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Full Length Research Paper

Assessment of diversity and plant growth promoting attributes of rhizobia isolated from *Cajanus cajan* L.

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Eight (8) strains of rhizobia from Pigeon pea *(Cajanus cajan)* were isolated and identified based on their physiological and biochemical characteristics using standard methods. Five (5) of the isolates (KCC1 to KCC5) were subjected to partial 16S rDNA gene sequencing for phylogenetic analysis. Genetic diversity was investigated using restriction patterns produced by amplified rDNA restriction analysis (ARDRA) and were placed into different clusters. They were identified as *Ensifer sinorhizobium* hence named as *Ensifer* spp. KCC1 to KCC4. KCC5 is placed in *Ensifer fredii* clade. Nodulation and nitrogen fixing abilities of the isolates were confirmed by amplification of *nod*C and *nif*H genes. All of them showed indole-3-acetic acid production and phosphate solubilization activity. Only two isolates (KCC2 and KCC5) produced siderophore on CAS medium and showed strong antagonistic effect against *Fusarium udum*. None of the isolates produced cyanogen. The study showed that there is a considerable homogeneity amongst *C. cajan* root nodule isolates and can be exploited for plant growth promotion of *C. cajan* with effective antagonism against *F. udum*.

Key words: Diversity, *Ensifer* (*Sinorhizobium)* spp., pigeon pea (*Cajanus cajan*).

INTRODUCTION

Pigeon pea *(Cajanus cajan)* is one of the important pulse crops and a very popular food in developing tropical countries. India is a principal pigeon pea-growing country contributing nearly 90% of total world's production. It is widely grown in almost all the states of the country.

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Abbreviations: PGPR, Plant growth promoting rhizobacteria; **IAA,** indole-3-acetic acid; **YEMA,** yeast extract mannitol agar; **EDTA,** ethylenediamine tetra acetic acid; **PCR-RFLP,** polymerase chain reaction-restriction fragment length polymorphism; **NCBI,** national center for biotechnology information; **CAS,** Chrome-azurol S; **PDA,** potato dextrose agar; **HAB,** Hoffer's alkaline broth; **GPA,** glucose peptone agar; **ARDRA,** amplified rDNA restriction analysis; **PGP,** plant growth-promoting; **DGGE,** denaturing gradient gel electrophoresis; **LCOs,** lipochito-oligosaccharides.

Symbiotic nitrogen fixation by *Rhizobium* strains with legumes is important for agricultural productivity. All rhizobia belong to alpha *Proteobacteria,* based on the sequences of the gene coding for small subunit (16S) rRNA (Martinez and Mellado, 1996). The classification of fast growing rhizobia nodulating tropical legume, *C. cajan* has been paid little attention in past decades. Earlier, slow growing tropical rhizobia of pigeon pea have been reported (Vincent, 1970). There have been several proposed changes in rhizobial classification in recent years, which were largely based on analysis of physiological, biochemical and morphological data complemented with DNA-DNA hybridization. The emergence of new powerful tools of systemic bacteriology has been influencing the radical changes in rhizobial classification over the last few years. Phylogenetic relationships based on 16S rRNA sequences and phenotypic variations have been implicated to ascribe isolates into distinct species. However, a significant research is required to establish and understand the diversity of a wide range of fast growing rhizobia from various legume species before any

effective classification system can be developed. To form an effective symbiosis, rhizobia require several classes of specific genes. These include *nod* genes, encoding the production of Nod factors which stimulate the plants to produce symbiotic nodules, and *nif* genes which produce the nitrogen-fixing nitrogenase enzymes (Haukka et al., 1998). Rhizobia are important members of plant growth promoting rhizobacteria (PGPR) showing several plant growth promoting activities (Glick et al., 1994). Direct plant growth promoting activities include production of indole-3-acetic acid (IAA), siderophore, and phosphate solubili-zation (Arora et al., 2001; Deshwal et al., 2003).

The present study investigates the diversity of fast growing rhizobia of pigeon pea nodules based on analysis of morphological, physiological and biochemical data, variations in partial sequences of the 16S rRNA gene and restriction fragment length polymorphism (RFLP). The *nod*C and *nif*H gene analysis have been used to assess the diversity of rhizobial populations in pigeon pea growing in central part of India, and also reports the plant growth promoting properties for *C. cajan* and antagonistic properties against *Fusarium udum*.

MATERIALS AND METHODS

Isolation of bacteria from root nodules of pigeon pea

Root nodulating bacteria were isolated from nodules of pigeon pea plants grown in farmer's field of Betul district (M.P.), India in July and August. The area has an average temperature range of l0°C (in winters) to 41°C (in summers). The method of Vincent (1970) was followed for isolation of root nodulating bacteria. *C. cajan* seedlings were uprooted carefully and root nodules were collected and washed with sterile distilled water followed by surface treatment with 95% ethanol (2 ml) and further rinsing with sterile distilled water. Properly washed nodules were surface sterilized quickly (2 to 3 min) with 0.1% mercuric chloride ($HgCl_2$) and again cleaned for at least 6 times with sterile distilled water so as to remove the traces of $HgCl_2$. The nodules were crushed in a half filled culture tube with saline water (0.85% NaCl) with the help of sterile glass rod. A milky bacterial suspension obtained was serially diluted and streaked on sterile yeast extract mannitol agar (YEMA) plates (Vincent, 1970). The inoculated plates were incubated at 28 ± 1°C for 24 – 48 h and observed for specific features of rhizobia. The eight isolates obtained from *C. cajan* were named as KCC *(C. cajan* rhizobia) 1 to 8 and maintained separately on YEMA slants at 4°C for further study.

Phenotypic characterization of isolated bacteria

The fast growing rhizobial strains were morphologically and biochemically characterized according to Bergey's Manual of Determinative Bacteriology by Holt et al. (1994).

DNA isolation for genotypic characterization

For the isolation of genomic DNA, the rhizobia were separately grown in yeast extract mannitol (YEM) broth. The bacterial pellets were washed with 50 mM ethylenediamine tetra acetic acid (EDTA, pH 8.5). The washed cells were lysed by suspending in 0.5% sodium dodecyl sulphate (SDS) for 10 min at 60°C. The resulting lysate was cleared from cell debris by centrifugation at 10,000 rpm for 10 min. The supernatant was taken and protein was removed by adding proteinase K followed by addition of ethanol to collect the DNA. The DNA solution was prepared by the addition of 1 ml phenol-chloroform mixture. RNase (50 µg ml^{-1}) was added to DNA extract and mixture was incubated at 37°C for 30 min. This was followed by the addition of tris-phenol and centrifugation at 12,000 rpm for 15 to 20 min at 4°C. Sodium acetate (0.3 M) 100 µl and cold isopropanol (1 ml) were added with continuous vortex to the heat sterilized supernatant. Finally, the DNA was purified by the addition of phenol-chloroform followed by centrifugation at 15000 rpm (Sambrook and Russel, 2001).

16S rDNA gene amplification

Universal eubacterial primers fD1 5'CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGCTCAG3' and rD1 5'CCC GGG ATC CAA GCT TAA GGA GGT GAT CCA GCC3' (Kumar et al., 2006) were used for amplification of 1492 bp region of the 16S rDNA gene on PTC 100 (MJ Research USA) thermal cycler. 50 µl reaction mixture included 5 to 10 ng of bacterial DNA as template, 1 µl of each primer, 1 U of *Taq* DNA polymerase (Banglore Genei, India) and 100 µM dNTPs. The reaction conditions were: Initial denaturation of 7 min at 94°C followed by 29 cycles of denaturation of 1 min at 94°C, extension of 1 min at 72°C and annealing temperatures 54°C for 7 cycles; 53 and 52°C for one cycle each, 51°C for 20 cycles and a final extension of 10 min at 72°C. Amplified gene was visualized in 0.8% agarose after electrophoresis.

Polymerase chain reaction (PCR)-RFLP analysis of 16S rDNA

The PCR amplified products of 16S rRNA gene were digested with three restriction endonucleases *Alu*I, *Hae*III and *Msp*I. Aliquotes of purified PCR products (12.3 µl) were digested with 3 U of restriction endonucleases (0.3 µl) in 14 µl reaction volume using the manufacturer's recommendation buffer (1.4 µl of 10X) and temperature. The digests were separated slowly by electrophoresis in 2% agarose gels. Electrophoresis was carried out at 80 V for 2 h and 30 min with standard gels (11 × 14 cm). The restricted patterns were visualized under UV illumination and photographed with gel documentation system (Vilber Lourmet, Germany). Average linkage unweighted pairs group method with averages (UPGMA) method was used to construct dendogram using the statistical program NTSYS2.

16S r DNA gene sequencing

Partial 16S rDNA gene sequencing was performed in the same reaction mixture and following the same amplification conditions as described in full 16S rDNA. The only difference was in primers. In the partial gene amplification, primers f1 and r1 were used. The PCR products were analyzed on 1.2% agarose gel in TAE buffer, run at 50 V for 2 h. Gels were stained with ethidium bromide and visualized as described above. The amplicons were purified with Banglore Genei, PCR purification kit and quantified spectrophotomatrically at 260 nm compare with calf thymus DNA. The cleaned partial 16S rDNA amplicon was sequenced with DNA sequencing system.

Analysis of 16S rDNA sequences using bioinformatics tools

The PCR products were purified and sequenced. All the sequences were subjected to phylogenetic analysis. The homology of partial sequences were compared with the sequences from the DNA databases and similar sequences showing above 95% were retrieved by nucleotide Basic Local Alignment Search Tool (BLAST) program at the National Center for Biotechnology Information (NCBI) BLAST server (www.ncbi.nlm.nih.gov/BLAST). Multiple sequence alignment of retrieved sequences was done by EBI ClustalW server (www.ebi.ac.uk.clustal/index.html). Phylogenetic tree was constructed by using *gene bee* server (www.genebee.msu.ru/services/phtree-reduced.html). Phylogenetic tree was obtained with bootstrap values in cluster algorithm, phylip format and topological algorithm.

Amplification of *nif*H gene of the rhizobial strain

For amplification of *nif*H region (781 bp), two primers, *nif*H1 5' CGT TTT ACG GCA AGG GCG GTA TCG GCA 3' and *nif*H2 5' TCC TCC AGC TCC TCC ATG GTG ATC GG 3' were used as described by Perret and Broughtn (1998): 50 µl reaction mixture contained 5-10 ng of bacterial DNA template, 1 µl of each *nif*H primer, 1 U of *Taq* DNA polymerase (Banglore Genei, India) and 100 µM dNTPs. The conditions of thermal cycler (PTC 100, M.J. Research, USA) for *nif*H gene amplification were: Initial denaturation of 30 s at 95°C, 5 cycles of 30 s at 94°C, 30 s at 55°C and 1 min at 72°C, 30 cycles of 30 s at 94°C, 30 s at 65°C and 1 min at 72°C with a final extension of 10 min at 72°C (Pandey et al*.,* 2004). An aliquot of 10 µl of amplified product was visualised on 1.2% agarose gel after electophoresis. Visualization was captured on a gel documentation system (Vilber Lourmet, Germany).

Amplification of *nod*C gene from nodule isolates

The *nod*C gene from genome and plasmids of root nodule isolates was amplified using the *nod*C gene primers *nod*C1 5' GCC ATA GTG GCA ACC GTC GT 3' and *nod*C2 5' 'TCA CTC GCC GCT GCA AGT C 3' as described by Jacob et al. (1985). 50 µl reaction mixture included 5 to 10 ng of bacterial DNA as template, 1 µl of each primer, 1 U of *Taq* DNA polymerase (Banglore Genei, India) and 100 µM dNTPs. The reaction conditions for PCR (PTC 100, M.J. Research, USA) were: Initial denaturation of 3 min at 95°C followed by 30 cycles of denaturation of 45 sec at 94°C, annealing temperatures 55°C for 30 sec, extension of 1 min at 72°C with a final extension of 10 min at 72°C. Amplified gene was visualized in 0.8% agarose after electrophoresis (Kumar et al., 2006).

Determination of plant growth-promoting mechanism

Siderophore production

Siderophore production was determined on chrome-azurol S (CAS) medium following the method of Schwyn and Neilands (1987). The log phase culture of bacterial strains spotted separately on CAS medium and plates were incubated-at 28 ± 1°C for 48 h. Formation of orange to yellow halo around the colonies showed the production of siderophore.

IAA production

To observe IAA production, exponentially grown cultures (10^8 cells ml^{-1}) of the strains were incubated separately on broth medium for 24 h (Bric et al., 1991). Supernatant of the strains were collected by centrifugation at 10,000 rpm for 15 min at 4°C and 2 ml supernatant of each was transferred separately to a fresh tube to which 100 µl of 10 mM O-phosphoric acid and 4 ml of Salkowaski reagent (1 ml of 0.5 mM $FeCl_3$ in 35% $HClO_4$) were added. Mixtures were incubated at room temperature for 25 min and observe for pink colour production.

Phosphate solubilization

Phosphate solubilization ability of isolated strains were detected by spotting separately on Pikovskya's agar plates. Plates were then incubated at 28 ± 1°C for 3 d, and observed for the clearing zone around the colonies (due to the solubilization of inorganic phosphate by bacteria) (Pikovskya, 1948).

Antifungal activities of rhizobial isolates

Antagonistic properties of rhizobial isolates were tested against *F. udum* on potato dextrose agar (PDA) plates using dual culture technique (Skidmore and Dickinson, 1976). Five days old mycelial discs (5 mm diameter) were placed in four corners of solidified medium in plates containing modified PDA by addition of 2% sucrose. Exponentially grown cultures (10^8 cells ml^{-1}) of rhizobial isolates were spotted 2 cm juxtaposed from the fungal disc and incubated at 28 ± 1°C for 5 d. Growth inhibition was calculated by measuring the distance between the bacterial and fungal colonies as compared to control.

RESULTS

Isolation of bacteria from root nodules of pigeon pea and phenotypic characterization

A total of 8 bacterial isolates were screened from root nodules of *C. cajan*. All the isolates (KCC1 to KCC8) were gram-negative, non-sporing, and non-capsulated, motile rods with single sub-polar flagellum. The isolates formed red, semi-translucent, rounded, smooth, mucoid colonies (2 to 4 mm diameter) on CrYEMA after 48 h of incubation.

All the isolates were fast growers with average mean generation time of 2.9 h. Isolates were positive to catalase and oxidase activities and produced yellow colour on bromothymol blue which indicated acid production. Almost all the isolates were positive for urease and gelatinase production, utilized citrate and precipitated calcium glycerophosphate. Except KCC5, all the isolates gave negative results for starch hydrolysis. The isolates failed to grow on glucose peptone agar (GPA) but grew in Hoffer's alkaline broth (HAB) and could tolerate 8% KNO_3 and 2% NaCl (Table 1).

The characters of isolates were compared with standard rhizobial strains viz; *Sinorhizobium meliloti* MTCC-100, *Rhizobium leguminosarum* MTCC-99 and *Mesorhizobium loti* MTCC-2378 and most of them possessed characteristics similar to *S. meliloti* MTCC-100 (Table 1).

Table 1. Morphological, physiological and biochemical characters of root nodulating bacteria from *Cajanus cajan* var. Manak isolates.

Characteristic	KCC1	KCC2	KCC3	KCC4	KCC5	KCC6	KCC7	KCC8	MTCC100	MTCC 99	MTCC 2378
Gram reaction	-	-	-	-	-	-	-	-	-	-	-
Growth at: 28°C	+	+	+	+	+	+	+	+	+	+	+
41°C	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+
Capsule	+	+	-	-	+	+	+	-	+	+	+
Endospore	-	-	-	-	-	-	-	-	-	-	-
PHB accumulation	+	+	+	+	+	+	+	+	+	+	-
Generation time (h)	1.5	1.5	1.8	1.5	1.5	1.4	1.5	1.5	2.7	2.9	1.6
Catalase	+	+	+	+	+	+	+	+	+	+	-
Oxidase	+	+	+	+	+	+	+	+	+	+	+
H_2S	-	-	-	+	-	-	-	+	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-	+	+	-
Starch hydrolysis	-	-	-	-	+	-	-	-	-	-	-
Citrate utilization	+	+	+	+	+	+	+	+	+	+	+
Growth on GPA	-	-	-	-	-	-	-	-	-	-	+
Growth in HAB	+	+	+	+	+	+	+	+	+	+	-
2% NaCl tolerance	+	+	+	+	+	+	+	+	+	+	+
8% KNO_3 tolerance	-	-	-	-	-	-	-	-	+	+	-
Ca- glyc	-	-	-	-	-	-	-	-	+	+	-
Utilization of:											
Arabinose	+	+	+	-	-	+	+	+	+	+	+
β alanine	-	+	+	+	+	+	+	-	-	+	+
Sucrose	+	+	+	+	+	+	+	+	+	+	+
Glucose	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+
meso-Inositol	-	+	+	+	+	+	+	+	+	+	+
Lactose	+	-	-	+	+	+	-	-	+	+	+

+, Positive; -, negative; GPA, glucose peptone agar; HAB, Hoffer's alkaline broth; PHB, poly hydroxyl butyrate; Ca glyc; precipitation in calcium glycerophosphate; *Sinorhizobium meliloti* MTCC-100; *Rhizobium leguminosarum* MTCC-99; *Mesorhizobium loti* MTCC-2378.

Amplification of 16S rRNA gene in *C. cajan* nodulating isolates

After amplification of 16S rRNA gene of all the isolates with universal fD1 and rD1 primers, the amplified products were compared with low range ruler DNA (Banglore Genei, India). The total length of amplicons observed was approximately 1500 bp (1.5 kb) that represents the amplification of nearly full-length 16S rRNA gene from all the *C. cajan* isolates. The size of the PCR products corresponded to the size of the 16S rRNA genes among all eight isolates.

PCR-RFLP analysis of 16S rDNA amplicons

Analysis of the genetic relatedness amongst eight *C. cajan* isolates was done employing amplified rDNA restriction analysis (ARDRA) of 16S rDNA-RFLP. Restriction analysis of 16S rDNA with all three endonucleases, *Alu*I, *Hae*III and *Msp*I was carried out. The product from ARDRA varied in molecular mass from approximately 5 kb to 200 bp (Figure 1). Based on variation in number and size of bands, it was possible to identify identical strains of *C. cajan* KCC1, KCC2 and KCC3 belonging to a single cluster, whereas reference strain formed cluster with KCC5. KCC5 and reference strains were 100% similar and showed a similar homogeneity but were distinct from the other isolates. It is clear from the dedrogram that the *C. cajan* isolate share a great homogeneity (Figure 2).

Phylogenetic analysis

Phylogenetic analysis of 16S rDNA sequences of *C. cajan* nodulating isolates (KCC1 to KCC5) with other reference strains revealed that KCC1 and KCC2 belong

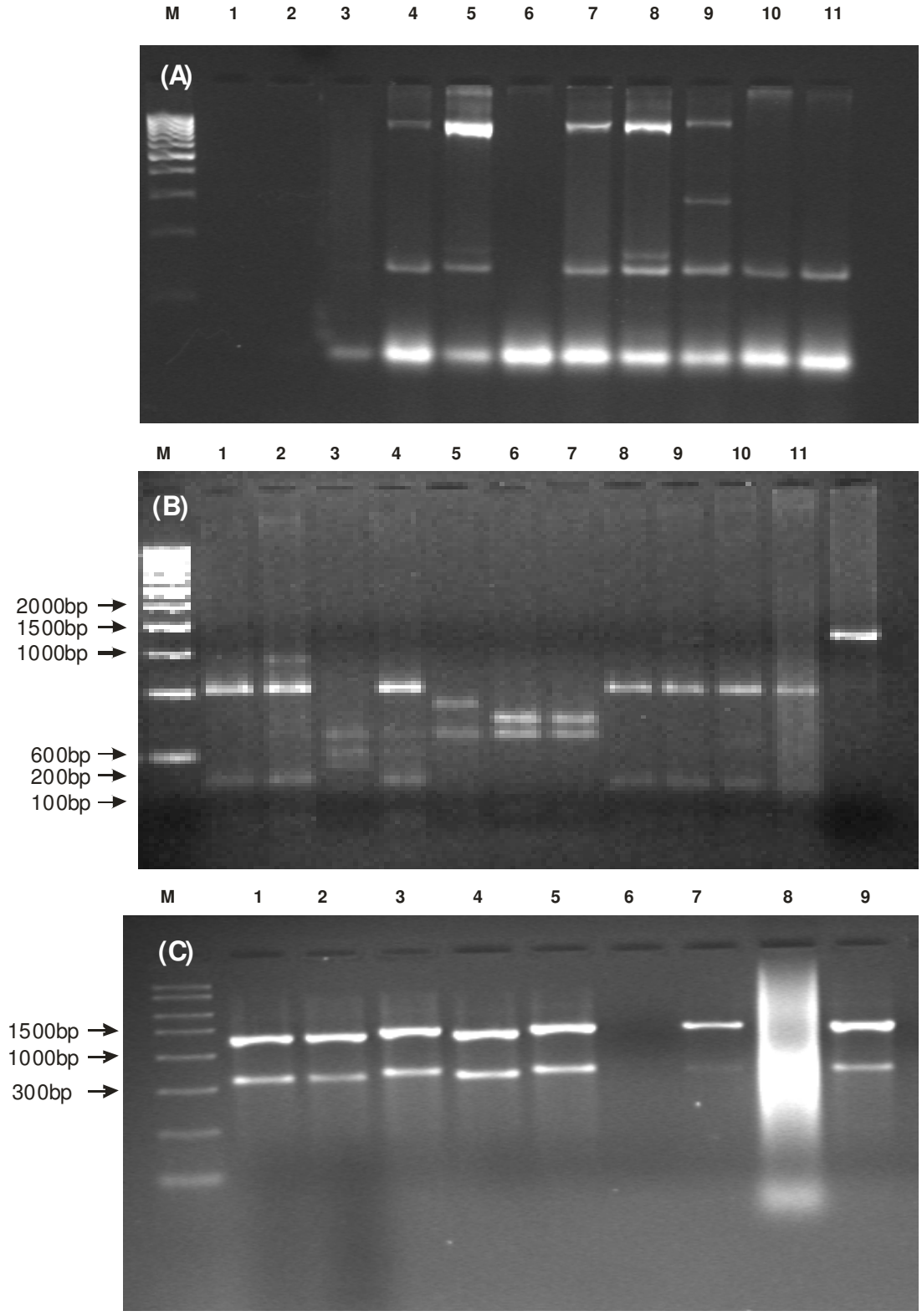

Figure 1. Restriction patterns of PCR-amplified 16S rDNA digested with (A) *AluI*; (B) *HaeIII* and (C) *MspI* of *C. cajan* isolates. Lane 1, KCC1; Lane 2, KCC2; Lane 3,KCC3; Lane 4, KCC5; Lane 5, KCC5; Lane 6, KCC6; Lane 7, KCC7; Lane 8, KCC8; Lane 9, *Sinorhizobium meliloti* MTCC100; Lane 10, *Mesorhizobium loti* MTCC 2378; Lane 11, *Sinorhizobium fredii*; Lane M, DNA ruler (marker).

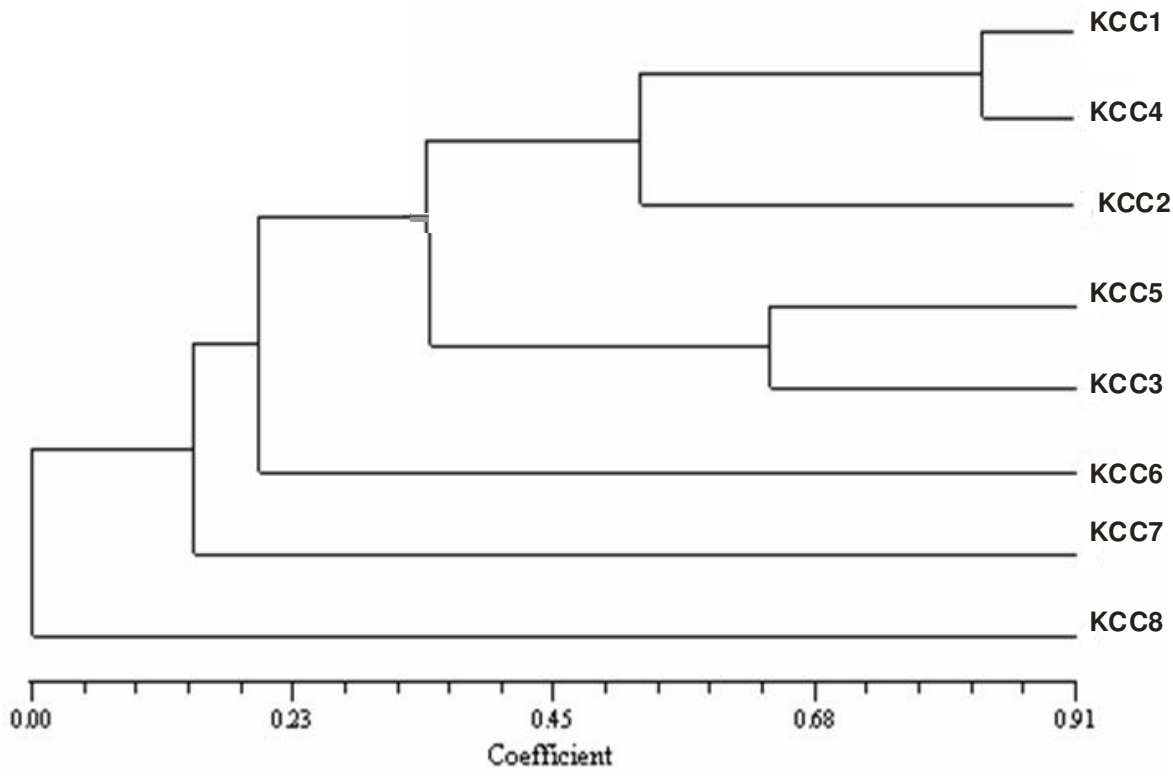

Figure 2. UPGMA cluster of nodule isolates of *C. cajan* on the basis of 16S ARDRA with *AluI, HaeIII and MspI.*

to same cluster with 100% bootstrap value. KCC3, KCC4 and KCC5 also belong to same cluster (Figure 3). KCC4 and KCC5 were more distinct with 55% bootstrap value (Figure 3); a neighbour joining dendogram was generated using the sequences from *Rhizobium* spp. KCC5 and representative *Sinorhizobium fredii*.

Amplification of *nod*C and *nif*H gene in rhizobial strains

When the genomic DNA of the isolates were amplified with the primers of *nod*C gene viz, *nod*C1 and *nod*C2, amplicons of 500 bp were observed (Figure 4A) and compared with low range ruler DNA (Banglore Genei, India). The same products were amplified both from bacterial DNA and plasmid DNA. After amplification of *nif*H gene by specific primers viz, *nif*H1 and *nif*H2, and comparison with low range DNA ruler, 781 bp long amplified products were obtained (Figure 4B).

Plant growth-promoting (PGP) activities of root nodulating bacteria of *C. cajan*

Except KCC1, all strains of *Sinorhizobium* spp. (KCC1 to KCC8) were positive for IAA production as observed by the development of pink colour in the cell free supernatants (Table 2). *Sinorhizobium* spp. KCC5 produced maximum IAA and its production was induced in the presence of tryptophan. In KCC5, the IAA production commenced after 18 h of inoculation.

During phosphate solubilization, all the strains of *Sinorhizobium* spp. formed clear halos around their spot inoculation by solubilizing tricalcium phosphate on the Pikovskya's agar. All the strains solubilized inorganic phosphate (Table 2). In the profile, it was found that phosphate solubilization started after 24 h of incubation in all the strains and maximum in *Sinorhizobium* spp. KCC5 at the 7th day of incubation (Table 2).

None of the strains of *Sinrhiobium* spp. produced cyanogens (Table 2). All the strains were screened for siderophore production on CAS agar. *Sinorhizobium* spp. KCC2 and *Sinorhizobium* spp. KCC5 showed siderophore activity as revealed by orange halo around their colonies. Secretion of siderophore by both the strains started after 12 h of incubation. On further incubation for 5 d at 28°C, siderophore production increased but later on, production was declined. To know the type of siderophore, 48 h old cell-free culture supernatant of the strains (*Sinorhizobium* spp. KCC5) scanned at 400 nm revealed major peak. These peaks corresponded with

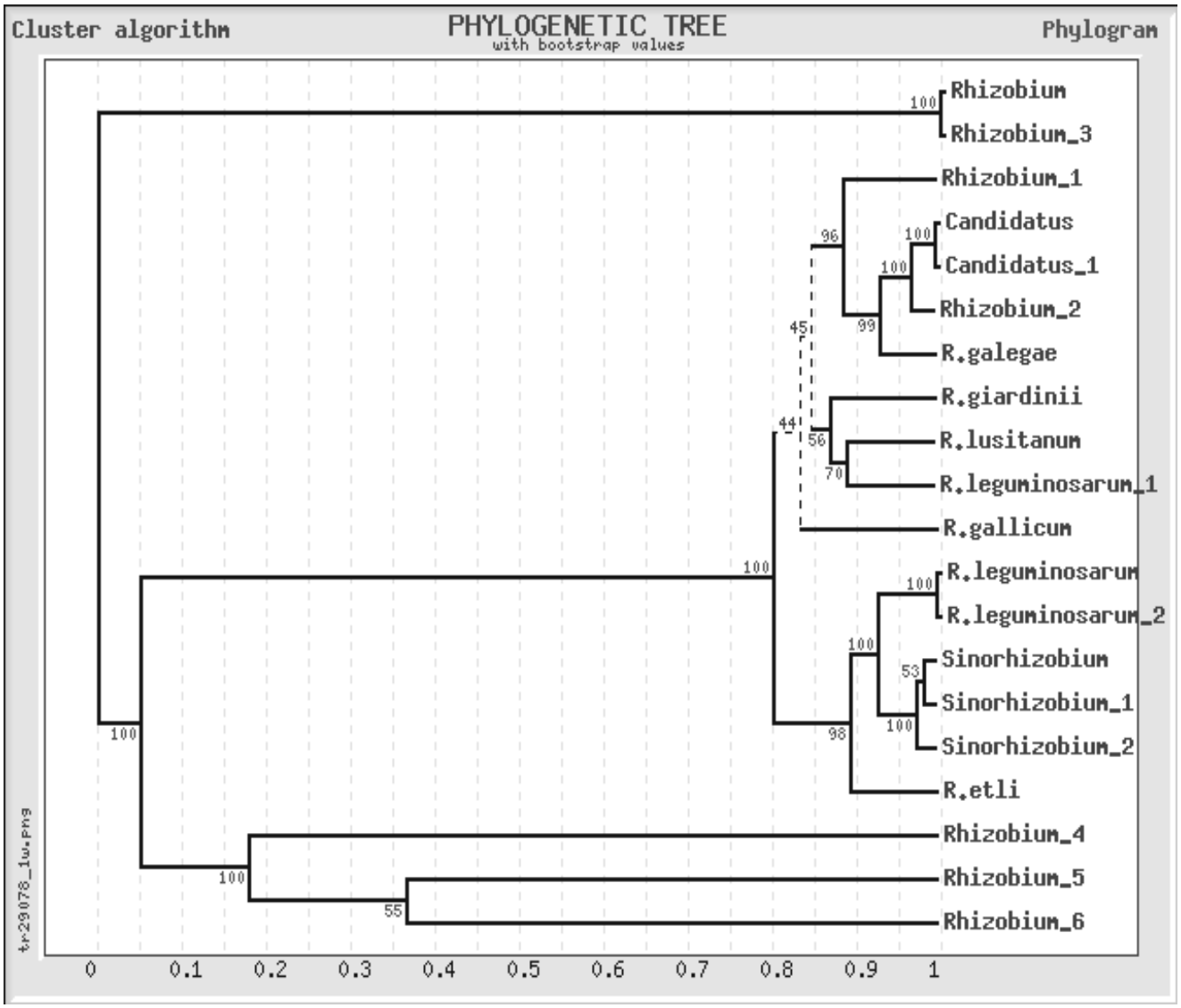

Figure 3. Phylogenetic relationship between bacterial strains of *Rhizobium* and representing species based on partial length 16S rDNA sequences constructed using cluster algorithm. KCC1, *Rhizobium*; Rhizobium-3, KCC2; Rhizobium-1, *Rhizobium sp*. Esparseta 3; Candidatus and Candidatus-1, Candidatus *R. massiliae*; Rhizobium-2, Rhizobium sp. TANU 14; *R. galgae*, *Rhizobium galegae* strain Desai 2028; *R. giardinii*, *Rhizobium giardinii* strain R-4385; *R. lusitanum*, *Rhizobium lusitanum* p1-7; *R. gallicum*, *Rhizobium gallicum* PB2; *R. leguminosarum*, *Rhizobium leguminosarum* strain DASA23002; *Sinorhizobium*, *Sinorhizobium fredii* S47; *Sinorhizobium*-1, *Sinorhizobium fredii* S25; *Sinorhizobium*-2, *Sinorhizobium fredii* S8; *R. etli, Rhizobium etli* B11; Rhizobium-4, KCC3; Rhizobium-5, KCC4; Rhizobium-6, KCC5.

hydroxamate type of siderophore. None of the strain was able to develop wine colour in the reaction mixture following the methods of Arnow (1937). Hence, possibility of presence of catechol-phenolic type of siderophore production is ruled out.

Results of screening for HCN and siderophore production (PGP activity) revealed that none of all the three standard rhizobial strains (*S. meliloti* MTCC-100, *R. leguminosarum* MTCC-99, *M. loti* MTCC-2378) were found positive. All the strains solubilized phosphate except *S. meliloti* MTCC-100 but none produce IAA (Table 2).

In vitro antifungal activities of *Sinorhizobium* spp. strains against *F. udum*

For determination of antifungal activity of *Sinorhizobium* spp., result showed that only *Sinorhizobium* spp. KCC2 and KCC5 exhibited antifungal activity against *F. udum*

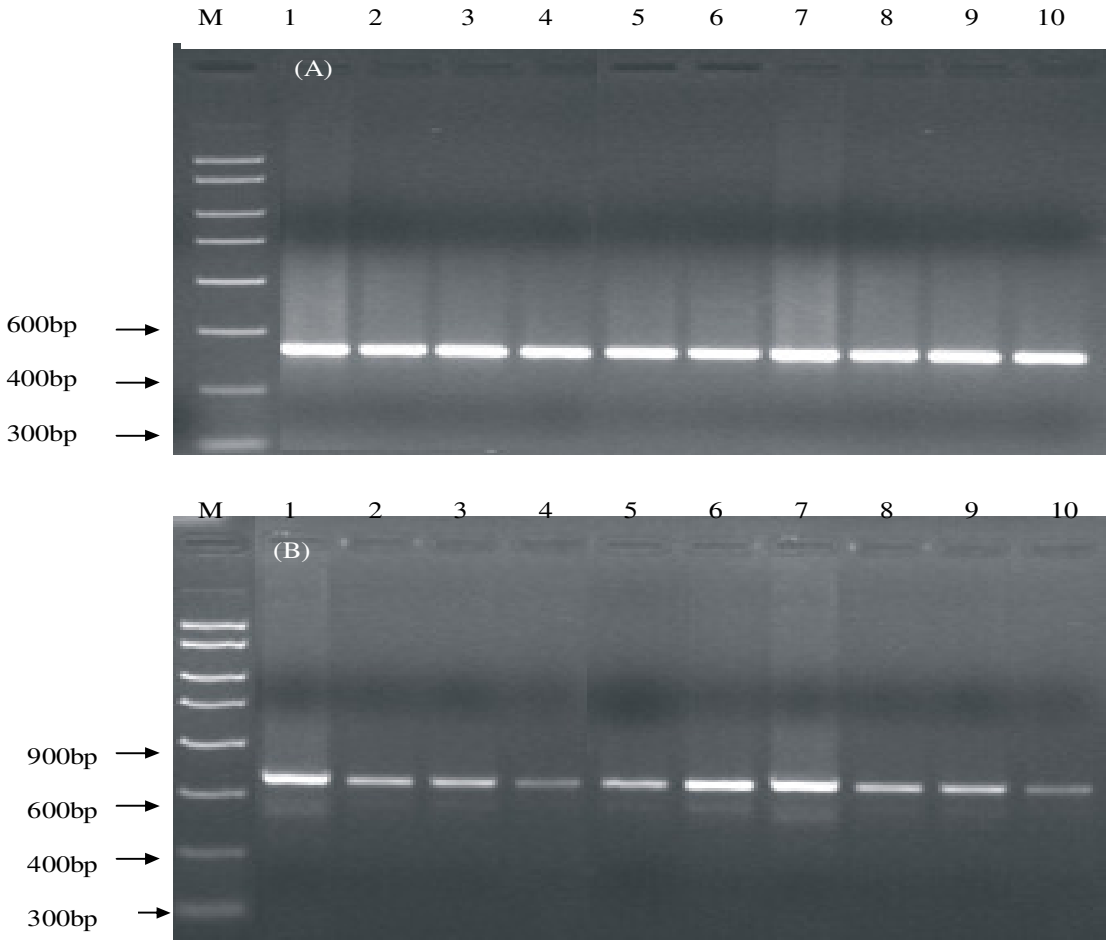

Figure 4. Amplicons of *nod*C gene (A) and *nif*H gene (B) of *C. cajan* nodulating bacterial isolates. Lane 1, KCC1; Lane 2; KCC2; Lane 3,KCC3; Lane 4, KCC5; Lane 5, KCC5; Lane 6, KCC6; Lane 7, KCC7; Lane 8, KCC8; Lane 9, *Sinorhizobium meliloti* MTCC100; Lane 10, *Sinorhizobium fredii* ; Lane M, DNA ruler (marker).

Table 2. Plant growth-promoting and antifungal properties of *Sinorhizobium* strains.

Strain	IAA [A]	Phosphate solubilization[B]	HCN[c]	Siderophore[D]	Antagonism against *F. udum*
KCC 1	-	++	-	-	-
KCC 2	++	+	-	+++	+++
KCC 3	+	+	-	-	-
KCC 4	+	+	-	-	-
KCC 5	+++	+++	-	+++	+++
KCC 6	+	+	-	-	-
KCC 7	+	+	-	-	-
KCC 8	+	+	-	-	-
Standard strain					
MTCC 100	+	+	-	-	-
MTCC 99	-	+	-	-	-
MTCC 2378	-	+	-	-	-

A , IAA negative; +, IAA positive; B, phosphate solubilization negative; +, phosphate solubilization positive; C, HCN negative; +, HCN positive; D, absence of halo formation; +, small halos <0.5 cm wide surrounding colonies; ++, medium halos > 0.5 cm wide surrounding colonies; +++, large halos >1.0cm wide surrounding colonies; *Sinorhizobium meliloti* MTCC-100; *Rhizobium leguminosarum* MTCC-99; *Mesorhizobium loti* MTCC-2378.

(Table 2). Results also showed that antifungal activity was proportional to incubation period with inhibition rate of 70% by Sinorhizobium spp. KCC5 after 6 d of incubation (regression correlation (r^2) values of 0.9745).

DISCUSSION

Rhizobia are α-proteobacteria which establishes symbiotic relationship with leguminous plants leading to the formation of root nodules where they fix atmospheric nitrogen. All the isolates from *C. cajan* were fast growing with average generation time of 2.6 h (Table 1). All of them were Gram-negative, aerobic, non-spore forming and cocci (Holt et al., 1994). Colony morphology of *Rhizobium* has been demonstrated to play an important role in the process of symbiotic nitrogen fixation (Vincent, 1970). Most of the strains were positive for catalase and oxidase activity and utilized citrate. The strains precipitated calcium glycerophosphate. The characteristic features reported in the present study of all bacterial strains are similar to those as described by Holt et al. (1994).

The strains utilized a wide range of carbohydrates including hexoses, pentoses, disaccharides and trioses (Table 1). This is also in agreement with the earlier findings that S*inorhizobia* with other fast growing rhizobia are capable of growing on a variety of carbon substrates (Jordan, 1984; Elkan, 1992). Further, the morphological and physiochemical characters of the strains were found to be similar to that of the genus *Sinorhizobium* (Holt et al., 1994).

Phylogenetic relationship between the isolates, KCC1 to KCC5, is shown in Figure 3. The representing species on the basis of partial length 16S rDNA sequences was constructed by using cluster algorithm. In phylogenetic tree based on 16S rDNA *Rhizobium* sp. KCC5, showed close homology with the *S. fredii* clade. PCR amplification of rDNA genes from environmental DNA samples, combined with fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE), terminal (T)-RFLP, ARDRA, cloning and sequencing provide the detailed information. It is realized that 16S rDNA homology allows a first phylogenetic affiliation of new isolate. Recently, Pandey et al. (2005) also characterized *Burkholderia* spp. MSSP from root nodules of *Mimosa pudica* on the basis of 16S rDNA analysis.

The DNA based methods (RAPD and RFLP) are most suitable for the measurement of genetic relatedness (Sikora and Redzepovic, 2003). In the present study, polymorphism was observed in the combination of 3 endonucleases (*Msp*I, *Alu*I and *Hae*III): *Msp*I produced comparable patterns for *Sinorhizobium* spp. KCC1, KCC2 and KCC4, whereas *Sinorhizobium* sp. KCC5 had the similar profile as of KCC3 (Figure 1). *Alu*I was the most discriminatory restriction enzyme among the three enzymes used for restriction digestion of all the eight isolates. Two clusters were detected among eight isolates and strains KCC1 and KCC4 shared a particularly close homology of approximately 84%. Laguerre et al. (1994) reported that a combination of any two endonucleases *Msp*I and *Nde*II differentiated between *Rhizobium* species such as *Rhizobium loti* and *Rhizobium huakuii, Rhizobium etli* and *Rhizobium tropici.* Furthermore, *Hae*III could discriminate between *Rhizobium* strains nodulating *Leucaena luceocephala* and *Phaseolus vulgaris.* ARDRA generates species specific patterns (Heyndrickx et al., 1996). On the basis of ARDRA, strain KCC1, KCC4 and KCC2 clustered together, while KCC3 and KCC5 clustered distantly (Figure 2).

Nitrogen fixation and nodulation abilities of *Sinorhizobium* strains (KCC1 to KCC8) were confirmed by amplification of *nif*H and *nod*C fragments (Figure 4). The *nod* genes were present in all the rhizobia (Lindstrom et al., 1995). Bacterial genes that function in nodulation are termed nodulation or *nod* genes, and their expression is induced by flavonoid signals from the plant host. To form an effective symbiosis, rhizobia require several classes of specific genes. These include *nod, nol, noc* genes, which encode the production of Nod factors, which stimulate the plants to produce symbiotic nodules, and *nif* genes, for the nitrogen fixing 'nitrogenase' enzymes. The nodulation gene has a key role in the initiation of legume nodulation. The *nod*ABC are responsible for the synthesis of the chitin backbone and mutation in these genes for the loss of production of *nod* signals. The common *nod*ABC and the regulatory *nod*D1 and *nod*D2 genes of *S. fredii* have been characterized (Appelbaum et al., 1988; Krishnan and Pueppke, 1991). *Nod* factors, also known as lipochito-oligosaccharides or LCOs, have also been isolated from *S. fredii* and characterized (Bec-Ferte et al., 1996). These signal molecules, which are synthesized by the protein products of the *nod* genes, regulate nodule initiation and morphogenesis (Denarie et al., 1996).

Amplification of nod genes have also been reported by earlier workers and proved their role in nodule formation by several species of rhizobia (Haukka et al., 1998). In the present study, we found that the strains of *Sinorhizobium* spp. nodulated their host plants as proved by the amplification of *nod*C genes by the PCR techniques. Rhizobia are well known nitrogen fixers due to the presence of *nif*H genes. By the amplification of some specific *nif* genes such as *nif*H, the nitrogenase activity of the rhizobia is confirmed (Young and Haukka, 1996; Pandey et al., 2004). The *nif*H primers used in this study were designed by aligning the *nif*H sequences of *Bradyrhizobium japonicum* 110, *Rhizobium phaseoli* CFN 42, *Rhizobium trifolii* 329 and S. *meliloti* 41 (Perret and Broughton, 1998; Pandey et al., 2004). *Nif* H gene codes for Fe-protein subunit of nitrogenase enzyme and for denitrogenase reductase (Dean and Jacobson, 1992). *Nif* genes are found in many bacteria besides rhizobia and

are a part of genome that are considered as part of the normal bacterial genome (Young and Haukka, 1996).

Earlier, Carson et al. (2000) showed the production of siderophore by root nodulating rhizobial strains. Various strains of rhizobia have been reported to produce a wide range of siderophores such as rhizobactin, citrate, catechol, anthranalate under iron- deficient conditions (Arora et al., 2001 and Deshwal et al., 2003). In rhizobia, the ability to synthesize siderophores is restricted to a limited range of strains, rather than wide distribution (Carson et al., 2000). These results were also complementary with the study of Arora et al. (2001) who found that only 20% of rhizobial isolates produced siderophores.

In vitro fungal growth inhibition assay using S. *fredii* KCC5 revealed significant reduction in radial growth of *F. udum.* Inhibition zones between the test organisms viz, S. *fredii* KCC5 against *F. udum* was mainly due to production of siderophore and nutrient competition. One of the mechanisms for the inhibition of *F. udum* was due to the involvement of siderophores produced by *S. fredii* KCC5. Similarly, reduced inhibitory activity of rhizobacteria due to siderophores was observed under iron rich conditions as supported by Lynch (1990). However, role of other inhibitory metabolites such as toxins and proteolytic enzymes in the inhibition process of fungal pathogens can not be ruled out (Dunne et al., 1998). Antagonistic strains of *Rhizobium* sp. (Perdomo et al., 1995), *B. japonicum* (Deshwal et al., 2003), S. *meliloti* (Arora et al., 2001) inhibited *Macrophomina phaseolina* and its disease incidence in different crop plants. Inhibition of *Fusarium oxysporum* causing wilt disease in chickpea have also been reported by the involvement of *Rhizobium s*p. NBRI9513 (Nautiyal, 1997) and S. *meliloti* (Antoun et al., 1998).

It may be concluded that fast growing rhizobia of pigeon pea showed great homogeneity and the strain of *S. fredii* KCC5 was the best strains that showed maximum IAA and siderophore production and strong antagonism against *F. udum*. Further, the strains can be exploited for plant growth-promotion of *C. cajan* with effective biocontrol of *F. udum* with great possible and ecological importance.

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