INDIAN JOURNAL OF ARID HORTICULTURE
2009, Vol. 4 (1) : 26-36

Evaluation of rhizobial isolates from pigeonpea nodules for characterization of fast growing antagonist of *Fusarium udum*

Kamlesh Choure*[1], Rakesh Bhargava[2], R. C. Dubey[3] & D. K. Maheshwari[3]
[1]Department of Biotechnology, Faculty of Engineering, Life Sciences & Management,
IASE Deemed University, Sardarshahr-331 401 (Rajasthan), India
[2]Central Institute for Arid Horticulture (ICAR), Beechwal, Bikaner (Rajasthan), India
[3]Department of Botany and Microbiology, Gurukul Kangri University,
Haridwar-249404 (Uttarakhand) India

Abstract

Eight isolates of rhizobia were isolated from pigeonpea nodule and identified and characterized by physiological and biochemical tests. Isolate KCC5 showed maximum potential antagonistic characters. Phylogenetic analysis of 16S rDNA sequences of KCC5 with other reference strains. Form this analysis, KCC5 aligned to same cluster (100% bootstrap value) with reference strains of *Sinorhizobium fredii*. All the strains of *Pseudomonas* spp. were positive to IAA production and phosphate solubilisation. Only KCC2 and KCC5 were produced siderophore. KCC5 produced maximum quantity of siderophore. In dual culture technique, maximum inhibition of *F. udum* recorded in the case of KCC5. Cell free culture filtrate of KCC5 also restricted the hyphal growth of *F. udum*. Morphological abnormalities in the hyphae of *Fusarium udum* obtained from the zone of interaction during dual culture were observed by light microscopy. Fragmentation and degradation of mycelia and loss of structural integrity of conidia of *F. udum* were clearly observed under scanning electron microscopic analysis. In iron supplemented (30 µM Fe^{+3}) medium inhibition of the pathogenic fungi was reduced. It was found that under iron-deficient condition KCC5 inhibited maximum vegetative growth of *F. udum* as compared to under iron-sufficient conditions.

Key words: *Fusarium, Rhizobium, Pseudomonas, phosphate solubilisation, IAA production.*

Introduction

Pigeonpea (*Cajanus cajan*) is one of the important pulse crops and a very popular food in developing tropical countries. India is a principal pigeonpea-growing country contributing nearly 90% of total world's production and it is widely grown in almost all the states. *F. udum* is the most important soil-borne disease of pigeon pea causes fusarial wilt. Biological control of *F. udum* has attracted the attention throughout the world. In general, nowa-days, the idea of controlling soil borne plant pathogens including *F. udum* with chemical pesticides or fungicides has been shifted to the option that biological control can have an important role in agriculture. The beneficial effect of *Rhizobium* in legumes in terms of biological nitrogen fixation has been a main focus in the recent years. Obviously, rhizobia are known to increase nodulation and nodule weight in legumes along with increase in host plant growth and development besides, Rhizobia are reported to inhibit several soil-borne phytopathogenic fungi including *Macrophomina phaseolina* (Perdomo *et al.*, 1995; Arora *et al.*, 2001), *Fusarium oxysporum* (Antoun *et al.*, 1987; Nautiyal, 1997), *Rhizoctonia bataticola* and *Pythium* sp. (Nautiyal, 1997). The antagonistic activities of rhizobia are mainly due to the production of secondary metabolites such as siderophores (Rioux *et al.*, 1986; Arora *et al.*, 2001; Deshwal *et al.*, 2003), antibiotics (Breil *et al.*, 1996), HCN (Beauchamp *et al.*, 1991; Antoun *et al.* 1998), and phytoallexin production (Chakraborty and Chakraborty, 1988; Deshwal *et al.*, 2003).

The present study investigates the diversity of fast growing rhizobia of pigeonpea nodules based on variations in partial sequences of the 16S rRNA gene, analysis of morphological, physiological, biochemical data, assessment of secondary metabolite production and antagonism against *Fusarium udum*.

Materials and methods

Rhizobium isolates

Pigeonpea nodulating bacteria were isolated from nodules of pigeonpea plants grown in farmers field of Betul district (M.P.), India in July and August. This area comes under a temperature range of 18°C (in winters) to 41°C (in

*Corresponding author e-mail:
kamlesh.chaure@gmail.com

summers). For the isolation of the root nodulating bacteria, method of Vincent (1970) was followed. *C. cajan* seedlings were uprooted carefully and root nodules were collected and washed with the sterile distilled water followed by surface treatment with 95 % ethanol (2 ml) and again rinsed with sterile distilled water. Washed nodules were surface sterilized quickly (2-3 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 pre-incubated yeast extract mannitol (YEM) agar (Vincent, 1970) plates. Plates were incubated at 28 ± 1°C for 4-5 days and observed for specific features of rhizobia. In total eight isolates were obtained from *Cajanus cajan* which were maintained separately on YEM agar slants at 4°C, for further. These were named as KCC (*Cajanus cajan* rhizobia).

Phenotypic characterization of isolated bacteria

Morphological and physiological characteristics were determined for eight test isolates and the type strains of *Sinorhizobium meliloti*. Gram's staining of the isolates was done to provide information on presumptive tests of isolates. Colony morphology of nodule isolates (colony color, colonial form, elevation and colonial margins) were determined on YEM agar plates containing congo red dye using standard bacteriological techniques (Vincent, 1970). For the biochemical tests of log phase cultures (10^8 cells ml^{-1}) of root nodule isolates were used. They were separately inoculated in YEM broth as well as on YEMA and incubated at 28°C. All the tests were carried out in triplicates and at least three times. Motility was examined following hanging drop method. Generation time of each isolates was measured. The isolates (single colony of each) were inoculated in 50 ml of YEM broth in conical flasks and incubated at rotary shaker (150 rpm at 28±1°C) for 48 h. The growth (turbidity produced) was measured by O.D. method by O.D. at 610 nm after every 6 h in UV-VIS spectrophotometer (Shimadzu, Model UV-1601. The generation time was calculated by following formula: Generation Time= (T2 - T1)/3.3 ($^{10}\log OD_2$-OD_1), where, (T2 - T1) is time interval taken at any two points in the log phase of growth and ($^{10}\log OD_2$ - $^{10}\log OD_1$) is difference between the log values of OD at time T_2 h to $^{10}\log$ value of OD at time T_1 h. Growth on/in glucose peptone agar (GPA) (Klezkowska *et al.*, 1968), Hofer's alkaline broth *(HAB)* (Hofer, 1935), 2% NaCl (Sadowsky et al., 1983), calcium glycerophosphate (Hofer, 1941), catalase activity (Graham and Parker, 1964), Oxidase Activity (Kovaks, 1956) gelatin hydrolysis (Sadowsky *et al.*, 1983), hydrolysis of urea (Lindstrom and Lehtomaki, 1988), citrate utilization (Kosar, 1923), in presence of KNO_3 (El- Idrissi *et al.*, 1996), acid production (Arora *et al.*, 2000), hydrogen sulfide production (Zobell and Feltham, 1934), MR-VP test, utilization of carbon sources (El-Idrissi *et al.*, 1996), poly hydroxy butyrate (PHB) accumulation (Navarini *et al.*, 1992) were checked for each isolated bacteria.

Isolation of fungal pathogen

The cropping areas of Badgaon, Betul district in Madhya Pradesh, India were surveyed and wilt prone area were marked. Samples of diseased root parts and seeds were randomly collected and fungal pathogen *Fusarium udum was* isolated from infected roots of pigeon pea (*Cajanus cajan*) following the water agar technique (Musket and Malone, 1941). This technique was used for the isolation of external and internal mycoflora of the diseased seeds and infected roots showing reddish brown lesions on the hypocotyls. The external surface mycoflora was isolated by directly placing the seeds and infected plant material on the water agar medium containing 2% agar and penta-chloro-dinitrobenzene (PCNB). For the isolation of internal mycoflora, seeds and diseased roots were surface sterilized by soaking them in 0.5% sodium hypochlorite solution for 3-5 min. Samples were then rinsed in several changes of sterile water to remove the traces of sodium hypochlorite, and placed on the surface of pre-incubated water agar plates. After incubation at 28°C for one week, plates were observed for the appearance of various fungi.

Identification of fungal pathogens

Different fungal isolates from infected seeds and plant materials were grown on the potato dextrose agar (PDA) medium. The fungal cultures were maintained on potato dextrose agar (PDA) medium and characterized following illustration and description of standard mycological literature and comparing with standard cultures obtained from the division of Plant Pathology, Indian Institute of Pulses Research (IIPR), Kanpur, India. The morphological characters of *F. udum* such as colony growth, colour pigmentation in substrate, mycelial characters, and micro and macro conidia production were recorded and compared with those as described by Booth (1971). Isolated fungal cultures of *F. udum*, with their known cultures from IIPR, Kanpur, was maintained on PDA medium at 4°C.

IAA production

To observe IAA production exponentially grown cultures (10^8cells ml^{-1}) of the strains were incubated separately on broth medium for 24 h. 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. The mixture was incubated at room temperature for 25 min, and the absorbance of pink colour developed was read at 530 nm using UV-VIS spectrophotometer (Shimadzu, Model UV-1601. The concentration of IAA in cultures was determined by using a calibration curve of pure IAA as a standard following the linear regression analysis.

Phosphate Solubilization

Phosphate solubilization ability of isolated strains was detected by spotting them 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). The quantitative estimation of phosphate solubilization was carried out after modified method of Jackson, (1958). Exponentially grown (10^8cells ml^{-1}) cultures were incubated in Pikovskya's broth supplemented. After 48 h, supernatants were collected by centrifugation of cultures passing through 0.45 µm millipore filter membrane. Observations were made by comparing with the standard curve in the same conditions.

Siderophore production

Siderophore production was determined on Chrome-azurol S (CAS) medium following the method of Schwyn and Neilands (1987). For the preparation of CAS assay solution, 6 ml of 10 mM HDTMA solution was diluted upto 100 ml with double glass distilled water and a mixture of 1.5 ml iron (III) solution (1 mM $FeCl_3.6H_2O$ in 100 mM HCl) and 7.5 ml of 2 mM aqueous CAS solution was slowly added under stirring. Anhydrous piperazine (4.307 g) was dissolved in water and 6.25 ml of 12 M HCl was carefully added to it. This buffer solution (pH 5.6) was rinsed into the volumetric flask which was than filled with water to afford 100 ml of CAS assay solution. The CAS shuttle solution was obtained by adding 5-sulfosalicylic acid to the above solution at a concentration of 4 mM. The bacterial strains (24 h old cultures) spotted separately on CAS medium. Plates were incubated at 28±1°C for 48 h. Formation of orange to yellow halo around the colonies showed the production of siderophore. Presence of hydroxamate type of siderophores was determined following the method of Gibson and Magrath (1969) evaluating the absorption spectrum of 48 h supernatant at 400 nm, spectrophotometrically.

Siderophore Assay For siderophore production by bacteria, a special siderophore production liquid culture was prepared which contained MM9 salts, tris buffer, casamino acids (0.3%), L-glutamic acid (0.05%), (+) biotin (0.5 ppm) and sucrose (0.2%). Bacterial cultures were grown in this medium for 48 h at 28°C under rotating conditions and its cell density was monitored spectrophotometrically at 610 nm. After incubation, culture supernatant (0.5 ml) was mixed with 0.5 ml of CAS assay solution. An un-inoculated medium served as reference. After reaching colour equilibrium the absorbance of the mixture was measured at 630 nm. The concentration of siderophore in cultures was determined by using a calibration curve of hydroxalamine as a standard under the same conditions and following the linier regression analysis.

Time course of siderophore production: Following the above method of quantification of siderophore production, samples from the bacterial broth were withdrawn at every 24 h intervals up to 168 h for the quantitative determination of siderophore. A plot was prepared comparing siderophore production with respect to time.

DNA isolation

For the isolation of DNA from fluorescent *Pseudomonas* spp. were grown in Luria Bertani (LB) broth. The bacterial pellets were washed with 50 mM ethylenediamine tetraacetic 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 the addition of 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. To the DNA extract 50 µg ml^{-1} RNase was added and incubated at 37°C for 30 min. This was followed by the addition of tris-phenol and centrifugation at 12,000 rpm for 15-20 min at 4°C. The supernatant was heat sterilized and 100 µl, 0.3 M sodium acetate and 1 ml cold isopropanol was added with continuous vortex. Finally, the DNA was purified by the addition of phenol-chloroform followed by the centrifugation at 15000 rpm.

16S rRNA gene amplification

Universal eubacterial primers FD1 5'CCGAATTCGTCGACAACAGAGTTTGATCCTGGCTCAG3' and RD1 5'CCCGGGATCCAAGCTTAAGGAGGTGATCCAGCC3' were used for amplification of 1492 bp region of the 16S rRNA gene on PTC 100 (M.J. Research, USA) thermal cycler. A 50 µl reaction mixture included 5-10 ng of bacterial DNA as template, 1 µl of each primer, 1U 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°C 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.

16s rRNA gene sequencing

Partial 16S rRNA 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 ethedium bromide and visualized as described above. The amplicons were purified with Banglore Genei, PCR purification kit and quantified spectrophotometrically at 260 nm compare with calf thymus DNA. The cleaned partial 16S rDNA amplicon was sequenced with DNA sequencing system.

Sequence analysis of 16S rDNA sequences using bioinformatics tools

The PCR products were purified and sequenced. Theses all sequences were subjected for 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 BLAST (basic local alignment search tool) program at 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 constructed by using *genebee* server (www.genebee.msu.ru/services/phtree-reduced.html). Phylogenetic tree obtained with bootstrap values in cluster algorithm, phylip format and topological algorithm

Antifungal activities of Sinorhizobium fredii KCC5

Antagonistic properties of *Sinorhizobium fredii* KCC5 was tested against *F. udum* on 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 culture (10^8 ml^{-1}) of KCC5 was spotted 2 cm juxtaposed from the fungal disc and incubated at 28±1°C for 5 days. Growth inhibition was calculated by measuring the distance between the bacterial and fungal colonies as compared to control. Growth inhibition was calculated by measuring the distance between the edge of bacterial and fungal colonies. The zone of inhibition was recorded by using given below formula: Inhibition (%) = (C) – (T) / (C)× 100, where, C=Radial growth in control and T=Radial growth in dual culture.

Effect of culture filtrates on the inhibition of pathogenic fungi

Antagonistic bacteria were inoculated in their respective broth cultures and incubated in rotary incubator shaker at 150 rpm and 28°C for 7 days. After incubation broth culture of the strains were collected by centrifugation at 10,000 rpm for 15 min at 4°C and passed through Millipore filter (0.45 µm). Such culture supernatants were poured in the wells of the fungal challenged plates. After incubation at 28°C for 5-7 days, inhibition of the pathogenic fungi was recorded.

Antagonism in iron-deficient and iron sufficient conditions

In vitro test for antagonism of *S. fredii* KCC5 was made under iron-deficient and iron-sufficient (30 and 60 µM Fe^{3+}) conditions towards the *Fusarium udum*. Iron deficient medium was prepared by treating bacterial broth (250 ml) with 8-hydroxyquinoline solution in 100 ml chloroform (up to final concentration (0.1%). It was then vigorously shaken. Excess of hydroxyquinoline was removed by chloroform extraction until the disappearance of yellow color. Medium was boiled to remove the smell of chloroform. Agar (2%) was added and the content autoclaved at 15 lb /inch^2 (121°C) for 20 min. Antagonism was carried out by following the methodology mentioned as before. Bacterial broth supplemented with 30 and 60 µM Fe^{3+} was used to carry out *in vitro* fungal assay under iron sufficient condition. Methodology for antagonism study was adopted as mentioned above.

Compound microscopy of post-interaction events in fungal mycelia

Fungal mycelia growing towards the zone of inhibition were processed for microscopy. Fungal mycelium were picked up from the zone of inhibition with the help of sterile needle and transferred to a drop of lacto-phenol on the clean glass slide. Specimens were examined under a light compound microscope (Olympus BX 51 TRF) for fungal morphological abnormalities occurred due to antagonism mediated by the rhizobial strains. Images of fungal deformities were captured by using Image Analyser (Biovis).

Scanning electron microscopy (SEM) of post-interaction events in fungal mycelia

The antagonist and the test organism are grown in pure cultures. Agar disc (5mm diameter) are to be cut from the actively growing margin of the colonies and placed 3cm apart on the surface of the Petri dishes containing freshly prepared, sterilized and solidified potato Dextrose agar medium. Incubated Petri dishes at 25°C with continuous light after incubation of 4 days collect mycelial sample from the region of interaction. For preparation of SEM samples, the mycelia were collected with the help of micro-niddle in sterile environment from the zone of interaction with bacteria on the surface of gelled agar in Petri plate. Then it were fixed overnight at 4°C in 4% glutaraldehyde in 0.05 M phosphate buffer (pH 7.3) and washed three times (10 min each) in phosphate buffer. After three 10 min rinses in distilled water, samples were dehydrated through 70, 80, 90 and 100% ethanol (5 min in each stage) and three changes in 100% ethanol at room temperature. Ethanol was then replaced by liquid CO_2 and the samples were air dried. Then samples were mounted on stubs and coated with gold. These coated specimens were observed at 15 kV in a *LEO 485 VP* Scanning Electron Microscope. Photo-micrographs were recorded by the same machine.

Results

All the isolates of pigeonpea nodules were characterized by morphological, physiological and biochemical means (Table 1). All the isolates (KCC1 to KCC8) were Gram-negative, non-sporing, and non-capsulated

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

Characteristics	KCC 1	KCC 2	KCC 3	KCC 4	KCC 5	KCC 6	KCC 7	KCC 8	MTCC 99	MTCC 2378	MSSP	MTCC 100
Gram reaction	-	-	-	-	-	-	-	-	-	-	-	-
Growth at 28°C	+	+	+	+	+	+	+	+	+	+	+	+
41°C	-	-	-	-	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+	+	+	+	+
Capsule	+	+	-	-	+	+	+	-	+	+	+	+
Endospore	-	-	-	-	-	-	-	-	-	-	-	-
PHB accumulation	+	+	+	+	+	+	+	+	+	+	-	+
Generation time (h)	2.6	2.5	2.8	2.5	2.6	2.4	2.5	2.5	2.7	2.9	2.6	2.4
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	+	+	-	+	+	+	-	-	+	+	+	+

Abbreviations: +, 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; *Burkholderia* sp. MSSP (Pandey and Maheshwari, 2005)

which were motile with single sub-polar flagellum. The isolates formed red, semi-translucent, rounded, smooth, mucoid colonies on CrYEMA with 2 to 4 mm diam after 48 h of incubation.

All the isolates were fast growers with average mean generation time of 2.9 h showing catalase and oxidase activities and produced yellow colour to bromothymal blue 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 were negative for starch hydrolysis. Isolate KCC5 was negative for urease, while KCC4 was unable to utilize citrate and arabinose. The isolates failed to grow on GPA but were able to grow in HAB and could tolerate 8% KNO_3 as well as 2 % NaCl (Table 1). All the isolates utilized dextrose, glucose and glycerol when the carbon sources replaced mannitol in YEMA. Isolates KCC1 and KCC2 were unable to utilize cellobiose and KCC2 and KCC3 were negative for lactose utilization. The characters of isolates were compared with standard rhizobial strains viz., *Sinorhizobium fredii* MTCC-100, *Rhizobium leguminosarum* MTCC-99 and *Mesorhizobium loti* MTCC-2378 and most of them possessed characteristics similar to *S. fredii* MTCC-100 (Table 1).

After amplification of 16S rRNA gene with universal fd1 and rd1 primers, the amplified products were compared with low range ruler DNA (Banglore Genei, India) and observed that total length of amplicons was approximately 1500 bp (1.5 kb) which represented the amplification of nearly full length 16S rRNA gene from all the *Cajanus cajan* isolates. The size of the PCR product corresponded with the size of the 16S rRNA genes among all eight isolates.

Neighbour-joining dendograms were generated (based on cluster algorithm and topological algorithm) using 16S rDNA sequences of *cajanus cajan* root nodulating isolates and representative rhizobial sequences from EMBL/Gen Bank/DDBJ/PDB (Fig. 4). The 16S rDNA sequence of the following strains (type strains unless otherwise indicated) were obtained from GenBank: *Sinorhizobium fredii* S8 (EF506208), *Sinorhizobium fredii* S25 (EF506206), *Sinorhizobium fredii* S42 (EF506204). A neighbour-joining dendogram was generated using the sequence from *Rhizobium* sp. KCC5 and representative *Sinorhizobium fredii* sequences from GenBank (Fig. 5). Form this analysis, KCC5 aligned to same cluster (100% bootstrap value) with other reference strains (Fig. 5). This analysis has revealed that *Cajanus cajan* nodulating isolate KCC5 can be named now as *Sinorhizobium fredii* KCC5.

Except KCC4 all strains of *Rhizobium* spp. (KCC 1 to KCC8) were positive for IAA (indole acetic acid) production as evidenced by the development of pink colour of the cell free supernatants (Table 2). *Rhizobium* sp. KCC5 produced maximum IAA and its production was induced in

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

Strains	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 strains					
MTCC 100	+	+	-	-	-
MTCC 99	-	+	-	-	-
MTCC 2378	-	+	-	-	-

Abbreviations: 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

presence of tryptophan. In KCC5, the IAA production was started after 18 h of inoculation. The IAA production increased corresponding to incubation period and reached maximum after 168 h of incubation (Fig. 2). During phosphate solubilization all strains of *Rhizobium* spp. formed clear halos around their spot inoculation by solubilizing tricalcium phosphate on the Pikovskya's agar. All strains solubilized inorganic phosphate (Table 2A). Lowest pH recorded was 3.8 subjected after 7 d incubation. No acidic phosphatase activity was recorded. Phosphate solubilization was started after 24 h of incubation in all the strains and maximum in *Rhizobium* sp. KCC5 at 7th d of incubation (Fig. 2B). None of the strains of *Rhizobium* spp. was able to produce cyanogens (Table 2). All the strains were screened for siderophore production on CAS agar, *Rhizobium* sp. KCC2 and *Rhizobium* sp. KCC5 showed siderophore activity as revealed by orange halo around their colonies (Table 2). Secretion of siderophore by both the strains started after 12 h of incubation. On further incubation up to 5 days, siderophore production got increased but later on production got declined. To know the type of siderophore, 48 h old supernatant of the strain (*Rhizobium* sp. KCC5) scanned at 400 nm showed major peak. This peak corresponded with hydroxamate type of siderophore. None of the strain was able to develop wine colour in the reaction mixture following the methods of Arnow (1937) which ruled out the possibility of catechol-phenolic type siderophore.

All the *Rhizobium* strains were screened for antagonistic activity against *Fusarium udum*. Only *Rhizobium* sp. KCC2 and *Rhizobium* sp. KCC5 inhibited the growth of both pathogenic fungi on PDA plates at 28°C

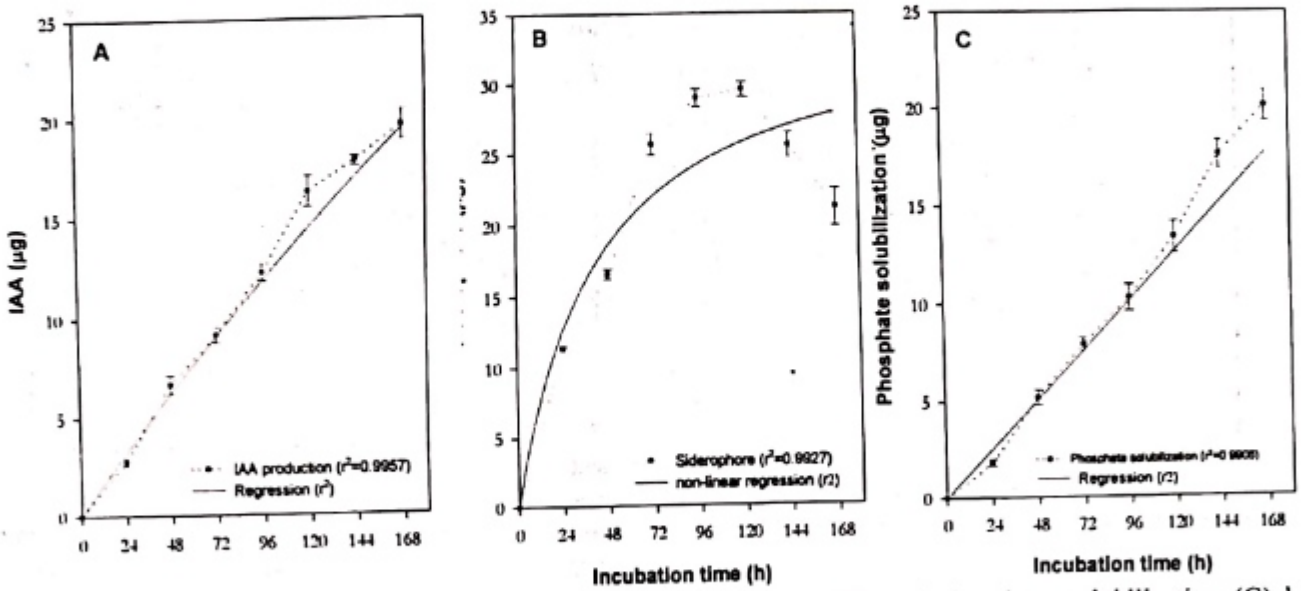

Fig 1. Time course of IAA production (A), siderophore production (B) and phosphate solubilization (C) by *Sinorhizobium fredii* KCC5 (C) (Values are means of standard deviation of three replications)

(Table 2). Increase in fungal inhibition corresponded to incubation period. Strain KCC5 was able to inhibit 56 % of *F. udum* after 6 days of incubation. (Fig. 3). None of the standard strains *Sinorhizobium meliloti* MTCC-100, *Rhizobium leguminosarum* MTCC-99, and *Mesorhizobium loti* MTCC-2378 inhibited *F. udum* (Table 2). Cell free culture filtrates of *Sinorhizobium fredii* KCC5 also restricted development of *F. udum*. It was observed that fungal inhibition by *Sinorhizobium fredii* KCC5 was more pronounced in dual culture conditions in comparison to that of cell free culture filtrates (Fig. 3).

To find out the siderophore-mediated inhibition of *F. udum*, *Sinorhizobium fredii* KCC5 was examined to inhibit it on modified YEM medium supplemented with 30 and 60 µM Fe^{+3}. In this experiment, the mycelial growth of the pathogen significantly got decreased by the antagonist with respect to control in iron-deficient conditions. Inhibition of the pathogen was reduced in iron supplemented (30 µM Fe^{+3}) medium. However, inhibition was less pronounced on the 60 µM Fe^{+3} supplemented medium in comparison to normal conditions. *Sinorhizobium fredii* KCC5 showed 56% inhibition of *F. udum* in 30 µM Fe^{+3} supplemented medium (Fig. 4) after 6 days of incubation. Under iron-deficient conditions remarkable inhibition of *F. udum* was observed and under such prevailing conditions, *Sinorhizobium fredii* KCC5 inhibited 48% vegetative growth *F. udum*, respectively (Fig. 4).

Morphological abnormalties in the hyphae of *Fusarium udum* obtained from the zone of interaction during dual cultures was observed by light microscopy and in scanning electron microscopy, revealed loss of structural integrity of the mycelium of *F. udum*. Twisting, hyphal perforations, lysis and empty cell formation, septation, fragmentation and degradation of mycelia of *F. udum* were observed under light microscopy (Fig 5). Fragmentation and degradation of mycelia and loss of structural integrity of

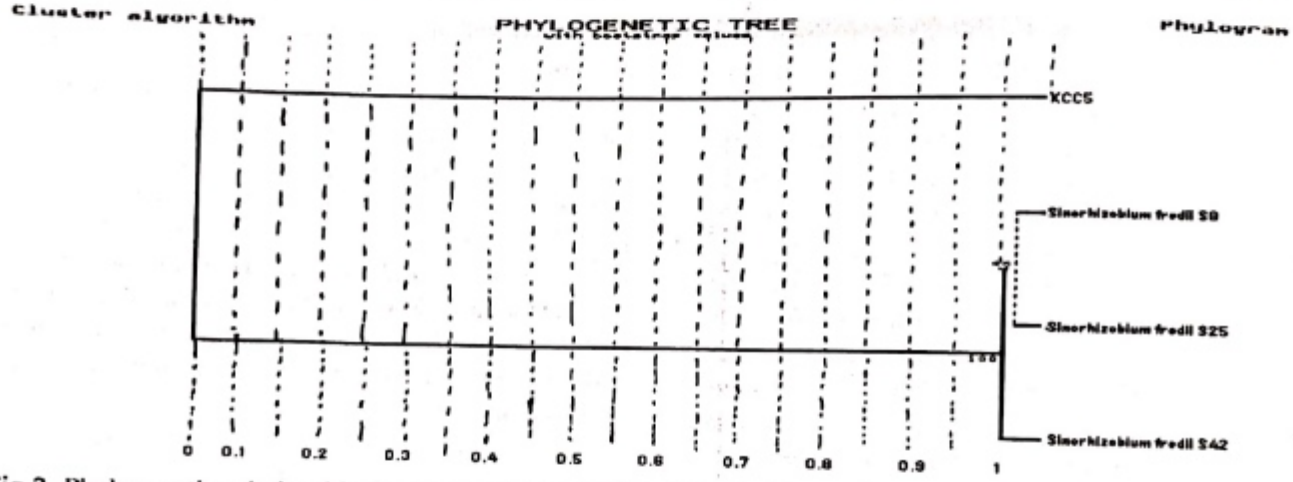

Fig 2. Phylogenetic relationship between bacterial strains of *Rhizobium* and representing species (A) and *Rhizobium* sp. KCC5 and representing species (B) based on partial length 16S rDNA sequences constructed using cluster algorithm. Bootstrap values are indicated (from 1000 replication).Accession numbers are mentioned in text.

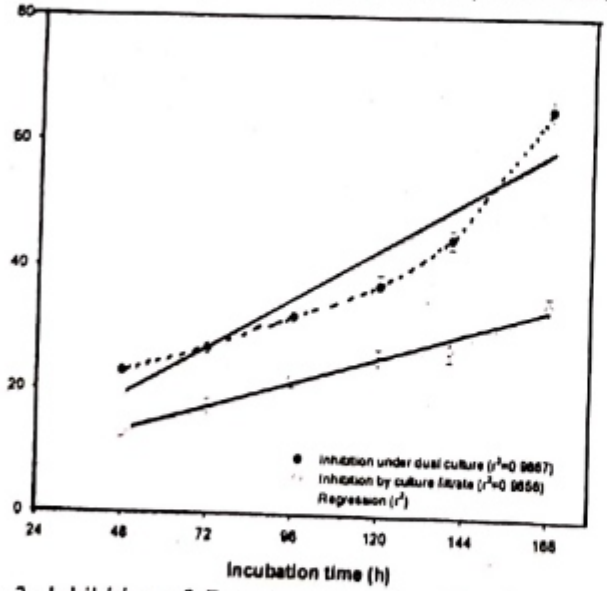

Fig 3. Inhibition of *Fusarium udum* by *Sinorhizobium fredii* KCC5 under dual culture and by Cell free culture filtrate (Values are means of standard deviation of three replications)

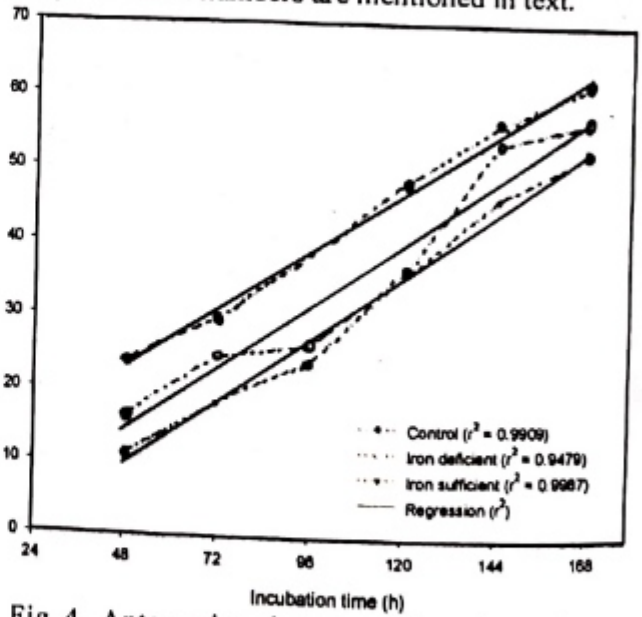

Fig 4. Antagonism between *Fusarium udum* and *Sinorhizobium fredii* KCC5 under iron sufficient and iron deficient conditions (Values are means of standard deviation of three replications)

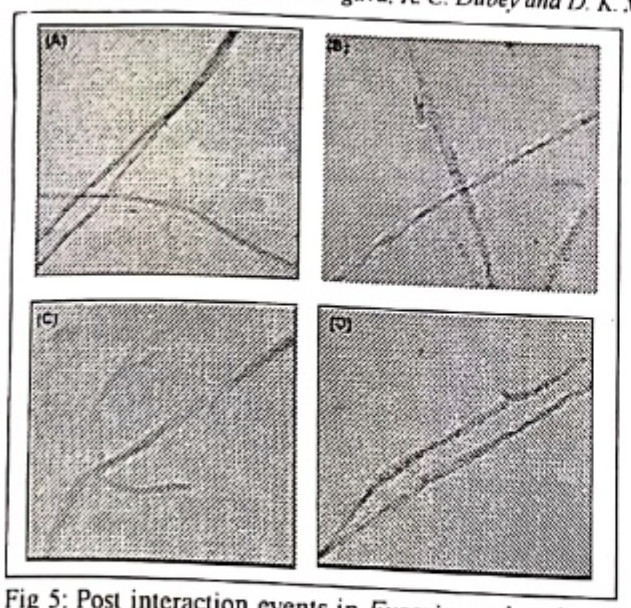

Fig 5: Post interaction events in *Fusarium udum* due to *Sinorhizobium fredii* KCC5, control (A) hyphal degradation (B) halo cell formation, hyphal perforation and digestion of fungal cell wall (C) cytoplasm coagulation (D) (see arrows)

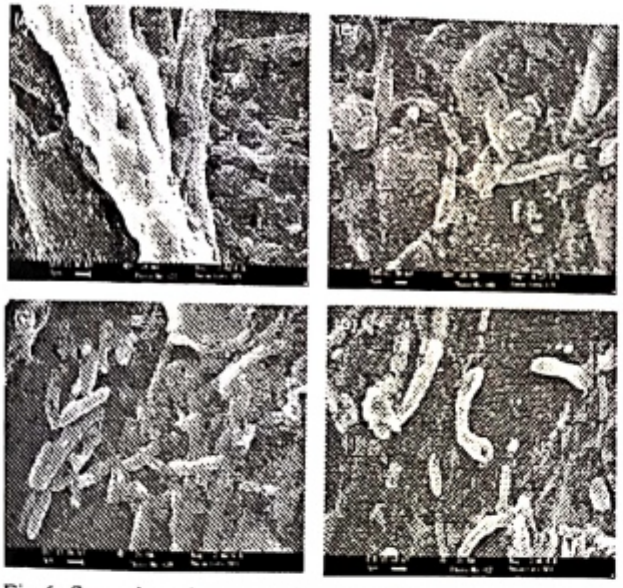

Fig 6: Scanning electron microscopic photographs showing mycelial and conidial destruction by antagonistic interaction effects of *Sinorhizobium fredii* KCC5; hyphal lysis (A and B), lysis of conidia (C and D) (see arrows)

conidia of *F. udum* were clearly observed under Scanning electron microscopic analysis (Fig 6). These features of mycelia may be due to production of siderophore by strains *Sinorhizobium fredii* KCC5.

Discussions

Rhizobia are α-proteobacteria able to establish symbiotic interactions with leguminous plants leading to the formation of root nodules where they fix atmospheric nitrogen. All the isolates from *Cajanus cajan* were fast growing with average generation time 2.6 h. All isolates of from *Cajanus caja n* were Gram-negative, aerobic, non-spore forming and cocci in shape (Holt *et al.*, 1994). Colony morphology of *Rhizobium* has been demonstrated to play an important role in process of symbiotic nitrogen fixation (Vincent, 1972). Most of the strains were positive for catalase and oxidase activity and utilized citrate. The strains precipitated calcium glycerophosphate. All strains failed to grow on GPA but able to grow in HAB and tolerated 8% KNO_3 and 2% NaCl. The characters reported in the present study of all bacterial strains are similar to those as described by Holt *et al.*, (1994).

The strains were able to utilize wide range of carbohydrates including hexoses, pentoses, disaccharides and trioses. This is also in accordance with the earlier findings that sinorhizobia with other fast growing rhizobia are capable of growing on 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 genus *Sinorhizobium* genera Holt *et al.*, (1994).

Out of eight strains of *Rhizobium* spp. (KCC1 to KCC8) isolate KCC5 was found most potential antagonist against *F. udum*. Therefore, it was further characterized by 16S rDNA sequence homology. In Phylogenetic tree based on 16S rDNA homology *Rhizobium* sp. KCC5 is placed in the *Sinorhizobium fredii* clade.. The use of rDNA gene analysis has become promising. Polymerase chain reaction (PCR) amplification of rDNA genes from environmental DNA samples, combined with fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE), terminal restriction fragment analysis (T-RFLP), amplified rDNA restriction analysis (ARDRA), cloning and sequencing, provide detailed information about the species composition of whole communities (Nicol *et al.*, 2003; Torsvik and Ovreas, 2002). This is realized that 16S rDNA homology allows a first Phylogenetic affiliation of new isolate. Molecular microbiological characteristics have been carried out to characterize different groups of bacteria by using 16S rDNA sequencing. Recently, Pandey *et al.* (2005) have also characterized *Burkholderia* spp. MSSP from root nodules of *Mimosa pudica* on the basis of 16S rDNA analysis.

Siderophore production by root nodulating rhizobial strains has been well documented (Smith *et al.*, 1985 and Carson *et al.*, 2000). Various strains of rhizobia have been reported to produce a wide range of siderophores such as rhizobactin, citrate, catechol, anthranalate under iron deficient conditions (Smith *et al.*, 1985; Guerinot *et al.*, 1990 and Deshwal *et al.*, 2003). In the present study, *Sinorhizobium fredii* KCC5 produced hydroxamate type of siderophore. Earlier, Arora *et al.* (2001) and Deshwal *et al.* (2003) reported hydroxamate and catechol type siderophores from *S. meliloti* RMP1 and

Bradyrhizobium (*Arachis*) sp. respectively. Hydroxamate type of siderophore production appears to be confined to *R. leguminosarum* as reported earlier by Patel *et al.* (1988) and in *Bradyrhizobium* by Guerinot (1991). Gibson and Magrath, (1969) stated the presence of hydroxamate type of siderophore in majority of rhizobia. Probably this was due to study of limited host range of leguminous plants (Young, 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 only 20% of rhizobial isolates produced siderophores.

Inhibitory effect of *S. fredii* KCC5 was observed against *F. udum* under iron sufficient conditions. It was found that inhibitory activity of strains *S. fredii* KCC5 was strongly neutralized when iron was supplemented to their respective medium. Significant reduction in the fungal inhibition has also been evidanced by Fuhrmann and Wollum (1989). Microbial siderophore production generally seems to be switched on when the Fe^{+3} concentrations is less than 20μM (Lynch, 1990), optimal at 5-10μM Fe^{+3} and inhibited beyond this concentration (Neilands, 1981). In the present study, KCC5 examined for inhibition of *F. udum* under iron (30 μM Fe^{+3}) supplemented conditions showed reduction in inhibition against both the fungal pathogens. On increasing the concentration of iron to 60 μM Fe^{+3}, more inhibition was recorded in comparison to normal iron condition. Similar findings were obtained by Derylo and Skorupska (1993) who reported that 60 μM Fe^{+3} suppressed the siderophore production in *Pseudomonas* sp. This gives evidence that 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).

Microscopic abnormalities in fungal pathogens under the influence of *S. fredii* KCC5 clearly showed degeneration and degradation of the hyphae of *F. udum*. Abnormal hyphal swelling, digestion of fungal cell wall, cytoplasm coagulation, hyphal perforation, hyphal tip degradation and halo cells formation appeared in mycelia of *F. udum* were caused *S. fredii* KCC5. Losses of structural integrity of conidia were also observed. Thus, it showed the hyphal wall interaction with bacterial cells rapidly lysed the hyphae, causing leakage of the fungal cytoplasm.

In vitro, fungal growth inhibition assay by *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* mainly due to production of siderophore and nutrient competition. 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 *M. 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* sp. NBRI9513 (Nautiyal, 1997) and *S. meliloti* (Antoun *et al.*, 1998). *S. fredii* KCC5 showed inhibition of *F. udum* in cell free culture filtrates. Different bacteria imparted varying degrees of inhibition of growth and conidia germination of phytopathogenic fungi due to production of antifungal compounds in culture filtrates (Skidmore and Dickinson, 1976; Upadhyay and Rai, 1987). There are several factors involved in the microbial antagonism which including staling growth products, pH of media, growth rate of interacting microbes, competition for nutrients and space, competitive colonization, nature and chemical composition of growth media, ionic or nutrient imbalance caused due to nutrient impoverishment and physical factors. Isolate of pigeonpea nodule, *S. fredii* KCC5 have been proved a great potential in controlling phytopathogenic fungus *F. udum*. Further work is in progress on interaction studies for preparation of combination of two or more than two biocontrol agents for better control of *F. udum*.

Acknowledgements

The authors wish to thank TMOP & M-CSIR, New Delhi and IASE Deemed University, Sadarshahr for financial assistance.

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