Genetic diversity of plant growth-promoting rhizobia isolated from a medicinal legume, *Mucuna pruriens* Linn.

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Genetic diversity of five Sinorhizobia from a medicinal legume, i.e. *Mucuna pruriens* was investigated using ARDRA analysis. All five strains were isolated on YEMA which showed pink colour on CrYEMA, tolerated 2% NaCl and precipitated calcium glycerophosphate. The isolates were analysed using restriction patterns produced by amplified DNA coding for 16S rDNA (ARDRA) and were placed into two genotypes. All five isolates belonged to a single cluster. A distinct similarity was observed between the RAPD and 16S rDNA RFLP analysis. Nodulation and nitrogen-fixing abilities of the strains were confirmed by amplification of 500bp *nod*C and 781bp *nif*H fragments respectively. All five isolates solubilized inorganic insoluble phosphate, produced IAA and did not produce HCN. Strains MPR3 and MPR4 produced siderophore and inhibited fungal pathogens, *Macrophomina phaseolina* and *Fusarium oxysporum*. The study showed that there is considerable homogeneity amongst *M. pruriens* root-nodule isolates.

Keywords: Fungal pathogens, genetic diversity, *Mucuna pruriens*, rhizobia, siderophore.

DURING the last decade, assessment of diversity within rhizobial natural populations in various regions of the world has received increased attention[1–3]. Attempts have been made to determine the characteristics of indigenous strains isolated from different cultivated as well as less explored legumes like shrubs, herbaceous plants[4,5] and medicinal legumes[6] that have an important role in certain ecosystems.

Phylogenetic information of bacteria has increased over the last two decades primarily through the use of molecular methods for the measurement of genetic relatedness. The availability of several sensitive and accurate PCR-based genotyping methods has enabled differentiation among closely related bacterial strains and the detection of higher rhizobial diversity than previously considered[7,8]. Laguerre *et al.*[9] applied restriction fragment length polymorphism (RFLP) analysis of PCR-amplified 16S rDNA gene for identification of rhizobia. This technique has also been utilized by several workers for the identification of several novel species[8]. Randomly amplified polymorphic DNA (RAPD) profiles have provided new tools for investigating genetic polymorphism. This method was used by van Rossum *et al.*[10] for genetic analysis of *Bradyrhizobium* strains nodulating *Arachis hypogea* and nodule isolates of *Acacia* sp. by Khbaya *et al.*[4].

Rhizobia are important members of plant growth-promoting rhizobacteria (PGPR) showing several plant growth-promoting (PGP) activities[11]. Direct PGP activities include production of IAA and siderophore, phosphate solubilization, etc.[12,13], and as the biological control agent for phytopathogens such as *Macrophomina phaseolina*, *Fusarium oxysporum*, *F. solani*, *Rhizoctonia solani*, *Pythium* spp., etc. by producing secondary metabolites such as antibiotics, siderophore, HCN and phytoalexins[14]. Some of them have been commercialized, but their use as biological control against fungal pathogens so far has not reached this stage[14].

In the present study, amplified 16S rDNA restriction analysis (ARDRA), RAPD fingerprinting, *nod*C and *nif*H gene analysis have been used to assess the diversity of the rhizobial populations in *Mucuna pruriens* growing in the sub-Himalayan tract of Uttaranchal, besides their potential to act as biological controlling agents against phytopathogens.

Mucuna pruriens Linn. belongs to Fabaceae, commonly known as cowhage or kavach or velvet bean, and one of the most popular drugs in the Ayurvedic system of medicine. The roots serve as nerve tonic and diuretic and bear nodule-inhabiting bacteria. Plants of *M. pruriens* were collected in July and August from the banks of streams of River Ganga, at Haridwar in Uttaranchal. This area comes under deciduous rainforest with a temperature range of 4°C (in winter) to 45°C (in summer). For recovery of the root-modulating bacteria, the method of Vincent[15] was followed. Morphological, physiological and biochemical properties of the strains were evaluated as described in *Bergey's Manual of Systematic Bacteriology*[16].

Genomic DNA of the bacterial isolates was isolated using standard phenol–chloroform–isoamyl (25 : 24 : 1) extraction and ethanol (70%) precipitation in the presence of sodium acetate (3 M). The concentration and purity of DNA were estimated spectrophotometrically at 260 and 280 nm respectively. For RAPD fingerprinting analysis, 15 arbitrarily chosen primers (OPA-1, OPA-2, OPA-20; OPB-8, OPB-10, OPB-17; OPC-1, OPC-3, OPC-4, OPC-5, OPC-11; OPE-1, OPE-2, OPE-3, OPE-4) were used. Amplification reactions were performed in a volume of 25 µl: Tris HCl, 10 mM (pH 8.3); KCl, 50 mM; $MgCl_2$, 2 mM; dNTPs mix, 100 mM; primer, 1 µl (25 pM); template DNA, 100 ng; *Taq* polymerase, 0.5 U. The reaction conditions for PCR were: initial denaturation at 94°C for 2 min, 45 cycles of denaturation at 94°C for 1 min, annealing at 36°C for 1 min, extension of 2 min at 72°C and final extension of 4 min at 72°C. Amplification products were

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analysed by electrophoresis (1.5% agarose gels) after staining with ethidium bromide (1 µg/ml) and observed on a gel documentation system (Vilber Lourmet, Germany).

Universal eubacterial primers FD1 5′ CCG AAT TCG TCG ACA ACA GAG TTT GAT CCT GGC TC AG 3′ and RD1 5` CCC GGG ATC CAA GCT TAA GGA GGT GAT CCA GCC 3′ were used for amplification of the 1492 bp region of the 16S rRNA gene on thermal cycler (PTC 100, M.J. Research, USA). PCR reaction mix for 100 µl reaction contained PCR buffer (10X), 10.0 µl; dNTPs mix (10 mM), 1.0 µl; $MgCl_2$ (50 mM), 6.0 µl; primer FD1 (100 ng μl^{-1}), 1.0 µl; primer RD1 (100 ng μl^{-1}), 1.0 µl; *Taq* polymerase (1.5 U), 0.5 µl; template DNA (50 ng), 10.0 µl; milli Q water, 70.5 µl. DNA amplification was performed with the following thermal profile: an initial denaturation temperature at 94°C for 5 min; 30 cycles of denaturation for 30 s at 94°C, annealing temperature 54°C for 40 s, and extension 72°C for 90 s, and a final extension at 72°C for 7 min. For every PCR reaction a negative control (no template DNA) and positive control (template DNA giving amplified product) were maintained.

Aliquots 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. Three tetra cutting endonucleases, viz. *Alu*I, *Hae*III and *Msp*I were used for RFLP restriction. Restricted DNA was analysed by horizontal electrophoresis in 2% agarose gels. Electrophoresis was carried out at 80 V for 2 h and 30 min with standard gels (11 × 14 cm). Ethidium bromide was added in the gel and a pre-run was always given before loading the samples. The gels were visualized on a gel documentation system.

DNA bands were scored as 1 (present) and 0 (absent). All intense and reproducible bands were considered. The data were used to determine the genetic distance between strains. Average linkage unweighted pairs group method with averages (UPGMA) method[17] was used to construct dendrograms using the statistical program NTSYS2.

For amplification of *nif* H region (781 bp), two primers, viz. *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; the reaction mixture and PCR conditions were followed as described by Pandey *et al.*[6]. *nod*C gene from genome and plasmids of root nodule isolates was amplified using the *nod*C gene primers, e.g. *nod* C1 5′ GCC ATA GTG GCA ACC GTC GT 3′ and *nod*C2 5′ TCA CTC GCC GCT GCA AGT C 3′ following procedure of Watson *et al.*[18].

IAA production was observed in exponentially grown cultures (10^8 cells ml^{-1}) of the strains. Supernatants of the individual strains grown in YEM broth medium[15] having composition (g l^{-1}) mannitol, 10; K_2HPO_4, 0.5; $MgSO_4$, 0.2; NaCl, 0.4; yeast extract, 2.5 having pH 6.8, were collected by centrifugation at 6000 *g* 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 reagent (1 ml of 0.5 mM $FeCl_3$ in 35% $HClO_4$) were added. The absorbance of pink colour developed was read at 530 nm using UV–Vis spectrophotometer[19]. Phosphate solubilization was detected by spotting the bacterial cultures on Pikovskya's agar plates[20]. Plates were incubated at 28 ± 1°C for 3 days, and observed for clearing zone around the colonies. HCN production was determined by modified method of Bakker and Schippers[21]. For demonstration of HCN production, exponentially grown cultures (10^8 cells ml^{-1}) of strains were streaked on solid agar plates with simultaneous addition of filter paper soaked in 0.5% picric acid in 1% Na_2CO_3 in the upper lid of plates. Plates were sealed with parafilm and after proper incubation at 28 ± 1°C, development of colour from yellow to light brown, moderate brown or strong brown was examined for putative HCN production. Siderophore production was determined on Chrome-azurol S (CAS) medium following the method of Schwyn and Neilands[22]. Bacterial strains (24-h-old cultures) were spotted on CAS medium plates and incubated at 28 ± 1°C for 48 h. Formation of orange to yellow halo around the colonies showed the production of siderophore.

Dual culture technique of Skidmore and Dickinson[23] was followed to determine antagonistic activity of the isolates against fungal pathogens, e.g. *M. phaseolina* and *F. oxysporum*. In brief, an agar block (5 mm dia) from 5-day-old culture containing mycelia of the pathogens was placed at the centre of the assay plate; one loopful culture medium (24-h-old) of isolated strain was spotted 2 cm away from the pathogen. Plates were incubated at 28 ± 1°C for 5 days. Growth inhibition was calculated by measuring the distance between the edge of bacterial and fungal colonies.

All five strains (MPR1 to MPR5) were Gram-negative, non-sporing, and non-capsulated and motile with single sub-polar flagellum. All the strains formed white, semi-translucent, rounded, smooth, mucoid colonies with 2 to 4 mm dia after 48 h of incubation. Strains absorbed congo red from CrYEMA (congo red yeast extract mannitol agar) after 5 days of incubation at 28°C resulting in red colonies. All the strains were fast growing with average mean generation time of 2.9 h. Strains were positive for catalase and oxidase, and produced acid from glucose. All the strains were negative for gelatinase and starch hydrolysis (except MPR5). All strains failed to grow on GPA[24] (glucose peptone agar) but were able to grow in HAB (Hoffer's alkaline broth)[24] and tolerated 8% KNO_3 as well as 2% NaCl. Strains also precipitated calcium glycerophosphate. All the characters of *Mucuna* isolates were compared with standard strains, viz. *Sinorhizobium meliloti* MTCC 100, *Rhizobium leguminosarum* MTCC 99 and *Mesorhizobium loti* MTCC 2378.

The genetic relatedness amongst five root nodule isolates of *M. pruriens* was analysed employing RAPD technique.

RAPD was carried out with 15 primers for the five isolates along with reference strains, viz. *Bradyrhizobium japonicum* (USDA6, SEMIA 566 and SB 103); *B. elkanii* (SEMIA 5019) and *Mesorhizobium ciceri* CC4. The amplification products in RAPD were between 200 bp and 5 kb. Based on variations in number and size of bands it was possible to identify individual strains of *Mucuna* isolates. However, despite the polymorphism observed with some primers, the diversity index was low. All five isolates belonged to a single cluster (Figure 1), whereas the reference strains formed a second cluster. Isolates MPR2 and MPR4 were identical and shared 92% similarity with isolates MPR1. *Mucuna* isolates MPR3 and MPR5 shared 88% similarity but were distinct with the other three isolates by almost 34%. Thus all five *Mucuna* isolates shared considerable homogeneity. Of the 15 primers used, OPA1, OPA2 and OPC4 gave good polymorphism. Individual characterization of the isolates was possible with these primers.

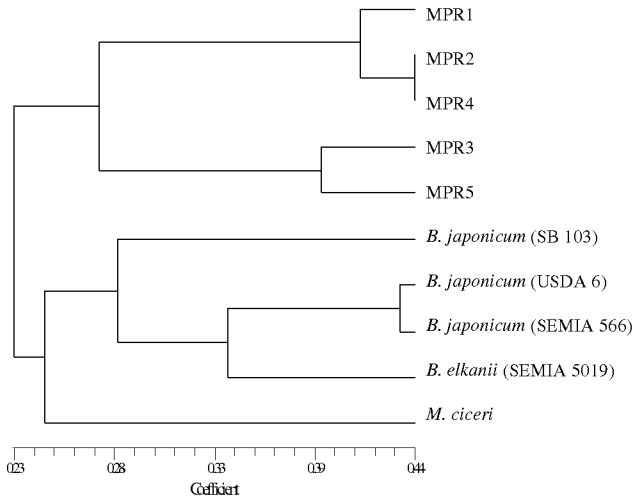

Figure 1. Dendrogram of *Mucuna pruriens* isolates derived from RAPD fingerprinting analysis generated using 15 different primers.

The 16S rDNA PCR amplified a product of 1.5 kb (Figure 2). Restriction analysis of the amplified 16S rDNA sequence was carried out with three tetra-cutter restriction endonucleases, *Alu*I, *Hae*III and *Msp*I. These three enzymes were selected on the basis of their usefulness in other studies[8]. Restriction analysis of 16S rDNA with these enzymes resulted in 3–4 different patterns for each enzyme. The sum of the estimated sizes of the digested fragment was close to that of the full size of the amplicon, which is 1.5 kb. A combination of three endonucleases permitted a good resolution level. *Msp*I produced comparable patterns for MPR1, MPR2 and MPR4, whereas MPR3 had a profile similar to MPR5. *Hae*III could differentiate between MPR1 and MPR2, whereas *Alu*I was the most discriminatory of all five isolates. Three genotypes were detected among five isolates (Figure 3).

When the genomic DNA was amplified with the primers of *nod*C gene, an amplicon of 500 bp was observed on comparison with low range ruler DNA (Banglore Genei, India). The same products were amplified both from bacterial DNA and plasmid DNA (Figure 4 *a*). After amplification of the *nif*H gene by specific primers and comparison with low-range DNA ruler, 781 bp long amplified products were observed in all but one isolate (MPR2) and the three standard cultures confirmed the nitrogenase activity of the isolates (Figure 4 *b*).

Except MPR5, all the strains (MPR1 to MPR4) were positive for IAA production. Clear halos around their growth were seen on Pikovskya's agar, which is due to dissolution of tricalcium phosphate, hence, all were positive for phosphate solubilization. None of the strains was able to change colour in the HCN determination test, whereas MPR3 and MPR4 were able to form an orange halo around their colonies on CAS agar medium, indicating siderophore production (Table 1).

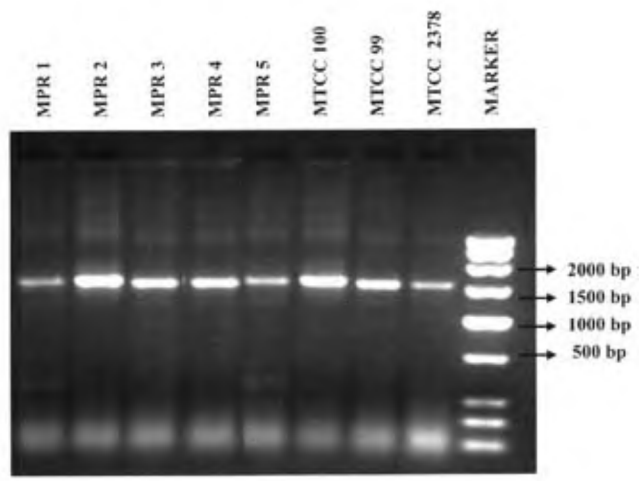

Figure 2. PCR amplification of 16S rDNA gene of isolates of *Mucuna pruriens*.

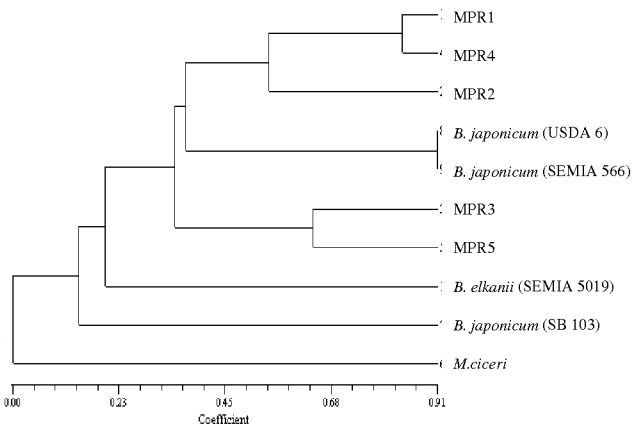

Figure 3. UPGMA cluster of nodule isolates of *Mucuna pruriens* on the basis of 16S ARDRA with *Alu*I, *Hae*III and *Msp*I.

Table 1. Plant growth-promoting and antifungal properties of isolated rhizobial strains isolated from *Mucuna pruriens*

Strain	IAA[A]	Phosphate solubilization[B]	HCN[C]	Antagonism against		
				Siderophore[D]	*Macrophomina phaseolina*	*Fusarium oxysporum*
MPR 1	+	+	–	–	–	–
MPR 2	+	+	–	–	–	–
MPR 3	+	+	–	+	+	+
MPR 4	++	++	–	++	++	+
MPR 5	–	+	–	–	–	–
Standard strains						
MTCC 100	+	+	–	–	–	+
MTCC 99	–	+	–	–	–	+
MTCC 2378	–	+	–	–	–	–

[A] –, IAA negative; +, IAA positive; ++, strongly positive; [B] +, Phosphate solubilization positive; [C] –, HCN negative; [D] –, Absence of halo formation; +, Small halos < 0.5 cm wide surrounding colonies; ++, medium halos > 1.0 cm wide surrounding colonies; *Sinorhizobium meliloti* MTCC–100; *Rhizobium leguminosarum* MTCC-99; *Mesorhizobium loti* MTCC-2378.

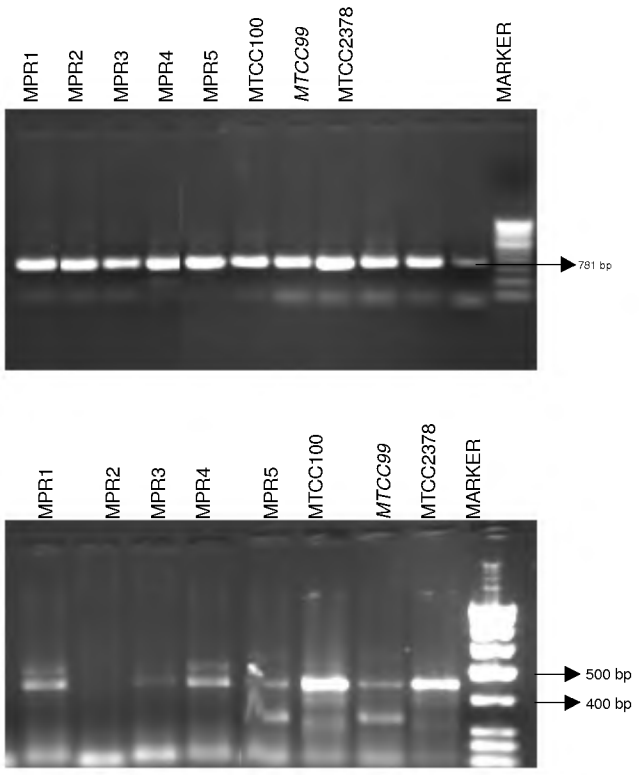

Figure 4. PCR amplification of *nod*C gene (***a***) and *nif*H gene (***b***) in *M. pruriens* isolates.

Among the five isolates, only MPR4 inhibited the growth of *M. phaseolina* and *F. oxysporum*. Strain MPR4 was able to inhibit 67 and 56% mycelial growth in *M. phaseolina* and *F. oxysporum* respectively with regression correlation (r^2) values 0.9653 and 0.9611. Cell-free culture filtrate of the strain also restricted hyphal as well as sclerotial development of *M. phaseolina* and vegetative growth of *F. oxysporum*. It was observed that fungal inhibition by strain MPR4 was more pronounced in dual culture conditions in comparison to that of cell-free culture filtrates (Figure 5).

All the strains were fast growers with average mean generation time of 2.9 h and gelatinase-negative. Except MPR4, all utilized citrate for growth. All strains failed to grow on GPA, but were able to grow in HAB and tolerate 8% KNO_3 as well as 2% NaCl. All characters belong to the species *Sinorhizobium meliloti*[16]. Strains precipitated calcium glycerophosphate, which is in accordance with the characteristics of *S. meliloti*[24].

Random primers have been used to determine variation among legume symbionts by RAPD[25]. Little polymorphism was observed among our isolates utilizing 15 different primers. Earlier, van Rossum *et al.*[10] observed greater genetic diversity by RAPD fingerprinting than that compared to rDNA sequence comparison while studying genetic diversity in *Bradyrhizobium* strains nodulating peanut. It is known that a large number of RAPD markers (7150), together with a sufficient number of products strains (710) are necessary to draw reliable (taxonomic) conclusion[26].

PCR–RFLP of *M. pruriens* isolates detected three genotypes among five isolates (Figure 3). Reference strains belonging to different genera were placed on separate lineages. Strains MPR1 and MPR4 shared a particularly close homology of approximately 84%. ARDRA generates species-specific patterns; exceptions showing variable banding patterns within a species have been reported in *Rhizobium etli*, *R. tropici* and *R. loti*[8]. In the present study, a combination of three restriction endonucleases, *Msp*I, *Hae*III and *Alu*I permitted a resolution level comparable to that reported by Laguerre *et al.*[9]. On the basis of ARDRA, MPR1, MPR4 and MPR2 clustered together, while MPR3 and MPR5 clustered distantly. The two clusters seem to share 50% similarity. The RAPD pattern also indicates the same. Two different species of *Bradyrhizobium* have earlier been reported from soybean[27], peanut[28] and *Phaseolus vulgaris*[29]; even rhizobia of two different

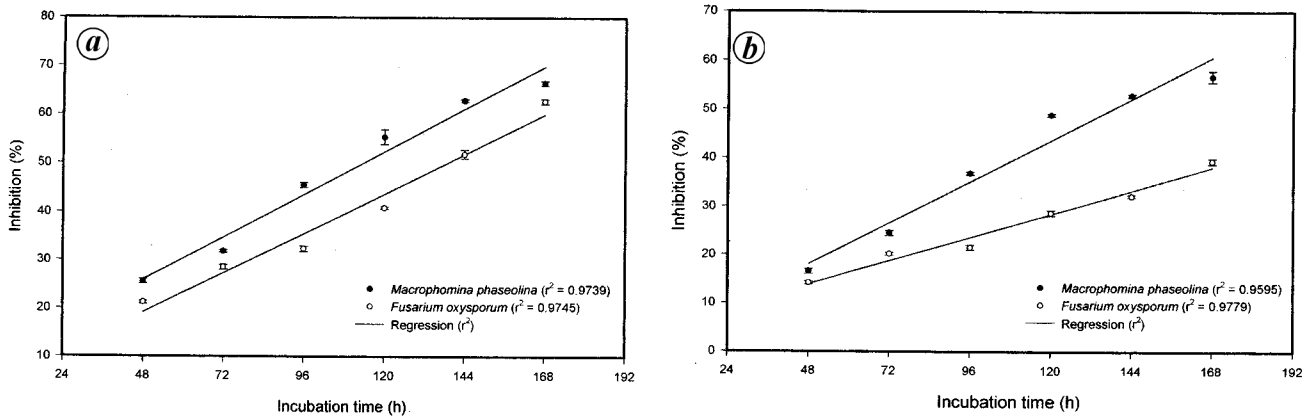

Figure 5. Inhibition of *Macrophomina phaseolina* and *Fusarium oxysporum* by strain MPR4 under dual culture (*a*) and inhibition by culture filtrates (*b*). Values are means ± SD of three replications.

genera have been reported from soybean nodules[30]. In view of these reports, two different rhizobia nodulating *M. pruriens* cannot be ruled out, which can be verified by sequencing of 16S rDNA of these isolates.

We did not correlate the ARDRA and RAPD profiles as the former provides single-locus analysis, whereas the latter assesses genetic variability in the whole genome using a large number, in our case 15 random primers. Furthermore, 16S rDNA is only 1500 bp long (albeit a much conserved one) compared to the whole genome. Earlier, rDNA clustering was not always found in correspondence to clustering based on RAPD[10]. It was concluded that high rDNA homology is a prerequisite but not an assurance for high similarity values obtained with other techniques.

The positive amplification of 781 and 500 bp amplicons in our isolates confirmed the presence *nif* H and *nod*C genes for nitrogen fixation and nodulation respectively. The *nif* H primers used in this study were designed by aligning the *nif* H sequences of *B. japonicum* 110, *R. phaseoli* CFN 42, *R. trifolii* 329 and *S. meliloti* 41. Other PGP attributes like phosphate solubilization and IAA production were also found in *Mucuna* isolates. Along with these, siderophore production and fungal inhibition by MPR4 makes it a good PGP agent.

The study shows that there is great homogeneity amongst *Mucuna* root nodule isolates. Also, the strains can be exploited for PGP activities.

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Biochemical responses in transgenic rice plants expressing a defence gene deployed against the sheath blight pathogen, *Rhizoctonia solani*

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Diverse defence responses were studied in transgenic Pusa Basmati1 (PB1) rice lines engineered with rice chitinase gene (*chi11*) for resistance against the sheath blight pathogen, *Rhizoctonia solani*. Enhancement of phenylalanine ammonia lyase, peroxidase, and polyphenoloxidase enzyme activities in response to the pathogen challenge under controlled conditions resulted in reduced symptom development and containment of disease in transgenic rice lines compared to non-transgenic control plants. Loss of chlorophyll resulting from *R. solani* infection was comparatively less in transgenic plants. Our results provide new information on the biochemical basis of chitinase-based fungal resistance in transgenic plants.

Keywords: Defence gene, resistance, *Rhizoctonia solani*, transgenic rice.

PLANTS live in a milieu of potential pathogens under natural conditions. Though plants have no antibody-mediated resistance mechanisms, they defend themselves against pathogens with an arsenal of defence mechanisms. These include both passive and induced defence responses, wherein the latter plays a vital role in the active defence mechanism and it requires the host metabolism to function[1,2]. Further, these factors appear to play an important role in transgenic plants engineered with defence genes. This study reports the role of the introduced chitinase gene in triggering the defence pathway in response to sheath blight pathogen.

Rice (*Oryza sativa* L.) is an important food crop, providing a major source of sustenance to over half of the world's population. Rice production is severely affected by several pests and diseases. Among the several devastating diseases, rice sheath blight (ShB) caused by *Rhizoctonia solani* Kühn, is a major limiting factor hampering the rice production[3]. Through genetic engineering approaches it is now possible to engineer ShB resistance in cultivated rice by introducing genes encoding chitinases[4] and thaumatin-like proteins[5,6]. These pathogenesis-related

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