community structure, physiological functions of each member of the community, and genetic interaction in nature have become easier. In the present study we have employed DNA fingerprinting techniques to decipher phylogenetic relationship among diazotrophic bacterial populations obtained from the rhizosphere of rice plant, specifically from Indo-Nepal border, after enrichment in modified JNFb$^-$ medium (Döbereiner, 1995).

Protein Profile of Selected Bacterial Isolates

In addition to studies on molecular diversity based on DNA fingerprinting and sequencing of various isolates, electrophoretic pattern of whole cell protein of eight isolates was performed to reveal diversity. Whole cell protein of all the isolates growing in diazotrophic condition was extracted and protein profile was obtained by running SDS-PAGE. It is evident from the electrophoretic pattern as shown in Fig. 2 that all the isolates showed different banding pattern. The maximum number of bands was noticed in *Serratia* sp. strain AF-5A and minimum in *Agrobacterium* sp. strain AF-1D. The phylogenetic tree constructed using UPGMA method revealed four clusters according to genera of the isolates. All the isolates showed 49-79% similarity among each other. The three members consisting species of *Klebsiella* showed 72% similarity to each other and two species of *Agrobacterium* showed 80% similarity to each other (Fig. 2).

In addition to DNA fingerprinting, protein profile of selected eight isolates was also analyzed to establish phylogenetic relationship. Clustering of isolates on the basis of SDS-PAGE pattern clearly demonstrated that they were clustered in 4 groups. Electrophoretic pattern showed subtle differences among all the isolates suggesting that they are not closely related species or strain of any individual bacterium. Results of DNA finger printing and protein profile allow us to conclude that molecular diversity does exist among all these rhizospheric diazotrophs.

Conclusion

In conclusion, it is reported here that diversity in PGPR of East Champaran, West Champaran, Varanasi district of India and Bara and Parsa district of Nepal showed 76 to 97% similarity to each other based on ERIC-PCR. *Serratia*, *Agrobacterium*, *Klebsiella*, *Pseudomonas* etc are predominantly present in the rhizosphere of rice fields.

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