Research Paper :

Analysis of Genetic Similarity of Rhizobacterial Strains Using RAPD-PCR Technique

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SUMMARY

Random amplified polymorphic DNA (RAPD) profiles using PCR for seven characterized, efficient rhizobacteria were generated with 30 random decamer primers. The primers generated 227 RAPD loci, of which 198 were polymorphic and exhibited 87.22 per cent polymorphism. The primers OPA-01, OPA-10, OPB-02, OPB-03 OPB-05 and OPB-06 showed 100 per cent polymorphism. Maximum genetic similarity was found between RB-31 and RB-50 (84.00%), while the lowest genetic distance was between RB-13 and RB-31 (40.00%). Dendrogram constructed using UPGMA showed two major clusters A and B at similarity coefficient of 0.52. The isolates RB-01, RB-10, RB-13, RB-22 and RB-43 in one cluster and RB-31 and RB-50 in other cluster.

Key words :
Genetic similarity, Rhizobacteria, Molecular marker, RAPD-PCR

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Seven rhizobacteria isolated from coleus rhizospheres were screened and tested *in vitro* and *in vivo* as biocontrol agents against different soil borne pathogens as well as for plant growth promotion by diverse mechanisms. It was difficult to distinguish these species using traditional morphological and biochemical differences. In recent years, limitations of morphological and biochemical markers have been overcome by molecular markers, some are relatively cheaper and simple to use in variety of applications in plant research. One of such markers is Random Amplified Polymorphic DNA (RAPD) and is one of the Polymerase Chain Reaction (PCR) based DNA markers, and defined as an assay based on the amplification of genomic DNA with single primer of arbitrary nucleotide sequence (Weising *et al*., 1995). RAPD can be used in studying genetic diversity, varietal identification *etc*. It is simple to operate and does not involve radio active labeling. With this in view, seven native isolates of rhizobacteria were isolated and screened against different soil borne pathogens causing root-knot and wilt complex in coleus were selected to understand more variation among the isolates of efficient rhizobacteria by subjecting them to RAPD analysis.

MATERIALS AND METHODS

Seven rhizobacteria isolates showing high efficacy against *Fusarium*, *Ralstonia* and *Meliodogyne in vitro* were studied during 2007. DNA was extracted by using rapid method from the rhizobacteria. Bacterial cultures were grown in 5ml NB with 10% glycerol (v/v) for 72 h at 28±2ºC. Eppendorf tube of 1.5 ml was used to centrifuge the cells at 13,000 rpm for 5 minutes. The pellet was suspended in 200 ml Tris 0.1 mol L^{-1} and added with 200 ml of lysis solution (NaOH 0.2 N and 1% SDS). Above solution was mixed and deproteinized with 700 ìl of phenol: chloroform: isoamyl alcohol (25:24:1 v/v/v) and centrifuged at 13000 rpm for 10 min. Top layer containing bacterial DNA was taken without disturbing the bottom layer and it was transferred to fresh 1.5 ml microcentrifuge tube. To this, 700 ml of ice cold 95% ethanol/isoproponol was added to precipitate the DNA and spinned. Final washing was given with 70% ethanol and centrifuged at 8000 rpm for 5 min. Precipitated DNA was dried at room temperature and resuspended in 100 ml of water. The DNA obtained was further quantified and electrophoresed on 0.8% agarose gel stained with ethidium bromide and photographed under UV light. The PCR amplification for RAPD analysis was performed according to Williams

Profile	Step	Temperature	Duration (mix)	Number of cycles
1	Initial denaturation	94°C	5	
2	Denaturation	94°C	1	40
3	Annealing	36°C	1	
4	Extension	72°C	2	
5	Final extension	72°C	10	
6	Hold temperature	4°C	Forever	

et al. (1990) with certain modifications. The optimum conditions for DNA amplifications used were as follows.

DNA from different rhizobacteria was used for RAPD analysis following the method recommended by Bhat and Jarret (1995) with required modification.

The amplified profiles for all the primers were compared with each other and the bands of DNA fragment were scored as '1' for presence and '0' for the absence of a band generating the '0' and '1' matrix and per cent polymorphism was calculated by using the formula.

$$\text{Per cent polymorphism } Z \frac{\text{No. of polymorphic bands}}{\text{Total number of bands}} \text{x100}$$

Pair wise genetic similarities between rhizobacteria were estimated by DICE similarity coefficient, clustering was done using the matrix of similarity coefficient and cluster obtained based on unweighed pair group arithmetic mean (UPGMA) using sequential agglomerative hierarical nested (SAHN) cluster analysis of NTSYS-PC program version 2.0 (Exeter software, New York, USA) as described by Rohlf (1998).

RESULTS AND DISCUSSION

Rhizobacteria isolated, screened and tested *in vitro* and *in vivo* for biocontrol as well as for plant growth promotion by diverse mechanisms were difficult to distinguish these species using traditional morphological differences. To understand more variation among the strains, a PCR based technique *i.e.* RAPD was used in the present investigation.

In the present investigation, OPA, OPB and OPF series of primers were used to determine genetic distance between strains and to construct a dendrogram (Fig. 1). Of the 30 primers tested for amplification, seven primers, *viz.* OPA-01, OPA-10, OPB-02, OPB-03, OPB-05, OPB-06 an OPB-07 showed cent per cent polymorphism (Table 1). This information helps us to identify efficient strains with diverse mechanisms directly from the soil, but need to be conducted several times to get repetitive results. Similarity coefficient ranged from 40 to 84 per cent. The

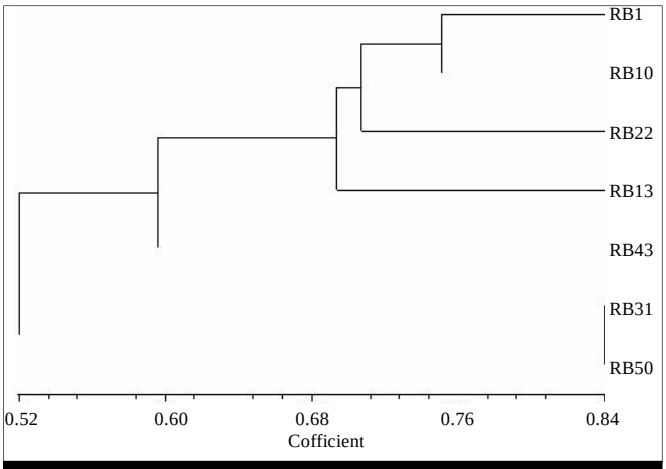

Fig. 1 : Dendrogram based on RAPD analysis of rhizobacterial strains

Table 1 : DNA banding profile of 30 random decamer primers in seven efficient rhizobacteria using PCR

Primer	Total no. of bands	No. of polymorphic bands	Per cent polymorphism
OPA-01	7	7	100.00
OPA-02	10	9	90.00
OPA-03	8	6	75.00
OPA-04	8	4	50.00
OPA-05	7	4	57.00
OPA-06	3	2	66.66
OPA-07	9	8	88.88
OPA-08	-	-	-
OPA-09	8	5	62.50
OPA-10	6	6	100.00
OPB-01	5	3	60.00
OPB-02	7	7	100.00
OPB-03	5	5	100.00
OPB-04	13	12	92.30
OPB-05	9	9	100.00
OPB-06	3	3	100.00
OPB-07	9	9	100.00
OPB-08	8	7	87.50
OPB-09	7	6	85.71
OPB-10	8	7	87.50
OPF-01	9	7	77.77
OPF-02	4	3	75.00
OPF-03	8	5	62.50
OPF-04	13	11	84.61
OPF-05	12	10	83.33
OPF-06	9	8	88.88
OPF-07	11	10	90.90
OPF-08	-	-	-
OPF-09	11	8	72.72
OPF-10	10	7	70.00
Total	227	198	87.22

Table 2 : Similarity co-efficient based on RAPD pooled over 30 primers in seven rhizobacteria

Rhizobacterial strains	RB1	RB10	RB13	RB 22	RB 31	RB 43	RB 50
RB1	1.00						
RB10	0.75	1.00					
RB13	0.73	0.66	1.00				
RB22	0.75	0.66	0.69	1.00			
RB31	0.49	0.54	0.40	0.48	1.00		
RB43	0.58	0.58	0.61	0.60	0.55	1.00	
RB50	0.56	0.55	0.44	0.54	0.84	0.60	1.00

highest similarity was observed between the strains RB31 and RB50.

The dendrogram obtained from the RAPD profiles revealed that the seven efficacious strains were differentiated into two clusters A and B. In cluster A, sub-cluster A4 comprised two strains RB1 and RB10 obtained from Bijapur district. Remaining strains belonged to different clusters obtained from different districts of Karnataka. But the strains RB31 and RB50, though geographically isolated (coming from a far-off locations - Uttara Kannada and Bangalore) showed 84 per cent similarity (Table 2).

The results revealed that the morphologically similar strains (short rods) with similar mechanism of strong siderophore, pyocyanin, IAA and volatile metabolites production. Wiedmann *et al.* (2000); Stafford *et al.* (2005) also demonstrated various molecular methods for subtyping rhizobacterial strains, including pulsed field gel electrophoresis (PEGE), PCR based typing methods (RAPD-PCR), DNA sequence based typing and ribotyping. Similarly, Ramesh Kumar *et al.* (2002) and Barriuso *et al.* (2005) also used PCR-RAPD analysis to PGPR strains which exhibited plant growth promotion and strong antifungal activity. Since differentiation by morphological, biochemical methods was limited.

In the present study also, the results obtained here showed the possibility of using RAPD technique to distinguish variability among the isolates of PGPR strains. The information could then be used to determine specific primers that would allow identification of PGPR strains directly from the soil. Strong siderophores, fluorescein, pyocyanin, IAA and volatile metabolites producing strains *viz.*, RB31 and RB50 belonged to the group differentiated by RAPD test which have 84 per cent similarity than other isolates. This indicates that the genetic variability revealed through RAPD test is authentic (Khan and Anwar, 2008).

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