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Surviving and Thriving in Terms of Symbiotic Performance of Antibiotic and Phage-Resistant Mutants of *Bradyrhizobium* of Soybean [*Glycine max* (L.) Merrill]

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Abstract Rhizobial inoculation plays an important role in yielding enhancement of soybean, but it is frequently disturbed by competition with bacterial population present in the soil. Identification of potential indigenous rhizobia as competitive inoculants for efficient nodulation and N_2-fixation of soybean was assessed under laboratory and field conditions. Two indigenous bradyrhizobial isolates (MPSR033 and MPSR220) and its derived different antibiotic (streptomycin and gentamicin) and phage (RT5 and RT6)-resistant mutant strains were used for competition study. Nodulation occupancy between parent and mutant strains was compared on soybean cultivar JS335 under exotic condition. Strain MPSR033 Sm^r V^r was found highly competitive for nodule occupancy in all treatment combinations. On the basis of laboratory experiments four indigenous strains (MPSR033, MPSR033 Sm^r, MPSR033 Sm^r V^r, MPSR220) were selected for their symbiotic performance along with two exotic strains (USDA123 and USDA94) on two soybean cultivars under field conditions. A significant symbiotic interaction between *Bradyrhizobium* strains and soybean cultivar was observed. Strain MPSR033 Sm^r V^r was found superior among the rhizobial treatments in seed yield production with both cultivars. The 16S rRNA region sequence analysis of the indigenous strains showed close relationship with *Bradyrhizobium yuanmingense* strain. These findings widen out the usefulness of antibiotic-resistance marked phage-resistant bradyrhizobial strains in interactive mode for studying their symbiotic effectiveness with host plant, and open the way to study the mechanism of contact-dependent growth inhibition in rhizobia.

Introduction

Soybean [*Glycine max* (L.) Merrill], originated from eastern Asia was domesticated ~4,000 years ago, and has been cultivated ever since. The high nitrogen (N) requirement of the crop is fulfilled by establishing a N_2-fixing nodule symbiosis with rhizobia. The effectiveness of symbiotic N_2-fixation may be an important factor to take into consideration for successful management of symbiosis between soybean cultivar and native rhizobia. Inoculation of soybean with bradyrhizobia that show high ability for nitrogen fixation is effective in increasing soybean production in soil that have never been under soybean cultivation. However, when the inoculation is performed in soybean fields, its efficiency is critical due to competition occurring between the introduced rhizobia and the indigenous rhizobia present at high densities. Maximum nodule occupancy by the inoculant strain is achieved using *Rhizobium* strains having more host cultivar specificity, better competitiveness and inoculum size. Competition for nodulation is usually measured by comparing the ability of introduced *Rhizobium* strains to form nodules on the chosen host [8, 24, 34, 35]. Antibiotic resistance has been frequently used in distinguishing the introduced inoculants strain from indigenous rhizobia and monitoring their survival and occupancy of legume nodules [5, 29]. Some of the rhizobial strains exhibit multiple antibiotic resistances [7, 16, 36] but some possess no detectable antibiotic

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markers [7, 28]. In that condition antibiotic markers could be introduced either by isolating spontaneous mutants or by transposition [26, 27].

Various biotic and abiotic factors influence the survival of *Rhizobium* in soil rhizosphere. Rhizobiophages are one of the important biotic factors to influence the survival and competitiveness of rhizobia in soil [3]. The presence of phage in soil may cause selection pressure on sensitive rhizobial strains, and thus their continued co-existence with the sensitive host genotype is interesting [12, 17, 25]. In such selection pressure, phage resistant form of *Rhizobium* may certainly create a better fitness in the rhizosphere. Owing to spontaneous mutation conferring resistance against phage infection in susceptible strains of rhizobia, the knowledge of associated changes in the symbiotic properties of such variants is important. Keeping this view in mind, the present investigation dealt with the competitive abilities of antibiotic and phage-resistant mutant for symbiotic effectiveness under laboratory and field conditions on soybean cv.

Materials and Methods

Rhizobium strains

Rhizobium strains MPSR033 and MPSR220, [15] isolated from soybean plant nodules collected from Bhopal (E 77.24, N 23.16) and Ashok Nagar (E 77.43, N 24.34) district of Madhya Pradesh, India, showing susceptibility with phage RT5 and RT6, respectively and were selected for present study. These strains were maintained routinely on yeast extract mannitol agar (YEMA) [33].

Isolation of Antibiotic Resistant Mutants

Mutant (MPSR033 Sm^r and MPSR220 Gm^r) strains of MPSR033 and MPSR220 were isolated spontaneously against antibiotics streptomycin (200 μg ml^{-1}) and gentamicin (200 μg ml^{-1}), respectively, on YMA plate supplemented with the respective antibiotics by employing double agar layer technique. Ten clones were picked up randomly from antibiotic containing YM agar plate. Growth of individual clones was then compared in the presence and the absence of respective antibiotics. Mutants showing comparable growth were stocked on agar slant for further study.

Isolation of Phage-Resistant Mutants

Antibiotic-resistant mutant (MPSR033 Sm^r and MPSR220 Gm^r) were used for further isolation of mutants, resistant to corresponding phages RT5 and RT6, respectively. Log-phase cells of the antibiotic-resistant mutants were incubated in broth with high phage titer (10^8 pfu ml^{-1}) in the ratio of 1:10 and were plated by double agar layer technique. Plates were incubated in culture room for 8–10 days, when resistant colonies appeared. Colonies were randomly picked up, grown in medium, and purified (freed from phages) by repeated streaking. Isolated clones were tested for resistance to respective antibiotic and phage strains. The isolated phage and antibiotic resistant mutants of both bradyrhizobial strains exhibiting nodulation efficiency similar to their parents were used for testing competitiveness of strains under laboratory condition.

Competition Experiment Under Lab Condition

Soybean plants were grown in earthen pots (two pots; each 9-cm height × 6-cm mouth diameter; upper pot having sterilized sand and gravel (3:1) cotton wicked with nutrient medium, present in lower earthen pot) under ideal growth condition. Thornton's nitrogen-free nutrient medium [33] was applied for proper growth. Seeds of soybean cv. JS 335 were germinated in water agar after surface sterilization. Single seedling was transplanted per pot and placed in a chamber at 28 ± 1 °C under a 16/8 h light/dark regime (S Fig. 1).

Four sets of rhizobial strain combinations were prepared to illustrate competitiveness between (i) two different phage-sensitive (MPSR033 vs. MPSR220) strains; (ii) phage sensitive (MPSR033) versus phage resistant marked with streptomycin-resistant (MPSR033 Sm^r V^r) strains; (iii) phage sensitive (MPSR220) versus phage resistant marked with gentamicin-resistant (MPSR220 Gm^r V^r) strains, and (iv) two different phage- and antibiotic-resistant (MPSR033 Sm^r V^r vs. MPSR220 Gm^r V^r) strains. The cells of two rhizobial strains in each set of combination were mixed in 1:1 proportion and used as inoculant. For preparation of mixed inoculum, the bacterial population of each strain was adjusted to 10^7 viable cells (cfu) ml^{-1} with sterile distilled water and then mixed in required proportions.

Detection of Rhizobial Strain in Nodule

For determining nodule occupancy by individual strains at 40 days after inoculation, nodules were carefully collected from plant root, washed with distilled water, and surface sterilized with 70 % alcohol for 30 s followed by sodium hypochlorite solution (1 % w/v) for 2–3 min. Then the nodules were thoroughly washed with sterile distilled water for 4–5 times. Each nodule was crushed separately with the

help of a sterilized forceps on a glass slide. A loopful of nodule exudates was dropped on selective agar medium [YMA; YMA + Sm (200 μg ml^{-1}); YMA + Gm (200 μg ml^{-1})] with the help of inoculation needle and high titer (10^8 pfu ml^{-1}) of specific phage strain was dropped over, as per clues for ascertaining particular strain are mentioned (Table 1). Plates were incubated in culture room and observation on growth was recorded after 6–8 days.

Field Experimental Trial

A field experiment was conducted at Agricultural Research Farm of Banaras Hindu University, India. Soil of experimental field was sandy loam with pH 7.4, organic carbon—0.51 %, and available N, P_2O_5, and K_2O were 148, 25, and 280 kg/ha, respectively. Two soybean cultivars, JS 335 released from Jawaharlal Nehru Krishi Vishwa Vidyalaya, Jabalpur, Madhya Pradesh, India during 1994, has a maturity period of 95–100 days retaining average yield of 25–30 Q/ha and DS 9712 released from Indian Agricultural Research Institute, New Delhi, India during 2005, has maturity period of 105–115 days with an average yield of 25–27 Q/ha, were taken for field trail with six bradyrhizobial strains. The experimental design was a completely randomized block design (CRBD) having plot size of 4×3 m^2 with four replications. Seeds were grown at 10 cm plant-to-plant distance and row-to-row distance of 30 cm. The seeds were wrapped with sterilized charcoal containing single strain inocula (5.2×10^7–6.8×10^7) with gum arbica as an adhesive. Data pertaining to nodule number, nodule dry weight, acetylene reduction activity (ARA), and plant dry weight were recorded after 45 days of plant growth and seed yield data were recorded at the time of harvest.

Molecular Techniques and Analyses

Genomic DNA of the isolates was extracted using the methods followed by Jaiswal and Dhar [14]. The 16S rRNA gene was amplified through Thermocycler GeneAmp PCR system 2720 (Applied Biosystem, USA) by using 27f, 5′AGAGTTTGATCMTGGCTCAG3′ and 1492r 5′TACGGYTACCTTGTTACGACTT3′ primers (Integrated DNA Technology, USA) according to Laguerre et al. [19]. PCR products of 16S rRNA were purified using PCR purification kits (QIAquick PCR purification kit, QIAGEN, USA) and sequenced using ABI 3010 (Applied Biosystems automated sequence analyzer). Sequences were submitted to the GenBank database (http://www.ncbi.nlm.nih.gov/blast) for significant

Table 1 Growth ascertaining particular strain residing in nodule under mixed inoculation

Rhizobial strains used in mixed inoculum	Growth in plate by nodule occupying strain							
	YMA	YMA + phage RT5	YMA + Sm (200 μg ml^{-1})	YMA + Sm (200 μg ml^{-1}) + phage RT5	YMA + phage RT6	YMA + Gm (200 μg ml^{-1})	YMA + Gm 200 μg ml^{-1}) + phage RT6
MPSR033 (A) and MPSR220 (C)	Heterogeneous growth; larger colonies of C	Only C	NC	NC	Only A	NC	NC
MPSR033 (A) and MPSR033 Sm^r V^r (B)	Nearly homogenous colonies	Only B	Only B	Only B	NC	NC	NC
MPSR220 (C) and MPSR220 Gm^r V^r (D)	Heterogeneous growth; larger colonies of C	NC	NC	NC	Only D	Only D	Only D
MPSR033 Sm^r V^r (B) and MPSR220 Gm^r V^r (D)	Heterogeneous growth; larger colonies of D	NC	Only B	Only B	NC	Only D	Only D

NC no colony

alignments. The phylogenetic analyses were conducted in MEGA4 [32]. The evolutionary history was inferred using the UPGMA method [30] and distances were computed using the maximum composite likelihood method [31]. The percentage of replicate trees in which the associated taxa are clustered together, replicate 1,000 in the bootstrap test [9].

Statistical Analysis

The data were analysed, using standard statistical procedure documented by Gomez and Gomez [10]. Significant effects were found at $P = 0.05$.

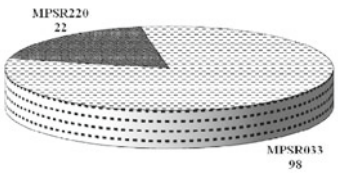
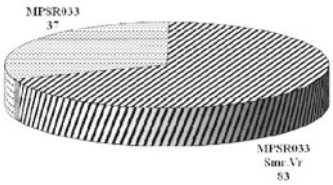
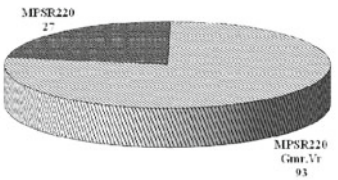
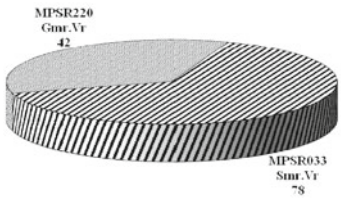
Results

Both strains, MPSR033 and MPSR220 were slow growers retaining generation time 16.2 and 11.0 h and showed susceptibility to different lytic phages, i.e., RT5 and RT6, respectively. They have ability to fix nitrogen *ex-planta.* Strain MPSR220 evidenced superior performance (107.6 nmol C_2H_4 produced h^{-1} mg^{-1} protein) in comparison to MPSR033 (21.7 nmol C_2H_4 produced h^{-1} mg^{-1} protein) for *ex-planta* nitrogenase activity at 24 h of incubation period. Strain MPSR033 Sm^r and MPSR220 Gm^r, producing colony morphology similar to their respective parental strains with susceptibility to respective phage strains, were used for isolation of phage-resistant variants.

All derived mutants of both rhizobial strains were tested for nodulation ability on soybean plants under laboratory condition. The symbiotically effective four bradyrhizobial strains namely MPSR033, MPSR033 Sm^r V^r, MPSR220, and MPSR220 Gm^r V^r were used for determining competitiveness among strains for nodule occupancy under different combinations.

The proportion of nodules formed by individual strains in mixed inoculation is presented in Fig. 1. Use of antibiotics (streptomycin and gentamicin) and phage in the YMA media served as highly sensitive detectable markers for identification of each strain in nodule (Table 1). Number of

Fig. 1 Nodule occupancy by individual strains in mixed inoculation on soybean cv. JS 335 under laboratory condition

Treatment		No. of nodules assayed	Nodule occupancy by the inoculant strains	
Used *Rhizobium* strains	Ratio in mixture		Number of nodules	Percentage
MPSR033 and MPSR220	1:1	120		81.7 / 18.3**
MPSR033 and MPSR033 Sm^r V^r	1:1	120		30.8 / 69.2**
MPSR220 and MPSR220 Gm^r V^r	1:1	120		22.5 / 77.5**
MPSR033 Sm^r V^r and MPSR220 Gm^r V^r	1:1	120		65.0 / 35.0**

nodule exudates showing growth on antibiotic-containing plates or lysis with respective phage strain formed the basis for determining nodule occupancy (Fig. 2).

A significant difference among used strains was observed for nodule occupancy (Fig. 1). In the first set of rhizobial strain combination, the parental strain MPSR033 dominated over MPSR220 and formed about 82 % total nodules. In second and third sets of strain combination, phage-resistant (MPSR033 Sm^r V^r and MPSR220 Gm^r V^r) strains were found superior over their corresponding parent strains with respect to nodulation. Strain MPSR033 Sm^r V^r occupied ~70 % nodule whereas ~78 % nodule occupied by MPSR220 Gm^r V^r when compare to their parents. The fourth set revealed that MPSR033 Sm^r V^r produced maximum number of nodules among both the phage-resistant strains.

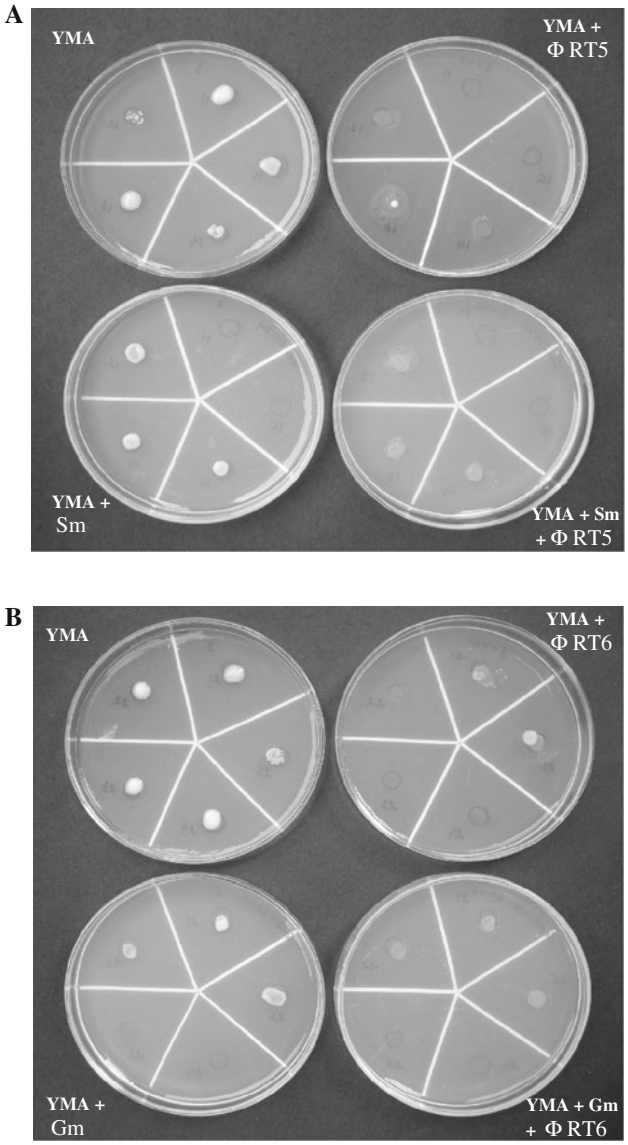


Fig. 2 Growth observed on respective agar nutrient plates from nodule in mixed strain treatment **a** MPSR033 × MPSR033 Sm^r V^r and **b** MPSR220 × MPSR220 Gm^r V^r

On the basis of results obtained in controlled conditions, strains MPSR033, MPSR033 Sm^r, MPSR033 Sm^r V^r, MPSR220, and two exotic strains USDA123 and USDA94 were used to study their symbiotic interaction with two soybean cv. JS335 and DS 9712 under field conditions. All strains showed nodulation on the tested soybean cultivars. However, host cultivars, strain treatments and their interaction were found to have significant effect on nodulation, nitrogenase activity, and seed yield. Both the soybean cultivars differed in symbiotic efficiency with used rhizobial strains, proving superiority of cultivar JS335 over the DS 9712 for all recorded symbiotic parameters. Among all rhizobial treatments, strain MPSR033 supported significantly higher nodule number (52.7) than USDA 123 with cultivar JS335. With cultivar DS 9712, maximum nodulation was observed with strain MPSR033 Sm^r (41.75), nearly equal to USDA 123 inoculant. The same was observed in the lowest i.e., under un-inoculated condition (Fig. 3a). Highest nodule dry weight was noticed with strain MPSR033 along with its mutants in cultivar JS335 and was significantly higher than both exotic strains (Fig. 3b). Both soybean cultivars expressed maximum nitrogenase activity with strain MPSR033 Sm^r V^r. On the basis of expressed ARA per plant, the comparative symbiotic efficacy of the strain is followed as MPSR033 Sm^r V^r, MPSR033 Sm^r, MPSR220, MPSR033, USDA123, and USDA 94 (Fig. 3c). Strain MPSR033 Sm^r V^r was also superior among all selected rhizobial strains in seed yield production with both the cultivars. In cultivar JS335, both streptomycin-and phage-resistant mutant strains (MPSR033 Sm^r V^r and MPSR033 Sm^r) supported significant increase in grain yield over all other inoculants. In DS 9712, highest seed yield (3.07 kg/plot) was also recorded with phage-resistant mutant and was at par with USDA123 (3.0 kg/plot) inoculant. Overall increase by strain MPSR033 Sm^r V^r was 8.7 and 21.0 % more than exotic strain (USDA123) and un-inoculated control (Fig. 3e). The results further clarified that interaction of strain MPSR033 Sm^r V^r × JS 335 and MPSR033 Sm^r V^r × DS 9712 supported highest ARA, total plant dry weight and seed yield per plot.

Phylogenetic relation of the strain MPSR033 and MPSR220 was examined on the basis of 16S rRNA sequence analysis. The tree of 16S rRNA gene phylogeny was built to infer the evolutionary relationship of both strains (Fig. 4). Result of sequence analysis showed 99 % similarity value in pairwise comparison with selected reference strain's sequence of *Bradyrhizobium*. The 16S rRNA sequences of both strains were deposited in NCBI GenBank for accession number which provided JF746896 and JF746897 for MPSR033 and MPSR220, respectively.

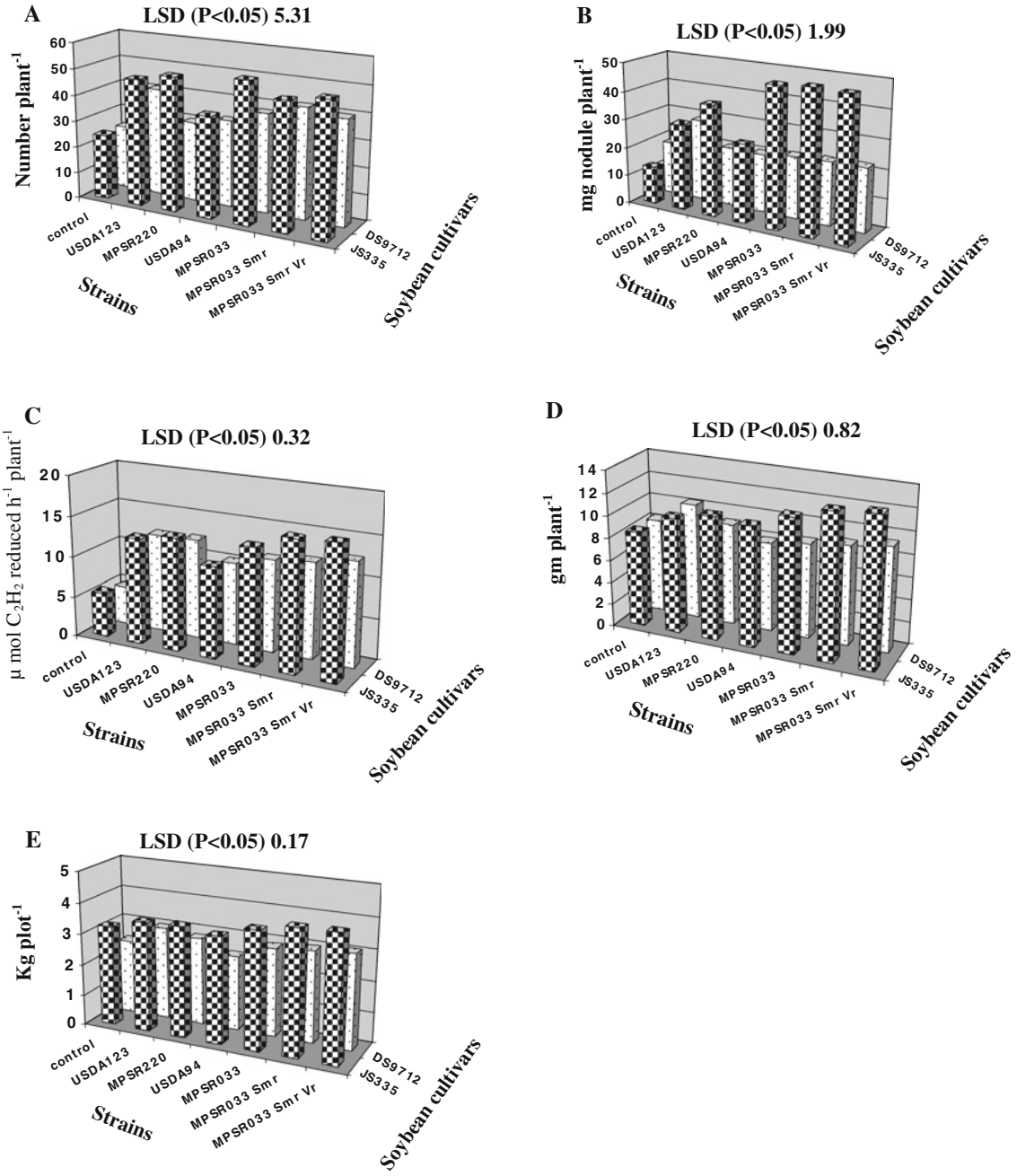


Fig. 3 Soybean cultivar *Rhizobium* interaction: **a** nodule number/plant; **b** nodule dry weight (mg); **c** nitrogenase activity; **d** plant dry weight (g) and **e** seed yield kg/plot

Discussion

All selected *Bradyrhizobium* strains have ability to form nodules with the host plant which is very necessary to study the competition for nodulation [8]. The obtained result in present investigation is congruent to the results of Mishra et al. [23] showing nodulation superiority of phage-resistant mutant of pigeonpea-*Rhizobium* strain IHP 195 over its parent strain for nodule number, nodule fresh weight, and nitrogenase activity on alternate hosts such as cowpea, soybean, and sirato but anti-parallel to the result of Kowalski et al. [18] that symbiotic properties of phage-resistant mutants R43 and 73c2 of *Sinorhizobium meliloti* on *Medicago sativa* and found no significant difference in nodulation and dry matter production with inoculated strains sensitive or resistant to phage. Buendia-Claveria and Ruiz-Sainz [6] also reported comparable symbiotic efficiency of rifampicin-resistant variant (RCR 3407-1) to its slow growing parental *R. japonicum* strain RCR 3407 on soybean. Gupta and Ramkrishna [11] found increased

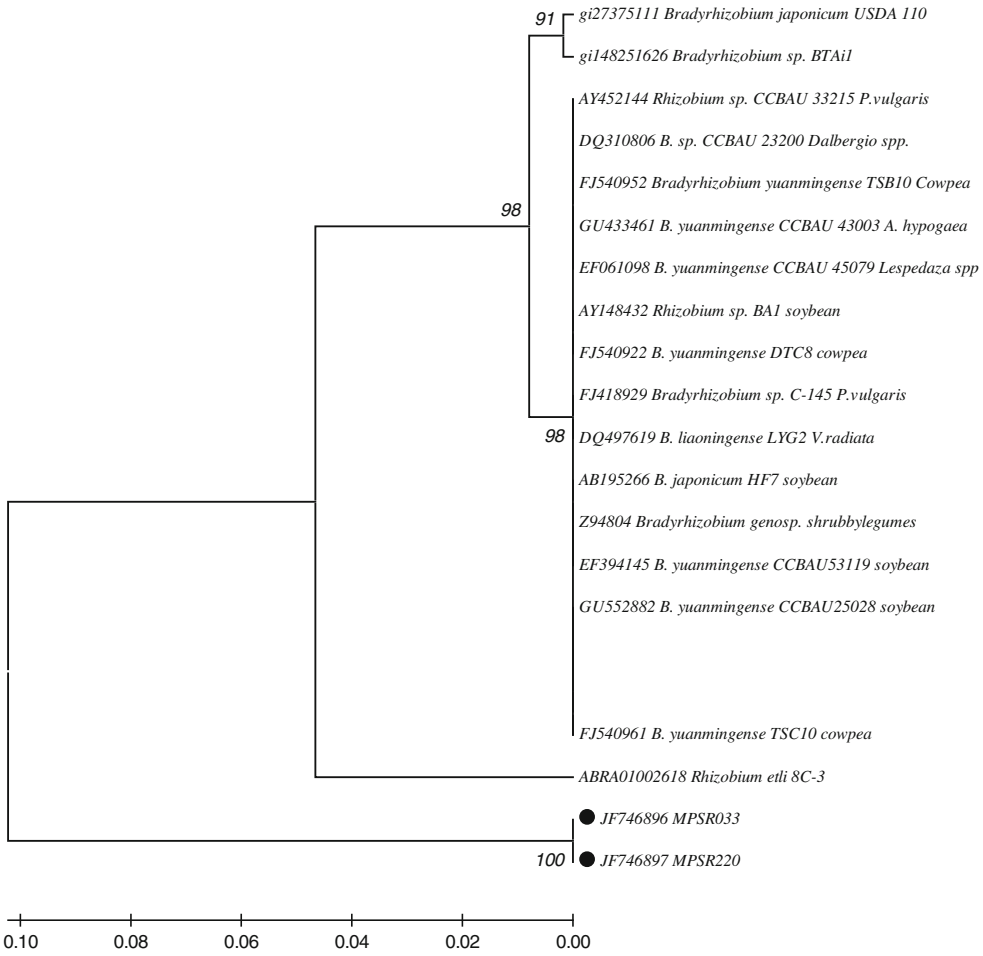


Fig. 4 Phylogenetic tree of 16S rRNA sequences of soybean *Bradyrhizobium*

nitrogen content per plant in *Vigna aconitifolia* inoculated with six different phage-resistant mutants as compared to its parental strain MR-41. Obtained results are not consistent with those reported earlier that virulent phages play a role in increasing the population of ineffective strains in rhizosphere [3, 17]. In present observation, strain MPSR033 Sm^r was symbiotically more effective to both soybean cv. in comparison with its parental strain which is contrary to the results of Bogino et al. [4] and Lochner et al. [20] that symbiotic properties were same for the parent and its antibiotic-resistant mutant strains, i.e., induction of antibiotic resistance had no effect on symbiosis. Our results indicate higher competitiveness of the phage-resistant mutants over their parental strains in soybean which is in contrast to the findings of Mishra et al. [22] who reported almost 100 % nodulation by phage-sensitive pigeonpea rhizobial strain when used in mixed inoculant on pigeonpea. May and Bohlool [21] also observed that most effective strains of *R. leguminosarum* varied greatly in competitiveness in mixed inoculant.

In the present result each individual strains performed well in field condition whereas in mixed inoculation only one strain displayed good symbiotic performance in comparison to others under controlled condition which may predict the possibility of contact-dependent toxin delivery system [1] in used strains. In this system, bacteria develop mechanisms to communicate and compete with one another [13]. This new form of contact-dependent inhibition (CDI) systems have been studied in *E. coli* [2] and open the way to investigate into the CDI system operative in *Rhizobium* too.

Symbiotic nodulation functions are encoded partly by accessory genes found in almost all rhizobia ("common nodulation genes") and partly by genes encoding host specificity. Thus, genes important for high-nitrogen fixation or responsible for competitiveness have not yet been properly pinpointed; they obviously vary depending on rhizobia, hosts, and the environment. This study on interaction of antibiotic marked phage-resistant bradyrhizobial strains with soybean widens out an idea on symbiotic effectiveness. Further, nodulation capacity of the rhizobial strains resistant to lytic phages is likely to be overlooked in field experiments but has great significance in ecological studies.

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