

nifH gene diversity and symbiotic effectiveness of *Bradyrhizobium yuanmingense* strains obtained from nodules of soybean cultivated in India

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ABSTRACT

Restriction fragment length polymorphism (RFLP) of *nifH* gene with three endonucleases revealed three different *nifH* genotypes among twenty one indigenous *Bradyrhizobium yuanmingense* strains. The interactive symbiotic effectiveness on soybean cv. JS335, nodulation and plant growth of these *B. yuanmingense* strains were comparatively evaluated with *B. japonicum* ASR011 under greenhouse conditions. Significant variation in symbiotic potential among native *B. yuanmingense* strains was observed and few of them exhibited significantly superior symbiotic performance over *B. japonicum* ASR011 and *B. yuanmingense* CCBAU 61071 type strain. A considerable difference in nitrogen-fixing characteristics was detected among *B. yuanmingense* strains which shared identical *nifH* gene sequence. This, to our knowledge, is the first study on symbiotic effectiveness of *B. yuanmingense* strains obtained from nodules of soybean cultivated in India. The symbiotically efficient *B. yuanmingense* strains may have applications in the formulation of appropriate inocula to improve soybean crop yield in Indian soils.

Keywords: *B. yuanmingense*, *Glycine max*, *nifH* gene diversity, Nodulation.

INTRODUCTION

Soybean (*Glycine max* L. Merrill) is a major grain legume grown worldwide. India ranks fifth in soybean production in the world. Soybean is one of the chief export commodities in India. The main soybean producing states are Madhya Pradesh, Maharashtra and Rajasthan with some regions in the states of Uttar Pradesh, Andhra Pradesh, Punjab, Tamil Nadu, Uttarakhand, Gujarat, Karnataka and Chhattisgarh. The mean soybean yield in India is about 1 t ha⁻¹ (<http://faostat.fao.org/> as on 15.07.2013) as compared to the world's average yield of 2.2 t ha⁻¹. Hence, improving the crop productivity is a major challenge in India. Successful management of symbiosis between soybean cultivars and native rhizobia is one of the cost effective approaches to improve the soybean productivity. Isolation of native symbiotically efficient isolates have proved to be useful in increasing the soybean yield (Melchiorre *et al.*, 2011).

Soybean plants are reported to form effective root nodules with *Bradyrhizobium japonicum*, *B. elkanii*, *B. liaoningense*, *B. yuanmingense* and unclassified bradyrhizobia (Vinuesa *et al.*, 2008; Appunu *et al.*, 2008a; 2009; Risal *et al.*, 2010). However, the most studied symbiotic performance in India is between *B. japonicum* strains and

soybean (Appunu *et al.*, 2008b; Meghvanshi *et al.*, 2010). Recent findings indicated the significant and wide spread presence of *B. yuanmingense* than *B. japonicum* strains in soybean grown soils in India (Vinuesa *et al.*, 2008; Appunu *et al.*, 2008a; 2009). However, symbiotic potential of *B. yuanmingense* in soybean is still unexploited in India. Therefore, the present study evaluates the symbiotic efficiency of *B. yuanmingense* strains under controlled conditions on soybean cv. JS335 which has been proved to be compatible to native *B. japonicum* ASR011 and *nifH* gene diversity in these strains.

MATERIALS AND METHODS

Experimental rhizobial isolates: Twenty one *B. yuanmingense* strains used in this study (Table 1) were isolated from the root nodules of soybean collected from nine soybean widely grown regions in India. The sites of isolation, morpho-physiological and genetic characteristics of these strains have been already described (Appunu *et al.*, 2008a; 2009). The native *B. japonicum* ASR011, a variant of *B. japonicum* USDA135, used in this study was isolated from soybean root nodule in India and its symbiotic efficiency with soybean cv. JS335 was established previously (Appunu *et al.*, 2008b).

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TABLE 1: Isolate number and agricultural-ecological-climatic characteristics of soybean nodulating *B. yuanmingense* isolated regions in India.

Rhizobia isolate ^a	Agro-eco-climatic and soil characteristics ^b of regions	Reference
<i>B. yuanmingense</i> isolate		
SR66, 69, 70, 71, 77	hot semi arid, with medium and deep black soils, coarse to fine loamy, highly alkaline, moderate to gentle slope	Appunu <i>et al.</i> 2008a
SR223, 257, 261, 268, 297	hot semi arid, with medium and deep black soils, coarse to fine loamy, highly alkaline, moderate to gentle slope	Appunu <i>et al.</i> 2009
SR84, 88, 91	hot sub humid, with red and yellow soils, moderate clay to deep clayey, slightly acidic to slightly alkaline, moderate to gentle slope	Appunu <i>et al.</i> 2008a
SR355, 375, 383, 401	hot sub humid, with red and black soils, deep loamy, neutral to slightly acidic, moderate to gentle slope	Appunu <i>et al.</i> 2009
SR105, 112, 113, 114	hot semi arid, with alluvium derived soils, loamy to clayey, slightly alkaline, gentle to very gentle slope	Appunu <i>et al.</i> 2008a
<i>B. japonicum</i> ASR011	hot sub humid, with red and black soils, deep loamy, neutral to slightly acidic, moderate to gentle slope	Appunu and Dhar, 2006; Appunu <i>et al.</i> 2008b

^aSites of isolation (states of India); more details are in Appunu *et al.* (2008a; 2009)

^bSource: National Bureau of Soil Survey and Land Use Planning, India and Indian Council of Agricultural Research -Hand Book of Agriculture, New Delhi, India. Hot semi arid: mean annual rainfall of 500-1100 mm; moisture index of (-) 33.3 to (-) 66.7; temperature > 22°C and length of growing period (LPG) of 90-150 d. Hot sub humid, mean annual rainfall of 1000-1500 mm; moisture index of (+) 20 to (-) 33.3; temperature of 15- 22°C and LPG of 150-180 d. Hot sub humid to semi arid, mean annual rainfall of 900-1600 mm; moisture index of (-) 66.7 to 0; temperature >15-22°C and LPG of 90-210 d. Soil pH: neutral, 6.6-7.5; slightly acidic, 6.1-6.5; slightly alkaline, 8.0-8.5; moderately alkaline, 8.6-9.0, highly alkaline, > 9.0. Soil topology (slope): level, <1-3%; very gentle, 1-3%; gentle 3-8%; moderate 8-15%.

Total DNA Isolation, nifH gene RFLP and Sequence analysis:

Total genomic DNA isolation, *nifH* gene amplification and *nifH* RFLP were done as described elsewhere (Appunu *et al.*, 2011). For performing *nifH* gene sequencing, PCR products were purified with the Perfectprep Gel Cleanup Kit (Eppendorf, Germany), and nucleotide sequence was determined using an ABI Prism dye terminator cycle sequence reaction kit recommended by the manufacturer's (Applied Biosystems, Foster City, CA, USA). Multiple sequence alignment was performed with Clustal_X (Thompson *et al.*, 1997) using the acquired sequences and related sequences extracted from the GenBank database by using the BLAST program. Phylogenetic tree was reconstructed with the aligned sequences using the Jukes-Cantor distances and neighbor-joining method, and was bootstrapped using 1000 replicates for each sequence using MEGA 5.1 software (Tamura *et al.*, 2011).

Symbiotic performance tests and plant measurements:

To assess symbiotic effectiveness on soybean cv. JS335, nodulation and plant growth of twenty one indigenous *B. yuanmingense* strains were comparatively evaluated with *B. japonicum* ASR011 under greenhouse conditions, and plants supplied with or without chemical N fertilizer served as the control. *B. yuanmingense* CCBAU 61071^T strain was also included in symbiotic performance study. Seed sterilization,

seedling inoculation and plant growth conditions were followed as explained elsewhere (Appunu *et al.*, 2009). All treatments received Thornton's nitrogen-free nutrient medium (Vincent, 1970) twice in a week for proper growth except N supplied plants [received 80 mg of N (as KNO₃) per plant⁻¹ week⁻¹]. Experiment repeated three times under completely randomised design and each treatment had four replications in all experiments during January-April 2012, May-August 2012 and September-December 2012. Data pertaining to symbiotic and vegetative characters were recorded as described previously (Appunu *et al.*, 2009). The nitrogen content of the shoot was estimated by the Kjeldahl method (AOAC, 1990). Data were analysed with the IBM SPSS program version 19 (SPSS Inc., Chicago, IL, USA) by one-way analysis of variance (ANOVA) and significant differences among treatment mean were determined by Tukey's test (*P* d' 0.05).

Nucleotide sequence accession numbers The *nifH* gene sequences of soybean *B. yuanmingense* isolates have been deposited in the GenBank database under accession numbers HF955508, HF955509 and HF955510, and for *B. japonicum* ASR011, EU357921.

RESULTS AND DISCUSSION

Restriction fragment length polymorphism (RFLP) of *nifH* gene with three endonucleases (*CfoI*, *HaeIII* and *MspI*) revealed three different *nifH* genotypes among twenty

one indigenous *Bradyrhizobium yuanmingense* strains. The *nifH* partial sequences of representatives of the three *nifH* genotypes were obtained. These *nifH* sequences showed 98-100% similarity with sequences of *B. yuanmingense* Genbank reference strains. Based on phylogenetic analyses, these *nifH* genotypes formed a monophyletic cluster including *B. yuanmingense* CCBAU 61071^T strain isolated from various host legumes (Figure 1). The *B. yuanmingense* species was originally created based on the description of nodule isolates from *Lespedeza* spp (Yao *et al.*, 2002). Since then, several studies have reported nodulation by *B. yuanmingense* strains in various legumes (Vinuesa *et al.*, 2008; Yang and Zhou, 2008; Appunu *et al.*, 2008a; 2009; Risal *et al.*, 2010). The occurrence of different biovarieties within the *B. yuanmingense* species in India had already been reported (Appunu *et al.*, 2008a; 2009).

Symbiotic performance test exhibited significant difference ($P \leq 0.05$) among the native *B. yuanmingense* isolates with soybean cv. JS335 and majority of the *B. yuanmingense* isolates significantly improved the shoot and root dry weights and shoot N content compared to non-inoculated plants (Table 2). This is in agreement with earlier

report that the inoculation of soybean with rhizobial strains significantly increased shoot and root dry weights and shoot N content (Hungria *et al.*, 2001; Pule-Meulenberg *et al.*, 2010; 2011). The highest increase in shoot N content was detected after inoculation with *B. yuanmingense* strains SR104, 223 and 401. Many *B. yuanmingense* strains shared identical *nifH* gene sequences but significantly differed in nitrogen-fixing characteristics (Table 2). The rhizobial isolates that share similar *nifH* sequences might differ in other composite genes due to frequent occurrence of horizontal transfer of nitrogen fixation (*nif*) genes between rhizobial strains in natural conditions. Horizontal transfer of nitrogen fixation genes among *Bradyrhizobium* strains have been well documented (Appunu *et al.*, 2008a; Vinuesa *et al.*, 2008). Moreover, native *B. yuanmingense* strains SR68, 87, 101, 257, 297, 355 and *B. yuanmingense* CCBAU 61071^T strain produced lower numbers of nodules per plant and other symbiotic characteristics.

It was interesting to observe that plants inoculated with certain *B. yuanmingense* isolates (SR112, 223 and 401) showed similar shoot N content to that of plants supplied with inorganic mineral nitrogen. This is in agreement with

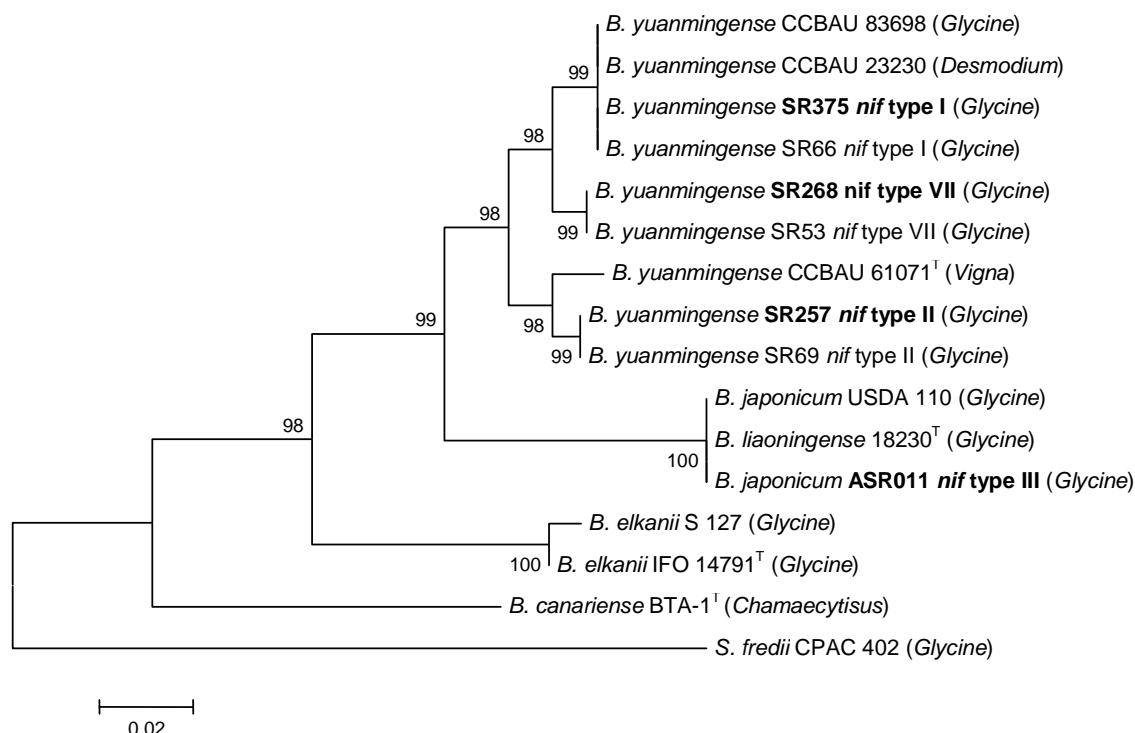


FIG 1: Phylogenetic tree based on nucleotide partial sequences of the *nifH* gene. The scale bar indicates the number of substitutions per site. The isolates are shown in bold, with their *nifH* genotypes indicated. The letter "T" indicates the type strain of the species. Accession numbers of sequences extracted from GenBank are as follows: CCBAU 83698, EU146010; CCBAU 23230, AY934871; SR66, EU357924; CCBAU 61071^T, EU113228; SR69, EU357925; USDA 110, BA000040; LMG 18230^T, EU818985; BTA-1^T, EU818926; S 127, DQ485701; IFO 14791^T, AB094963; and CPAC 402, DQ485714. The sequences determined in this study are indicated with bold letters (ASR011, EU357921; SR 375, FJ514062; SR268, EU357924; SR257, EU357925).

TABLE 2: Nitrogen fixation capacity of soybean cultivar JS335 inoculated with twenty two *B. yuanmingense* strains and a *B. japonicum* ASR 011 reference strain

<i>B. yuanmingense</i> strain (<i>nif</i> type)	Nodule		Root Dry (g plant ⁻¹)	Shoot	
	Number (no. plant ⁻¹)	Dry weight (g plant ⁻¹)		Dry weight (g plant ⁻¹)	Total N (mg N plant ⁻¹)
SR66 (I)	17.26 ^{cd}	0.12 ^d	1.26 ^d	3.51 ^c	84.02 ^d
SR69 (II)	12.46 ^{ef}	0.04 ^g	0.52 ^g	1.34 ^e	32.31 ^g
SR70 (I)	22.03 ^b	0.16 ^c	1.57 ^b	4.08 ^b	95.20 ^c
SR71 (I)	21.83 ^b	0.16 ^c	1.58 ^b	4.05 ^b	95.10 ^c
SR77 (VII)	15.07 ^{de}	0.10 ^e	1.00 ^f	2.78 ^d	59.64 ^e
SR84 (I)	21.13 ^{bc}	0.17 ^c	1.58 ^b	4.06 ^b	96.90 ^c
SR88 (I)	17.00 ^d	0.13 ^d	1.34 ^c	3.52 ^b	83.44 ^d
SR91 (II)	15.01 ^{de}	0.10 ^e	1.07 ^e	2.67 ^d	57.50 ^{ef}
SR105 (II)	14.33 ^{de}	0.04 ^g	0.53 ^g	1.25 ^e	33.44 ^g
SR112 (II)	39.61 ^a	0.35 ^a	1.69 ^a	4.67 ^a	129.54 ^a
SR113 (VII)	8.53 ^f	0.08 ^f	1.00 ^f	2.64 ^d	56.62 ^f
SR114 (II)	17.01 ^d	0.13 ^d	1.32 ^c	3.58 ^c	85.41 ^d
SR223 (II)	40.61 ^a	0.32 ^a	1.67 ^a	4.65 ^a	130.74 ^a
SR257 (II)	15.10 ^{de}	0.09 ^f	1.02 ^f	2.64 ^d	56.53 ^f
SR261 (I)	17.15 ^{cd}	0.12 ^d	1.06 ^e	3.50 ^c	84.44 ^d
SR268 (VII)	17.02 ^d	0.09 ^f	1.26 ^d	3.50 ^c	83.30 ^d
SR297 (I)	14.92 ^{de}	0.08 ^f	1.00 ^f	2.64 ^d	58.10 ^{ef}
SR355 (VII)	15.26 ^{de}	0.09 ^f	1.01 ^f	2.65 ^d	58.22 ^{ef}
SR375 (I)	17.04 ^d	0.13 ^d	1.58 ^b	4.06 ^b	95.50 ^c
SR383 (II)	14.94 ^{de}	0.09 ^f	1.01 ^f	2.69 ^d	59.56 ^e
SR401 (I)	41.83 ^a	0.33 ^a	1.68 ^a	4.66 ^a	129.64 ^a
CCBAU61071 ^T (IV)	17.00 ^d	0.12 ^d	1.31 ^c	3.47 ^c	85.37 ^d
<i>B. japonicum</i> ASR011 (III)	42.73 ^a	0.24 ^b	1.67 ^a	4.07 ^b	105.87 ^b
C-N ¹	0	0	0.39 ⁱ	1.20 ^f	14.39 ^h
C+N ¹	0	0	1.66 ^a	4.70 ^a	127.10 ^a

¹Control plants with (C+N) or without (C-N) mineral N (80 mg N plant⁻¹ week⁻¹); Different letters indicate significant different means for P<0.05.

previous report that inoculated rhizobium strains were able to fix atmospheric nitrogen that is higher or on par with that of shoot N content of inorganic nitrogen supplemented plants (Hungria *et al.*, 2001; El-Akhal *et al.*, 2008). All these results suggest that soybean nodulation by these efficient *B. yuanmingense* strains was low-cost in terms of energy for the plant with the best benefits in terms of nitrogen fixation yield. These are important criteria for isolate selection since use of mineral fertilizers can be significantly reduced which would contribute to economical and sustainable agricultural practises (Hungria *et al.*, 2003; El-Akhal *et al.*, 2008). *B. yuanmingense* strains (SR112, 223 and 401) exhibited significantly superior symbiotic performance than *B. japonicum* ASR011. In agreement with this result, *B. yuanmingense* isolates had significantly greater nitrogen-fixing potential with cowpea than *B. japonicum* and *B. elkanii* strains (Sarr *et al.*, 2009). The best symbiotic association depends on host genotypes, rhizobia and the environment. Therefore, selection of indigenous rhizobial strains with high

nitrogen-fixing capacity, specific to host and adapted to a range of environmental conditions at a specific site appears to be the way to harvest maximum benefit from legume-rhizobium symbiotic associations. These results suggest the existence of soybean nodulating and high nitrogen-fixing native *B. yuanmingense* strains in Indian soils. Soybean plants are widely nodulated by native *B. yuanmingense* strains in Indian soils (Appunu *et al.*, 2008b; Appunu *et al.*, 2009). Therefore, the results obtained in this study provide important leads for the development of effective native *B. yuanmingense* rhizobial inocula to be applied to the field for increasing the soybean productivity in India.

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