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Differential Symbiotic Response of Phage-typed Strains of *Bradyrhizobium japonicum* with Soybean Cultivars

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In this study, native *Bradyrhizobium* strains were isolated from the host plant, *Glycine max*, harvested from fields in Madhya Pradesh, India, and were typed by lytic rhizobiophages. Eight indigenous (Soy2, ASR011, ASR031, ASR032, MSR091, ISR050, ISR076 and ISR078) and two exotic strains (USDA123 and CB1809), all of which evidenced a distinct reaction with six phages, were employed in this study. The symbiotic interaction of these strains was studied initially using soybean cultivar JS335 in a sand culture in a controlled environment, and the efficiency was assessed based on the nodule number, nodule dry weight, plant dry weight, nitrogenase activity, and total accumulation of N per plant. Symbiotic effectiveness was found to be highest with the native phage-sensitive isolate ASR011, whereas it was at a minimum with the phage-resistant isolates, ISR050 and ISR078. Additionally, the effectiveness of these strains was evaluated using six soybean cultivars belonging to different maturity groups; namely, Bragg, Lee, Pusa20, PK416, JS335 and NRC37. Analysis of variance data evidenced significant differences due to both symbionts, for the majority of the tested parameters. The CB1809, USDA123, and ASR011 strains evidenced relatively superior symbiotic effectiveness with soybean cultivars Bragg, Lee and JS335. Strain ISR078 evidenced no significant responses with any of the cultivars. The ASR031 strain performed moderately well with all tested cultivars. The symbiotic response of all the strains was quite poor with cultivar PK416. Our studies showed that a significant relationship existed between the phage sensitivity and symbiotic efficiency of the bacterial strains with the host-cultivars.

Keywords: *B. japonicum*, cultivars interaction, phage types, soybean

Soybean has become the third major oilseed crop in India, as the result of a phenomenal increase in both its area and production over the previous three decades. Thus, the plant plays an important role in the diet of the population, partially as the consequence of its relatively high oil (18-20%) and protein (40-42%) contents. This crop is grown primarily under rainfed conditions. Legumes such as faba beans, peas, and cowpeas derive as much as 80-90% of their N requirement via N_2 fixation (Sims *et al.*, 1983) as compared to only 50% for the soybean (Pate and Atkins, 1983; Hardarson *et al.*, 1984). Native populations of *Bradyrhizobium japonicum* existing in soil vary with regard to their competitive ability to colonize soybean nodulation sites, thus effecting symbiotic N_2-fixation efficiency (Singleton and Travares, 1986). Native bacterial populations may, in some instances, nodulate plants, but cannot be relied upon to provide adequate BNF levels for commercial soybean production. Selections of host cultivar-compatible bacterial strains have been recognized as an important method for increasing N_2-fixation in the soybean (Hunt *et al.*, 1985; Israel *et al.*, 1986). Therefore, the success of this crop in the country relies on its efficient symbiosis with N_2-fixing bacteria. Differences in N_2-fixation among legume genotypes with different bacterial strains have been documented extensively (Date and Roughley, 1977). In the soybean, qualitatively inherited host plant genes have been identified to modulate nodulation response with the microsymbiont (Palmer and Kilen, 1987; Zhang and Smith, 2002). Bacteriophages that parasitize *Rhizobium* species strains are frequently detected in the rhizosphere soils of fields in which legumes are

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grown (Dhar *et al.*, 1979; Dhar *et al.*, 1980; Dhar and Ramkrishna, 1987; Dhar *et al.*, 1993; Dhar and Kumar, 1998), and perform an important role in determining the survival and fitness of rhizobial strains in soil. The phage-resistant mutants derived from phage susceptible strains are associated to lose their symbiotic effectiveness completely or partially (Kleczkowska, 1971; Patel, 1978; Barnet, 1979). However, insufficient information is currently available regarding the symbiotic performance of phage-susceptible / phage-resistant native rhizobial strains, as well as their interaction with the host cultivars. The primary objective of the current study was to evaluate the symbiotic effectiveness of indigenous and standard *B. japonicum* strains exhibiting distinct phage reactions with soybean cultivars, in different maturity groups under axenic conditions.

Ten *Bradyrhizobium japonicum* strains, including eight native (Soy2, ASR011, ASR031, ASR032, MSR091, ISR050, ISR076 and ISR078) and two standard (USDA123 and CB1809) strains, were used in this study. Data regarding the comparative growth properties and phage-reactions of all of these strains are given in Table 1. These strains evidenced marked differences in sensitivity with the six lytic rhizobiophages available in our laboratory; four strains, ASR011, ASR031, ASR032 and ISR076, were sensitive to phages SR3, SR4, SR5 and SR6, respectively; two strains, USDA123 and MSR091, were susceptible to phage SR1; whereas another two strains, CB1809 and Soy2, were found to be sensitive to phage SR2. Strains ISR050 and ISR078 were determined to be wholly resistant to all six phages. All bacterial strains were grown and maintained in Yeast Mannitol (YM) medium (Vincent, 1970) at 28 ± 1°C, in an incubation chamber.

Six soybean cultivars belonging to two broad maturity groups, namely PK416, Lee, Bragg and Pusa 20 (110-120 days) and NRC37, JS335 (90-100 days) were selected for use in this study. Genetically pure seeds from cultivars PK416, NRC37, and JS335 were acquired from the National Research Laboratory for Soybeans, in Indore, MP, India, whereas the Bragg, Lee and Pusa 20 cultivars were procured from the Pulses Research Laboratory, IARI, New Delhi, India.

The symbiotic effectiveness of the phage-typed *B. japonicum* strains was determined in plastic pots (13 cm ht × 10 cm mouth diam. × 6 cm base diam.) containing sterile sand and gravel (3:1). Thornton's nitrogen-free nutrient medium (Vincent, 1970) was applied for proper growth. The seeds were germinated on sterilized water agar (1%) plates, and transplanted into pots (single seed per pot). Each of the pots received 5 ml of approximately 10^7 cells/ml from 6-day old bradyrhizobial cultures. After the emergence of plantlets, the surfaces of each of the pots were covered with sterile aluminum foil, which functions as a dry barrier. The plants were grown under 14/10 h day/night conditions at 28°C in a plant growth chamber. The experiment was laid out in a randomized block design, with five replications. Uninoculated plants were used as controls.

After 45 days of plant growth, the tops of the plants were excised above the cotyledonary node, and dried for 2 days at 60°C. The detached root systems were employed for the measurements of C_2H_2 reduction (ARA), and expressed as micromoles of C_2H_4 plant^{-1} h^{-1} in accordance with the previously

Table 1. Phage sensitivity and growth characteristics of *Bradyrhizobium japonicum* strains

Strain	Sensitive to phage (strain)	Generation time (h)	Colony morphology on YMA (10 days)	
			Diam. (mm)	Characteristics
USDA123	S (SR1)	9.6	2.5	White, dome shaped
MSR091	S (SR1)	10.4	1.0	White, opaque, gummy
CB1809	S (SR2)	11.0	1.5	Milky, gummy, raised
SOY2	S (SR2)	15.3	1.0	White, gummy, raised
ASR011	S (SR3)	13.0	0.7	White, gummy
ASR031	S (SR4)	7.8	3.0	Dull, mucilaginous
ASR032	S (SR5)	10.1	2.5	Milky, gummy, compact
ISR076	S (SR6)	16.2	0.5	White, opaque, raised
ISR050	R	6.8	3.5	Dull, mucilaginous
ISR078	R	18.6	0.5	White, opaque, raised

Abbreviations: S, Susceptible to specific phage strain; R, Resistant to all the six phages.

described procedure (Somasegaran and Hoben, 1994). The nodules were counted after the C_2H_2 reduction assay, and then dried for 2 days in order to determine the nodule dry weights. After the estimations of total plant dry matter, including the shoot and root systems of each plant, the N contents of the plants were determined via the micro-kjeldahl digestion method (Somasegaran and Hoben, 1994).

The data was analyzed using a randomized block design. From analyses of variance, a least significant difference was utilized to make comparisons among the means at a level of significance of P (0.01).

All native soybean root nodule isolates were assigned to *B. japonicum* species on the basis of their Intergenic sequence restriction fragment length polymorphisms (data not shown). Differences in the symbiotic performance of ten phage-specific *B. japonicum* strains on soybean cv. JS335 grown under axenic conditions were observed (Table 2). All strains were shown to form nodules on the tested cultivars. However, a significant variation was noticed in the nodule numbers, nodule dry weights, C_2H_2 reduction activities (ARA), total plant dry weights, and N accumulation of the plants. The two rhizobial strains (USDA123 and MSR091) that were sensitive to phage SR1, and another two strains (CB1809 and Soy2) that were sensitive to phage SR2, exhibited similar trends in symbiotic effectiveness on cultivar JS335. The strains of the two phage groups evidenced different symbiotic response levels, but these values were not significant. The USA strain (USDA123) evidenced a significantly higher nodule dry weight (46.6 mg) than did the indigenous strain (MSR091), but the plant dry weight and total N accumulation values were lower in the USDA123 strain than in MSR091. The indigenous strains, ISR050 and ISR078, both of which were resistant to all six phages, evidenced inefficient symbiotic N_2-fixation activities, as compared to the phage-sensitive strains. The symbiotic traits of the phage-resistant mutants in culture from strains of *R. trifolii, R. leguminosarum,* and slow-growing lotus rhizobia, evidenced stable and inefficient N_2-fixation with their respective host plants (Kleczkowska, 1971; Patel, 1978). Significant differences were noted in the ARA values and total N contents per plant among the phage-sensitive strains. The phage-susceptible strains, ASR011 and ASR031, exhibited significant increases in symbiotic effectiveness for parameters including nodule dry weight, plant dry weight, N_2-ase activity, and N accumulation per plant, whereas the ASR032 and ISR076 strains evidenced superior performance with regard to N_2-ase activity and plant dry matter production. Nodule N_2-ase activity has previously been identified as a reliable marker for strain effectiveness in legume crops in general (Somasegaran and Hoben, 1994). In the soybean, significant differences between rhizobial strains for parameters including nodule number, dry weight and ARA activity, total dry weight, and total N accumulation, have been reported under growth room, green house and

Table 2. Symbiotic performance of *B. japonicum* strains on soybean cultivar JS335

Rhizobial strain	Nodule number plant^{-1}	Nodule dry weight (mg) plant^{-1}	Nitrogenase activity[a]	Total dry weight (g) plant^{-1}	N content (mg) plant^{-1}
USDA123	55.4	46.6*	6.89	1.78	22.2
MSR091	53.0	35.9	6.85	1.82	22.1
CB1809	53.4	34.2	6.22	1.80	22.6
SOY2	51.2	35.9	6.32	1.82	21.4
ASR011	82.3*	60.6*	14.88*	2.58*	48.8*
ASR031	58.4	51.3*	11.32*	2.34*	35.4*
ASR032	41.3	31.2	7.06*	2.12*	20.1
ISR076	61.3*	37.5	8.54*	2.01*	28.3
ISR050	26.4	18.7	3.24	1.35	10.8
ISR078	30.2	17.9	3.69	1.29	10.5
Uninoculated Control	0	0	0	1.21	9.3
CD (at 1%)	7.9	9.6	1.52	0.11	8.0

[a] Values are given in μ mol C_2H_2 reduced hr^{-1} plant^{-1}; CD- Critical Difference
Numbers are means of five replicates * Significant value at $P< 0.01$ level

phytotron conditions (Kucey *et al.*, 1988).

Interaction studies of these strains with the six cultivars belonging to medium and long maturity groups revealed significant differences (at $P < 0.01$) in nodule number, acetylene-reducing activity, and dry matter production (Table 3). A significant difference in symbiotic parameters was also noted between the soybean cultivars and bacterial strains. A highly-specific nodulation was detected in some cultivars, as was a great degree of variability in their ability to engage in effective symbiotic relations with cultivars. Although the highest nodule dry weight (166.5 mg) was recorded with cultivar JS335 in association with strain ASR011, the maximum N_2-ase activity (38.79 μg) and total plant dry weight (3.71 g) were measured in the Lee cultivar, in association with the USDA123 strain. The ISR078 strain evidenced a minimum nodule dry weight on the Lee cultivar, whereas N_2-ase activity

Table 3. Symbiotic interaction of strains of *B. japonicum* with selected soybean cultivars

Rhizobial strains	Soybean cultivars					
	Bragg	Lee	JS335	Pusa20	NRC37	PK416
(a) Nodule dry weight (mg) plant^{-1}						
USDA123	32.2	141.2^{ab}	33.8	28.3	18.7	20.7
CB1809	150.6^{ab}	29.9	26.1	36.3	25.7	22.2
ASR011	30.1	28.9	166.5^{ab}	33.3	22.5	21.5
ASR031	52.0	61.2^{a}	72.6^{b}	46.3	64.4^{a}	31.6
ISR076	34.5	35.2	40.8	36.5	20.8	24.1
ISR078	19.9	3.2	15.5	13.3	6.8	6.2
	Rhizobium strain (R)		Cultivar (C)		Interaction (R × C)	
CD (1%)	18.78		17.44		37.13	
(b) Nitrogenase activity (μ mol C_2H_2 reduced h^{-1} g^{-1} fresh nodule)						
USDA123	6.49	38.79^{ab}	6.92	7.16	3.30	4.12
CB1809	30.51^{ab}	6.66	6.90	6.80	4.83	3.05
ASR011	6.77	8.09	34.08^{ab}	8.51	5.17	5.67
ASR031	9.54	14.67^{ab}	13.26^{ab}	11.15^{ab}	5.38	4.34
ISR076	8.45	10.95^{b}	9.17^{b}	8.77^{a}	5.47	3.20
ISR078	1.75	2.95	3.08	2.64	2.23	1.16
	Rhizobium strain (R)		Cultivar (C)		Interaction (R × C)	
CD (1%)	1.47		1.40		2.90	
(c) Total dry weight (g) plant^{-1}						
USDA123	1.78^{ab}	3.71^{ab}	1.79	1.69	1.64	1.01
CB1809	3.08	1.86	1.71	1.61	1.59	1.08
ASR011	1.78	1.99	3.38^{ab}	1.95^{a}	1.95^{a}	0.97
ASR031	2.13^{a}	2.69^{ab}	2.35^{ab}	2.43^{a}	1.97^{a}	1.29^{a}
ISR076	1.88	2.36^{ab}	2.17	1.79	1.74^{a}	1.36
ISR078	1.03	0.97	1.03	1.09	0.99	0.97
	Rhizobium strain (R)		Cultivar (C)		Interaction (R × C)	
CD (1%)	0.085		0.079		0.172	

Significant value at $P < 0.01$ for ^{a}strain and ^{b}cultivar. Numbers are means of five replicates; CD; Critical Difference and total plant dry weight were lowest with cultivar PK416. The CB1809 strain exhibited its highest level of symbiotic performance for nodule dry weight and total plant dry matter content, in association with the Bragg cultivar. The total plant dry mass increased by 169, 168 and 192.8% with cultivars Bragg, JS335, and Lee, respectively. This may be attributable to the inoculation of these cultivars with their most compatible rhizobial strains. The influence of the host plant on symbiotic effectiveness, nodulation and N_2-fixation has previously been rather well documented (Caldwell and Vest, 1977), and strong cultivar-strain interactions have also been reported in the soybean (Israel, 1981). Strain ASR031 increased total plant dry weight by approximately 98% in all cultivars except for PK416 (only 9.7%). The phage-resistant strain ISR078 evidenced poor symbiotic efficiency with all six of the tested cultivars. The symbiotic performance of phage-resistant mutants developed from the phage-sensitive strains of *Rhizobium* species evidenced inefficient N_2-fixation with their respective host plants (Kleczkowska, 1971; Patel, 1978; Barnet, 1979). However, the exact molecular mechanism by which the phage sensitivity can be related to symbiotic effectiveness remains to be clearly elucidated. No significant variations in symbiotic effectiveness were observed among cultivars belonging to different maturity groups; however, long-duration cultivars evidenced performance superior to that of medium-duration cultivars at 45 days after planting.

To our knowledge, this study is the first report to compare phage-sensitive and phage-resistant strains belonging only to *B. japonicum* species, with regard to their symbiotic interactions with soybean cultivars from different maturity periods. It is clear, from the results of the present work, that the soybean and *Bradyrhizobium* genotype profoundly affects several aspects of symbiotic performance in controlled environments. Considerable host-cultivar specificity was detected among the phage-typed strains of *B. japonicum*. Strains CB1809, ASR011, and USDA123 evidenced the highest effectiveness in the following parameters: nodule dry weight, N_2-ase activity, and total plant dry weight, with the Bragg, JS335, and Lee cultivars, respectively. Similar results have been observed regarding symbiotic compatibility between native rhizobia from northern Thailand and soybean cultivars under laboratory conditions (Shutsrirung *et al.*, 2002). Further, native bradyrhizobial strains are symbiotically more effective on Indian soybean cultivars than are strains of foreign origin. Our study also indicated the existence of a relationship between the phage sensitivity of strains and selective host-cultivars with regard to symbiotic effectiveness. However, a variety of environmental and soil-related factors may also exert some influence on the efficiency of the *Rhizobium*-legume symbiosis, particularly under field conditions.

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