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Short communication

Genetic characterization of plant growth promoting rhizobacteria of two prominent leguminous trees in western Rajasthan

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Abstract

Plant growth promoting rhizobacteria (PGPRs) from the rhizosphere and root nodules of native *Acacia senegal* (L.) Willd. and *Prosopis cineraria* (L.) Druce trees in Western Rajasthan were molecularly characterized by direct sequencing of the *16S rRNA* gene. Of the nine isolates obtained from *A. senegal*, the lone rhizospheric isolate was identified as *Stephyllococus hominis* Kloos and Schleifer and the remaining eight from root nodules were characterised as *Sinorhizobium saheli* De Lajudie. Eight isolations from *P. cineraria* included *Lysinibacillus sphaericus* Meyer Neide and *Bacillus subtilis* (Ehrenberg) Cohn (one isolate each) from rhizosphere and *Sinorhizobium kostiense* Nick (one) and *S. saheli* (five) from root nodules. Eight RAPD primers detected intra-specific variations generating scorable amplicons and revealed 130 bands with 100% polymorphism. The phylogram based on multiple sequence alignment of 16S rRNA gene delineated not only different genera of PGPRs but also recognized four strains of *S. saheli*.

Keywords: *Acacia Senegal*, *Prosopis cineraria*, Polymorphism, 16S rRNA.

Plant-bacteria interactions in the rhizosphere are the determinants of plant health and soil fertility (Hayat et al., 2010). However, there is incomplete knowledge regarding the growth promoting microorganisms associated with the principal trees in the desert ecosystems of India. A study was conducted to molecularly characterize the diverse groups of plant growth promoting rhizobacteria (PGPRs) in the rhizosphere and root nodules of native *Acacia senegal* (L.) Willd. and *Prosopis cineraria* (L.) Druce trees of western Rajasthan by direct sequencing of *16S rRNA* gene to detect genetic diversity in field populations of PGPRs.

Three to four month-old plants of *A. senegal* and *P. cineraria* were procured from local nurseries of Jodhpur and Pali districts of Rajasthan and brought to the laboratory during August-September 2010. The soil adhering to the roots were collected after removing the polythene bags and the PGPRs isolated by serial dilution technique. The root nodules, collected with forceps after washing the roots under tap water, were surface sterilized using 0.1% mercuric chloride ($HgCl_2$) for 5 min. The sterilized root nodules were rinsed in sterilized water several times to remove the traces of $HgCl_2$. These nodules were treated with 70% ethanol for 2 min and allowed to dry for 10 min. The nodules were crushed with sterilized micro-pestles, serially diluted and 100 µl of the suspension was plated onto yeast extract mannitol agar (YEMA) supplemented with Congo red (31.83 g L^{-1}; HiMedia Laboratories) using spread plate technique. The plates were incubated at room temperature (25–30°C) for 5 days. Isolated colonies were picked and transferred to YEMA slants in test tubes and incubated for 5 days at room temperatures. Each bacterial culture was inoculated separately in conical flasks containing 50 ml of autoclaved Luria broth culture medium and kept on to rotary shaker for 48 h. The genomic DNA was isolated following

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HiPurA™ Bacterial and Yeast Genomic DNA purification spin kit.

The genomic DNA of each bacterial strain was used for amplification and sequencing of *16S rRNA* gene. The PCR primers EUB 1 (19 bp) and EUB 2 (20 bp) were used to amplify the 16S ribosomal DNA. Amplification by PCR was performed in a total volume of 50 µl containing: 1U Taq DNA polymerase (Bangalore Genei), 2.5 mM $MgCl_2$, 160 µM dNTP mix (Bangalore Genei), 50 pmol of each EUB 1 and EUB 2 primers, 50 ng genomic DNA in dH_2O. The reactions were performed in a gradient thermal cycler with following conditions: one min denaturation at 95°C, 30 s annealing at 50°C, 1 min 20 s elongations at 72°C for 36 cycles with a final elongation step of 72°C for 10 min. The PCR products were visualized on 1.5% agarose gel. PCR products were directly sequenced using big dye terminator method in ABI prism DNA sequencer. Nucleotide sequence comparisons were performed using BLAST network services of NCBI, USA database to identify each culture up to species level.

Multilocus genotyping by RAPD was performed using 13 decamer arbitrary primers supplied by Operon Technologies. Amplification was performed in a total reaction mixture of 25 µl. Each reaction mixture contained: decamer primer, 2 µl (50 pmol μl^{-1}); dNTP mix, 2 µl (2-mM each of dNTP); $MgCl_2$,1 µl (25 mM); Taq DNA polymerase, 0.5 µl (5U μl^{-1}); 10× PCR buffer, 2.5 µl, 13.0 µl of dH_2O and 4 µl of genomic DNA (50 ng). RAPD-PCR amplification was performed in a gradient thermal cycler with lid heating option at 110°C with an initial denaturation step of 94°C for 3 min followed by 36 amplification cycles of 94°C for 40 sec, 50°C for 40 sec and 72°C for 2 min and final elongation at 72°C for 10 min.

PCR amplification products were electrophoretically separated on 1.5% agarose gel prepared in 1× TAE (tris-acetic acid-EDTA) and scored for presence (1) and absence (0) of scorable bands. Dendrograms were constructed to establish the genetic relationship implying NTSYS-pc, Version 2.02 h. Polymorphic information content (PIC) was estimated using the formula of Kumar et al. (2003). Nucleotide sequence comparisons were performed by using the BLAST network services against the NCBI databases. The multiple sequence alignment of partial sequences of 16S rRNA gene region of the representative PGPR strains of different groups was performed using CLUSTAL X (1.8) software to determine the genetic relationships by construction of a phylogram.

Direct sequencing of amplified PCR products of 16S rRNA gene resulted in molecular identification of 17 PGPRs (Table 1). Isolates from *A. senegal* rhizosphere was identified as *Stephyllococus hominis* Kloos and Schleifer (single isolate) and those from root nodules were identified as *Sinorhizobium saheli* De Lajudie (eight). Isolates of *P. cineraria* included *Lysinibacillus sphaericus* Meyer Neide, *Bacillus subtilis* (Ehrenberg) Cohn and *Sinorhizobium kostinese* Nick (one each) and *Sinorhizobium saheli* (five). Out of 13, eight RAPD primers detected intra-specific variations generating scorable amplicons and revealed 130 bands in range of 200 bp to 8 kb (Table 2). Among these, all bands were polymorphic amounting to 100% polymorphism in banding pattern. The PIC values varied from 0.82 to 0.94. High PIC values provided a robust estimate of the discriminatory power of most of the loci by taking into account the relative frequencies of the expressed alleles. The combined dendrogram of eight primers data matrix delineated 17 PGPRs into five main clusters and three as out groups (Fig. 1).

One representative PGPR from each RAPD cluster and all distinct strains were subjected to multiple sequence alignment of 16S rRNA gene. The phylogram delineated not only different genera of PGPRs (*Staphylococcus hominis*, *B. subtilis*, *Lysinibacillus sphaericus*, *S. kostiense*) but also distinguished four representative strains of *S. saheli* (Fig. 2). The results showed that the genetic diversity of PGPRs of population 2 was greater than population 1 (Table 3). Furthermore, all isolates of *S. saheli* did not completely correlate with the host from which they were isolated and readily formed clusters representing both host species. Multiple sequence alignment of representative cultures from all

Table 1. Molecular identification of PGPRs isolated from *Acacia senegal* and *Prosopis cineraria* from two districts of western Rajasthan.

Strain	Host	District	Organism	16S Partial sequence (bp)	Gen accession no.
AS-1	*Acacia senegal*	Jodhpur	*Staphylococcus hominis*	987bp	HQ738493
AS-2	as above	Pali	*Sinorhizobium saheli*	923bp	HQ738494
AS-3	as above	Pali	as above	924b	HQ738495
AS-5	as above	Jodhpur	as above	926bp	HQ738497
AS-6	as above	Pali	as above	926bp	HQ738498
AS-7	as above	Pali	as above	928bp	HQ738499
AS-8	as above	Jodhpur	as above	931bp	HQ738500
AS-9	as above	Pali	as above	925bp	HQ738501
AS-10	as above	Pali	as above	923bp	HQ738502
PC-12	*Prosopis cineraria*	Jodhpur	*Lysinibacillus sphaericus*	987bp	HQ738504
PC-13	as above	Jodhpur	*Bacillus subtilis*	984bp	HQ738505
PC-14	as above	Pali	*Sinorhizobium kostiense*	918bp	HQ738506
PC-16	as above	Jodhpur	*Sinorhizobium saheli*	925bp	HQ738508
PC-17	as above	Jodhpur	as above	926bp	HQ738509
PC-18	as above	Pali	as above	925bp	HQ738510
PC-19	as above	Pali	as above	925bp	HQ738511
PC-20	as above	Jodhpur	as above	927bp	HQ738512

Table 2. Details of primer code, GC content and per cent polymorphism of RAPD primers.

Primer code	Primer sequence 5' to 3'	GC%	No. of bands	No. of polymorphic bands	PIC value
OPP-02	TCG GCA CGC A	70	18	18	0.89
OPP-09	GTG GTC CGC A	70	22	22	0.94
OPP-16	CCA AGC TGC C	70	14	14	0.90
OPB-06	TGC TCT GCC C	70	15	15	0.92
OPB-13	TTC CCC CGC T	70	15	15	0.92
OPB-09	TGG GGG ACT C	70	16	16	0.91
OPA-09	GGG TAA CGC C	70	19	19	0.92
OPA-16	AGC CAG CGA A	60	11	11	0.82
	Total		130	130	

Table 3. Summary of genetic variation statistics for all loci.

Locus mean	Sample size	*na*	*ne*	*h*	*i*
Pop 1	8	1.8308	1.3344	0.2147	0.3433
Pop 2	9	1.8538	1.3289	0.2177	0.3510
Mean of all loci	17	2.0000	1.3357	0.2238	0.3672

na = Observed number of alleles, *ne* = Effective number of alleles, *h* = Nei's gene diversity, *i* = Shannon's Information index

RAPD clusters and distinct PGPRs were delineated. Maximum genetic distance was observed in the isolates of *S. saheli* due to diversity in nucleotide sequences. The study revealed that *Sinorhizobium saheli*, *S. kostiense, L. sphaericus*, *B. subtilis* and S. *hominis* are the major PGPRs associated with the rhizosphere of *A. senegal* and *P. cineraria* in the arid region of western Rajasthan.

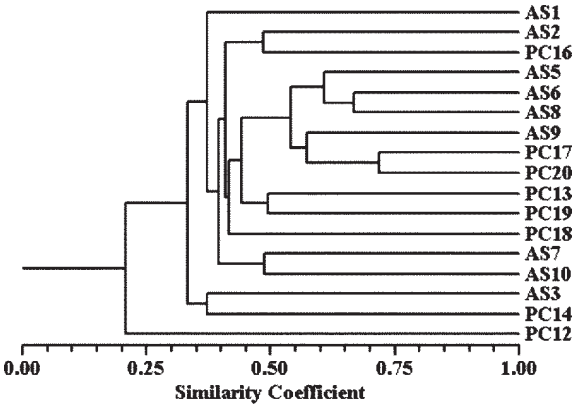

Figure 1. UPGMA dendrogram generated by eight RAPD primers data matrix.

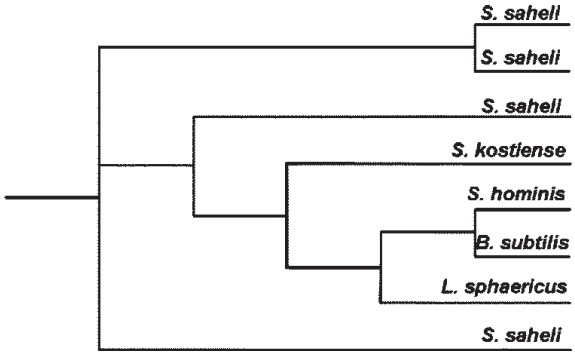

Figure 2. Phylogram generated using tree view of multiple sequence aligned 16S rRNA region of eight PGPRs.

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