Biochemical characterization and metabolic diversity of soybean rhizobia isolated from Malwa region of Central India

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ABSTRACT

Soybean cultivation in many zones of India shows occurrence of native rhizobia besides other exotically adapted strains. In the current study, 22 rhizobial isolates (recovered from 12 different soybean growing sites) and 8 reference strains were selected for biochemical and metabolic characterization. Of 22 isolates, 18 were recovered as fast growing isolates while the rest were slow growing based on bromothymol blue (BTB) test. Unlike earlier belief that rhizobia have no ability to grow on glucose peptone agar medium, in this study, some isolates and some reference strains grew well on this medium. Similarly, when all the isolates were subjected to ketolactose test, some of the isolates were found to show growth on the medium. In contrast, based on C-utilization pattern (15 carbohydrates) a remarkable metabolic diversity was observed among the rhizobial isolates recovered in the study. The clustering and matching analysis showed that most of isolates were matching with slow growing reference strains, a few were with fast growing reference strains and some were found to be unique and hence not matching with any of reference strains. Such analysis suggests the occurrence of metabolically distinct types of rhizobia besides commonly known types (*B. japonicum*, *B. elkanii* and *S. fredii*) of soybean rhizobia and further validation is suggested through 16SrRNA gene sequencing technique.

Keywords: soybean rhizobia; metabolic diversity; biochemical characterization

In India, soybean (*Glycine max* (L.) Merrill) is mainly concentrated in central part predominantly in Madhya Pradesh, Maharashtra and Rajasthan around a latitude range of about 16° to 26°N and longitude range of about 73° to 84°E (Tiwari 2003). When compared to other potential soybean growing countries (US, China, Argentina, Brazil) the unit area productivity of soybean is very low despite its yield potential. The reason for low productivity of soybean is largely erratic, uneven and inadequate rainfall and, among several other abiotic factors, occurrence of varying degrees/magnitude of drought is perhaps the most important abiotic factor limiting the productivity of soybean. Despite the considerable capacity for acquiring nitrogen from biological nitrogen fixation (BNF) (Wani et al. 1995), the inoculation of soybean with rhizobial strains does not necessarily result in yield increase. Nevertheless, the significance of rhizobia forming root nodules and growth enhancement in soybean was widely studied by many workers in the recent past (Saeki et al. 2006, Sharma 2006). However, selection of niche-based new elite strains adapted to local environmental conditions and to newly bred plant lines stays a need of an hour (Appunu et al. 2008).

Therefore, it is imperative to assess the diversity of the rhizobial communities in the rhizosphere soils of different soybean growing areas to tap the maximum strains for inoculation programme. A major limitation in the study of rhizobial populations is the difficulty to identify strains in their natural habitat (Zabaloy and Gómez 2005). However, several techniques for characterization and identification of strains have been developed, such as C source utilization pattern and fatty acid methyl esters (FAME) analyses (Kennedy 1994) or by means of Biolog system (Swelim et al. 1997) to

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assess bacterial community present in the soils. Amarger et al. (1997) classified the rhizobia based on C- source utilization pattern of carbohydrates. Phenotypic characterization of fast and slow growing rhizobia is based on biological reactions on yeast extract mannitol agar (Hungria et al. 2001). The fast growing rhizobia were found to be more salt tolerant than slow growers (Hua et al. 1982). Zabaloy and Gómez (2005) carried out a diversity analysis of 76 rhizobial strains isolated from Argentina agricultural soils using C- utilization patterns. Dendrogram analysis of their data showed that there were remarkable differences among the strains isolated from the same soil. The genetic diversity of slow- and fast-growing rhizobia nodulating soybean was reported in many parts of the world. Recently Appunu et al. (2008) studied the genetic diversity of soybean-nodulating *Bradyrhizobium* isolates from India and found that the diversity is wider than expected based on previous studies in various geographic areas and on the current taxonomy of soybean rhizobia.

In the current study we have characterized and evaluated the diversity of root-nodulating soybean rhizobial isolates collected from various soybean fields involving different cultivars and soil management practices of Madhya Pradesh state.

Table 1a. Details of the sites from where soybean root nodulating rhizobia were isolated

No.	Isolates code	Site	Soybean cultivar	Cultivation practice
1	3b	Betma village, Dhar district	JS 335	NPK fertilization in soybean (conventional), wheat-soybean rotation
2	3c			
3	4a	Near Utavad village, Dhar road, Dhar district	JS 335	cultivation without application of NPK fertilizers and manures; soybean-soybean
4	4b			
5	5a	Utavad village interior, Dhar district	JS 93-05	soybean was gown without NPK and manure application; NPK was applied regularly in wheat
6	6b	Palnaghar village, Dewas district	Samrat	soybean grown without NPK application but FYM applied at the rate of 8-10 t/ha. NPK was applied regularly in wheat
7	6c			
8	7c	Daulatpur village, Sehore district (near Sonkachh)	JS 71-05	soybean grown without NPK application but FYM applied at the rate of 8-10 t/ha. NPK was applied regularly in wheat
9	8b	Chinnota village/Dodighati (Near Sehore)	JS 95-60	soybean grown without NPK application but FYM applied at the rate of 8-10 t/ha. NPK was applied regularly in wheat
10	9a	MP Oil Regional Crop Research Centre, Amlah, Sehore district	NRC 7	conventional, soybean-wheat rotation
11	9b			
12	10a	MP Oil Regional Crop Research Centre, Amlah, Sehore district	NRC 37	
13	10c			
14	12b	Gwaloo village, Khargone district	JS 335	no fertilizers in soybean, NPK application in wheat
15	12c			
16	14b	Thapna village, Khargone district	JS 335	no fertilizers in soybean, NPK application in wheat
17	15a			
18	17b	Khajrana village, Ujjain district	JS 93-05	
19	18e		JS 93-05	
20	19a	Ramvasa village, Ujjain district	JS 335	conventional, soybean-wheat
21	19e			
22	20b	Bherugargh village, Ujjain district	JS 93-05	no fertilizers only FYM application in soybean

Table 1b. Chemical and nutrient characteristics of soils of sampling sites (pooled to district wise)

Site/district	pH (1:2.5, soil water ratio)*	Organic carbon (%)	Available P (mg/kg)	Mineral N (%)	DTPA extractable Zn (mg/kg)
Dhar	8.1	0.44	5.14	4.10	1.82
Dewas	7.8	0.67	6.10	12.10	2.42
Sehore	7.8	0.64	8.16	6.18	1.31
Khargone	7.6	0.49	4.88	4.84	1.62
Ujjain	6.8	0.61	6.12	8.36	1.34

MATERIALS AND METHODS

Field sites and sampling. Rhizosphere soil samples along with intact root nodules of soybean plants were collected during 2007 kharif (monsoon/rainy season) at 12 farmer fields located in Dhar, Dewas, Sehore, Indore, Khargone and Ujjain districts of Madhya Pradesh state of Central India (Table 1a). From each site, five soybean plants were randomly collected, totaling 60 plants in the study. Ten active nodules were randomly chosen from each sample of 60 plants. All the nodules were placed on cotton in screw cap plastic vials containing calcium carbonate as desiccant below cotton at the bottom and stored in refrigerator until isolation. Besides root nodules, the sub samples of rhizosphere soil of a district were pooled to three samples per district site; these 18 samples (6 districts × 3) were used for the analysis of chemical and nutrient characteristics (Table 1b).

Isolation of rhizobia from root nodules. Isolation of rhizobia from root nodules was done by the method of Somasegaran and Hoben (1985). From each sample, two-three nodules were picked up and washed thoroughly with sterile distilled water. After washing, nodules were surface sterilized in 95% alcohol for 30–40 s to remove wax coating if any and subsequently immerged in 4% sodium hypochlorite for 3–4 min. Then nodules were immediately washed 5–6 times with sterile distilled water to remove traces of sodium hypochlorite. The surface-sterilized nodules were transferred to sterile tubes containing 100 µl sterile distilled water. Nodules were crushed with the help of sterile glass rod and then were streaked one loopful of milky suspension on congo red yeast extract mannitol agar (CRYEMA) containing 25 µg/ml congo red, (0.5 g KH_2PO_4; 0.2 g $MgSO_4 \cdot 7H_2O$; 0.1 g NaCl; 0.5 g yeast extract; 10 g mannitol; 15 g agar; per litre) and were incubated at 28°C in dark until growth appeared. Single unique colonies were picked up and were re-streaked on CRYEMA medium until pure culture was obtained and authentication of strains was performed using nodulation test in soybean plants as described by Somasegaran and Hoben (1985).

Reference cultures used

Following reference cultures were used in the study (Table 2).

Biochemical characterization of rhizobial isolates. Gram-staining reaction was carried out by using a loopful of pure culture grown on YEM

Table 2. Cultures used in this study

No.	Code	Strain	Source
1	UDSA 110	*Bradyrhizobium japonicum*	Rhizobium germplasm, USDA/ARS, Beltsville, MD
2	USDA 31	*Bradyrhizobium elkanii*	
3	USDA 205	*Sinorhizobium fredii*	
4	FA-2	*Bradyrhizobium japonicum*	University of Miyazaki, Japan
5	CF10	*Sinorhizobium fredii*	
6	HA-1	*Bradyrhizobium japonicum*	
7	SEMIA 5079	*Bradyrhizobium japonicum*	Embrapa, Brazil
8	YCK 294	*Bradyrhizobium japonicum*	National Yeongnam Agricultural Exp. Station, RDA, Korea

broth (yeast extract mannitol broth) and stained as per the standard gram's procedure (Somasegaran and Hoben 1994). The purified isolates were characterized based on biochemical tests. The isolates were classified as fast (medium turn yellow) and slow growers (medium turn blue) based on their reaction on the yeast extract mannitol agar supplemented with bromothymol blue (Somasegran and Hoben 1994).

The conformity of *Rhizobium* was carried out by streaking each culture on glucose-peptone agar medium containing bromocresol purple indicator dye (100 µg/ml) (glucose 10 g; peptone 20 g; NaCl 5 g; bromocresol purple 100 mg; per litre) followed by incubation at 28°C for 3–6 days. Similarly, ketolactose test (lactose 10 g; K_2HPO_4 0.52 g; $MgSO_4 \cdot 7\ H_2O$ 0.2 g; NaCl 0.12 g; $CaCO_3$ 3 g; yeast extract 1 g; agar 15 g; per litre) was also performed on the ketolactose agar plate and overlay with Benedict's reagent (Solution A-sodium citrate 17.3 g; sodium carbonate (anhydrous) 10 g; distilled water 60 ml; Solution B-copper sulphate 1.73 g; distilled water 10 ml mixed both and make up 1 l with water) to distinguish *Agrobacterium* and *Rhizobium*.

Intrinsic antibiotic resistance (IAR) test was also conducted using specific antibiotics as per the method of Ladha and So (1994). The rhizobial isolates (22) and reference strains (8) were distinguished by their antibiotic pattern within the *Rhizobium* spp. Resistance and sensitivity of rhizobial isolates against designated antibiotics were carried out by spot inoculation of culture on tryptone yeast extract agar supplemented separately with seven different antibiotics: kanamycin (100 µg/ml), gentamycin (250 µg/ml), streptomycin (100 µg/ml), chloramphenicol (100 µg/ml), nalidixic acid (50 µg/ml), polymyxin-B sulphate (50 µg/ml) and trimethoprim (50 µg/ml).

Metabolic fingerprinting of isolates based on carbon source utilization pattern

Carbon source (C-source) utilization test was carried out in 2 ml vials containing specific carbohydrates (Amarger et al. 1997) using M_9 minimal medium. Fifteen C sources (adonitol, maltose, sucrose, lactose, trehalose, mannitol, glucose, D-mannose, D-galactose, L-arabinose, D-xylose, rhamnose, inositol, D-ribose and fructose) were used in the concentrations of 0.1% (w/v) for each isolates. Log phase culture was inoculated in the M_9 medium (1.5 ml) containing 25 µg/ml bromothymol blue dye (pH 7.0) and incubated at 28°C with 150 rpm for 4–6 days. A control was maintained without sugar. The change in colour from green to blue or bluish green (alkali production) or yellow (acid production) indicated the utilization of the respective C-sources and no change in colour of the broth was taken as non-utilization. Reference cultures were also included in this study for comparison. Software NTSys pc version 2.0e (Applied Biostatistics Inc. USA) was used to construct dendrogram for clustering the isolates to assess the diversity and similarity among the isolates.

RESULTS AND DISCUSSION

Based on the growth rate on the standard medium and acid and alkali production, out of 22 isolates, 18 isolates were found to be fast-growing and remaining 4 isolates were found as moderate- to slow-growing. The strategy for assessing rhizobial diversity by isolating from root nodules collected directly from field was found to be better than the plants inoculated with soil dilutions (Loureiro et al. 2007). The rhizobial diversity is depending on number of factors such as crop history and method of sampling (Grange and Hungria 2004). The rhizobial isolates in the current study were further tested on YEMA plates containing bromothymol blue which indicated that fast-growing isolates (18) were found to produce yellow colonies due to acid production on the medium with high mucous after 2 days of incubation (Table 3).

On the other hand, remaining 4 isolates produced blue colour colonies, which indicated the presence of alkali producers. These isolates were similar in terms of reaction on the YEMA (BTB) when compared with reference strains which produced yellow and blue colour in fast and slow growing strains, respectively (Hungria et al. 2001). The use of YMA-BTB medium for categorizing Vietnami indigenous soybean root-nodulating fast and slow growing rhizobia based on acid/alkaline production was also carried out by Saeki et al. (2005). While further confirming these isolates, out of 18 fast-growing isolates, 14 isolates showed either poor or no growth on the glucose peptone medium indicating character of rhizobia as a conventional rule. Remaining five isolates (4b, 5a, 8b, 19a and 20b) invariably showed growth on the medium. These isolates, (except 4b) and one reference culture (USDA-205), besides showing poor growth on the medium, were also found to turn the medium yellow. Amongest all the slow growing isolates

Table 3. Categorization of rhizobia based on acid and alkali production on YEM–BTB medium and growth on glucose peptone and ketolactose production by rhizobial isolates

Rhizobial isolates	Origin/collection	BTB**	Glucose peptone	Ketolactose test
Fast-growing				
3c	Dhar disctrict	yellow	+	–
4b		yellow	–	+
5a		yellow	–*	–
6b	Sehore district	yellow	+	+
6c		yellow	+	+
7c		yellow	+	+
8b		yellow	–*	+
9b	Khargone district	yellow	+	+
10a		yellow	+	+
10c		yellow	+	+
12b		yellow	+	+
14b		yellow	+	–
15a		yellow	+	+
17b	Ujjain district	yellow	+	+
18e		yellow	+	+
19a		yellow	–*	+
19e		yellow	+	+
20b		yellow	–*	+
YCK 294	Reference cultures	yellow	+	–
USDA 205		yellow	+*	+
CF10		yellow	+	+
Slow-growing				
3b	Dhar disctrict	blue	–	+
4a		blue	+	+
9a	Khargone disctrict	blue	+	+
12c		blue	+	–
USDA 110	Reference cultures	blue	+	+
FA2		blue	+	+
USDA 31		blue	+	+
SEMIA 5079		blue	+	+
HA 1		blue	+*	+

**BTB bromothymol blue; *yellow in colour; + positive, poor growth or no growth; $^{+}$no ring formed; – negative ring formed or growth recorded

except one, i.e. 3b, showed no growth on the medium, which confirms the rhizobial colonies. All the reference strains, the fast-growing (CF 10 and, USDA 205) and slow-growing strains (FA 2, USDA 31, HA-1, SEMIA 5079 and YCK 294) showed either poor or no growth on the medium, which confirms the purity of strains. When ketolactose test was performed, most of the isolates were found to be negative for the production of 3-ketolactose from lactose (Table 3). However, four fast-growing isolates (3c, 5a, 14b, 18e) and one slow-growing isolate (12c) were found positive (showed growth on the medium) which are in contradiction to earlier work carried out by Sadowsky et al. (1983). At present, the rhizobia are being authenticated using PCR-based molecular techniques; therefore

Table 4. Antibiotic sensitivity/resistance pattern of rhizobial isolates and reference strains

Isolates	Kanamycin (100 µg/ml)	Gentamycin (250 µg/ml)	Streptomycin	Chloram-phenicol	Nalidixic acid	Polymyxin B sulphate	Trimetho-prim
			(100 µg/ml)			(50 µg/ml)	
Fast-growing							
3c	+	–	+	+	+	+	–
4b	–	–	–	–	+	–	+
5a	+	–	–	–	+	–	+
6b	–	+	+	+	+	+	–
6c	–	–	–	–	–	–	+
7c	–	–	–	–	+	+	–
8b	–	–	+	+	–	–	+
9b	–	+	–	–	–	–	–
10a	–	–	–	–	+	–	–
10c	–	–	–	–	+	–	–
12b	+	–	+	+	+	+	+
14b	–	–	–	–	–	–	–
15a	–	–	–	–	–	–	–
17b	–	–	–	+	–	–	–
18e	–	–	–	+	+	–	+
19a	–	–	+	–	–	–	–
19e	–	+	–	+	+	–	+
20b	–	–	–	–	–	–	–
YCK 294	+	+	+	+	+	+	+
SEMIA 5079	+	–	+	+	+	–	+
USDA 205	–	–	–	–	+	–	–
CF10	–	–	–	+	–	+	+
Slow-growing							
3b	–	–	–	+	–	+	–
4a	+	+	+	+	+	–	+
9a	–	–	–	–	–	–	–
12c	+	–	–	–	+	+	+
16b	+	+	+	+	–	+	+
USDA 110	–	–	–	+	+	–	–
FA2	+	+	–	+	+	+	+
USDA 31	+	–	–	+	+	+	+
HA 1	+	–	–	+	–	+	+

+ growth recorded means resistance; – no growth, sensitive

the results obtained through biochemical methods should be revalidated for further work. Based on the intrinsic antibiotic resistance (IAR) test using seven antibiotics, most of isolates, except few, were found to be resistant to nalidixic acid (50 µg/ml), chloramphenicol (100 µg/ml) and trimethoprim (50 µg/ml) (Table 4). Out of all fast-growing isolates only three (3c, 5a and 12b) rhizobial isolates were found resistant to kanamycin (100 µg/ml). Whereas the slow-growing isolates, most of them except 3b, 9a and one reference strain USDA110 were found sensitive to kanamycin (100 µg/ml). Reactions of most of the isolates to gentamycin, streptomycin and polymyxin B sulphate were found

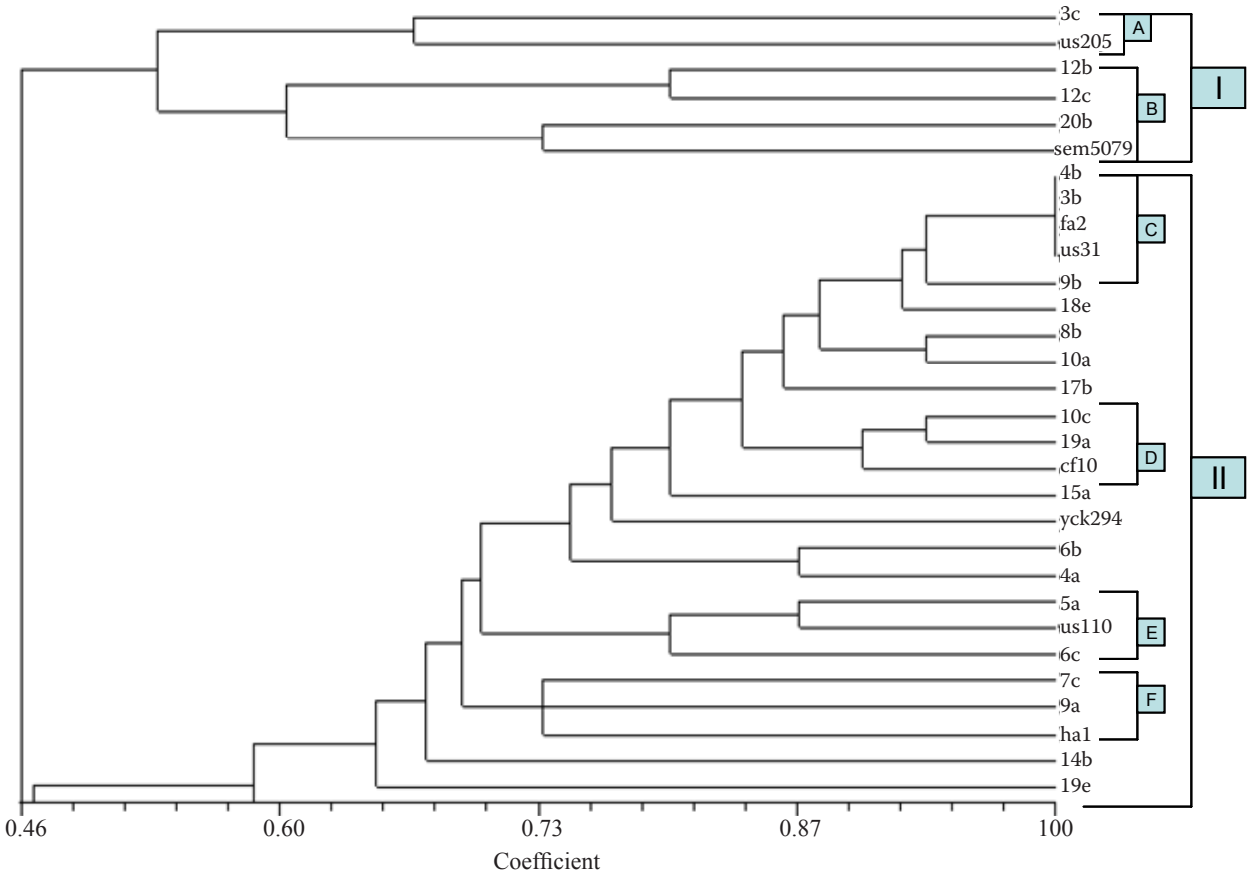

Figure 1. Dendrogram showing phenotypic relatedness, based on utilization of carbon sources and Euclidean distance, among 22 rhizobial isolates and 8 reference strains. Cluster analysis was performed using the Unweighted Paired Group with Arithmetic Average (UPGMA) method in the NTSys program

to be sensitive. Similarly, Padmanabham et al. (1990) studied the IAR pattern of fast-growing rhizobia and found lower IAR than that of slow-growing bradyrhizobia. Among slow-growing rhizobia, Kuykendall et al. (1992) observed relatively lower IAR among strains of *B. japonicum* when compared to *B. elkanii* USDA 31. Abaidoo et al. (2002) suggested that the antibiotic resistance profile method can be used as a simple means of assessing genetic variability and grouping of a large number of *Bradyrhizobium* spp. (TGx) isolates. Representative isolates from each group can then be selected for further characterization. However in this study authors attempted to assess the diversity based on C-source utilization assay.

Relationship among 22 rhizobial isolates and 8 reference strains was depicted in the Figure 1 based on 15 carbohydrate sources utilization pattern. The dendrogram analysis (Figure 1) shows that there are remarkable differences among the soybean rhizobia. The grouping shows that most of the fast-growing rhizobial isolates (18) and some slow-growing (4) were broadly classified into two major clusters, cluster I and cluster II, which were further divided into sub-clusters from A to F for further interpretation. Cluster I consisting of two sub-clusters dealt with *Bradyrhizobium japonicum* and *Sinorhizobium fredii* groups whereas cluster II dealt with four sub clusters 'a mixed group' comprising *B. japonicum*, *B. elkanii, S. fredii* groups and some isolates without any grouping. It is noteworthy that some isolates like 8b, 10a, 14b, 19e, 18e, 6b, 4a, 15a and 17b were not matching with any reference strains and thus indicating that these rhizobia are probably 'unique' and can be identified based on 16SrRNA gene sequencing technique.

In the current study based on matching with the cluster/sub-clusters of reference strains, out of 22 isolates, of the 18 fast growing rhizobia identified based on acid production only three matched with fast growing reference strains (3c-USDA205; 10c and 19a CF10), seven (4b and 9b- FA2, USDA 31; 5a and 6c-USDA,110; 7c; HA-1; 12b, 20b, SEMIA5079) matched with slow-growing reference strains, and

remaining eight (8b, 10a, 14b, 19e, 18e, 6b, 15a and 17b) did not match with reference strains used in the study. On the other hand, all the slow growing isolates except one (4a) were matching with slow-growing reference strains (3b-USDA 31, FA2; 9a-HA 1; 12c-SEMIA5079). The result shows congruence with genotypic fingerprinting on the basis of RFLP analysis of 16S rDNA and also reported occurrence of distinct type of soybean rhizobia in black soils of Malwa region (Sharma et al. 2006). In another study by Satya Prakash and Annapurna (2006) also reported occurrence of soybean rhizobia that are distinct from soybean rhizobia of USA and Brazilian origin based on BOX-PCR analysis. This was further supported by occurrence of distinct rhizobia in tropical soils of India (Appunu et al. 2008). Thus, remarkable differences among the rhizobial isolates observed in the current study based on C-sources utilization may have ecological significance for their exploitation in future inoculation programmes. However, in this study biochemical tests did not provide full proof to distinguish rhizobia. Also the C-source utilization patterns of some of rhizobial isolates used in this study do not match the similarity with reference strains; therefore the use of polyphasic approach involving biochemical and molecular techniques should be employed to characterize the native population of soybean rhizobia.

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