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## Existence and Characteristics of Rhizobiophages in Soybean Grown Fields in India

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**Abstract:** Rhizobiophages infective against *Bradyrhizobium* strains nodulating soybeans were detected in soils of soybean grown fields. Phages infective on two *Bradyrhizobium japonicum* strains and eight indigenous bradyrhizobia strains were isolated. Distributions of phages in rhizosphere soil were different on indicator strain. The field with soybean after wheat or gram or sorghum contained phages infective on more strains than soybean monocropping fields. Phages were also found in nodule exudates and maximum titre was  $4.5 \times 10^4$  particles/g nodule. Host range, lytic activity, plaque characteristics of four phage strains was studied. Competitive ability and symbiotic effectiveness test revealed that ASR011 strain was most compatible and competitive to soybean cv JS335. Phages can be used for an easy test of nodule occupancy by a particular phage susceptible strain at under laboratory and field conditions.

**Key words:** Rhizobiophage, plaque characteristics, soybean, *Bradyrhizobium* strains

### INTRODUCTION

Soybean (*Glycine max* L. Merrill) is an emerging momentum in India for its high oil (18-20%) and protein content (40-42%) in the seeds. The crop has the capacity to derive a considerable proportion of their nitrogen requirements from the atmosphere through symbiosis with *Bradyrhizobium* sp. and *Sinorhizobium* sp. (Satos *et al.*, 1999). The common presence of rhizobiophages in soil suggests that they can affect the out come of *Rhizobium*-legume symbiosis. However, presence of rhizobiophages in bacterial inoculum mixture did not affect the symbiotic efficiency (Kowalski *et al.*, 2004). Rhizobiophages were isolated from soils of many countries and are usually associated with legume plantation (Amarger, 2001). Existence of phages in soil suggests that they could play an important role in selective propagation or elimination of bacterial strains and can influence the evolution of bacterial populations (Vincent, 1977). The objective of the study was to isolate and characterize phages infective on *Bradyrhizobium* strains from root nodules of soybean and their distribution in soils of different soybean grown fields. We also report the competitive nodulation of cultivar-specific phage susceptible strain.

### MATERIALS AND METHODS

**Bacterial strains:** Rhizobia were isolated from root nodules of field grown soybean plants at Bhopal, Bina,

Ujjain and Indore, Madhya Pradesh, India. Host plant was inoculated with single cell clones of these isolates under aseptic conditions; clones were reisolated and maintained on yeast extract mannitol (YEM) slant (Vincent, 1970). In addition to these strains USDA31, 94, 110, 122, 123 and CB1809 were utilized as test strains for plaque assay.

**Rhizobiophages:** Phages were isolated from nodules and rhizospheric soil of various fields at four locations. Phages were isolated as single plaques as described (Dhar *et al.*, 1978), using double agar layer plates. Phage isolates were purified by three successive isolations of single plaque. High titre phage stocks were prepared using the confluent lysis method (Adams, 1959). Extracted phages were filtered through 0.22  $\mu$ m pore size membrane filters and stored in YEM broth at 4°C. The occurrence of phages in the fields was studied on indicator strains (Dhar *et al.*, 1993) and phage titre was expressed as plaque forming units (Pfu) g<sup>-1</sup> nodule or dry soil. The intact nodules were collected randomly from individual plants and surface sterilized (Vincent, 1970). Weighted (0.5 g) nodules were ground in tissue-homogenizer with 10 mL YEM broth, treated with 0.5 mL chloroform (1%), centrifuged (at 10,000×g for 10 min) and the supernatants were assayed for plaque formation.

**Distribution of phages in soil:** Frequency of distribution of phages in soil and nodules for soybean grown fields with different cropping systems was studied at four

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Table 1: Some properties of soils sampled from soybean grown fields

Location site	Soil type	Previous crop in the field	pH	CEC (C mol kg <sup>-1</sup> )	Organic C (%)	Sand (%)	Silt (%)	Clay (%)
Gorasonera, (Guna)	Clay	Soybean	7.4	51.2	0.58	14.2	32.8	53.0
Mendora, (Bhopal)	Sandy clay loam	Wheat	6.4	24.3	1.02	51.5	14.9	33.6
Ujjain, (Ujjain)	Sandy loam	Sorghum	6.6	11.3	1.73	76.5	7.2	16.3
Jindakheri, (Indore)	Clay	Gram	7.5	50.2	0.78	16.9	30.1	53.0

locations. Soil samples were analyzed for their physical and chemical properties (Jackson, 1976) and listed in Table 1. Sampling was done at late flowering stage of the plants. Ten random plants were uprooted from each field. Nodules and soil samples from root zone were ground separately and assayed for plaques on indicator strains. Phage host range was performed as described previously (Appunu and Dhar, 2004).

**Competitive ability and symbiotic effectiveness:** Surface sterilized bold and healthy 'JS335' soybean seeds were coated with *Bradyrhizobium* strains (Vincent, 1970). For competitive nodule occupancy test, equal sizes of populations of both rhizobial strains were mixed before seed treatment. Treated seeds were grown in earthen pots containing sterilized soil. Plant and polyhouse conditions were maintained as explained previously (Appunu and Dhar, 2006a). Plants were harvested after 5 weeks of sowing and data pertaining to symbiotic and vegetative characters were recorded as described (Appunu *et al.*, 2005). Nodule occupancy by rhizobial strains was tested by spot test as explained (Appunu and Dhar, 2004).

Data was analyzed by using completely randomized design. From analysis of variance LSD was used to make comparisons among the means at p (0.05) level of significance.

## RESULTS AND DISCUSSION

Soybean nodulating *Bradyrhizobium* strains, nearly thirty were tested for phage susceptibility to supernatants of soil suspensions. Plaques were appeared on ten strains, viz., ASR004, ASR005, ASR011, ASR031, ASR032, ISR076, MSR091, USR258 (indigenous strains), CB1809 and USDA123. On the basis of plaque morphology and host specificity, four phages were isolated and designated them as SR1, SR2, SR3 and SR5. Phages were frequently detected on four strains USDA123, CB1809, ASR011 and ISR076. These type strains were used to determine existence and distribution of phages in soils of soybean grown fields (Table 2). Phages infective on strains USDA123, CB1809, ASR011 and ISR076 were present in 2, 2, 4 and 3 soybean planted regions, respectively. In Guna region phages were found only infective against the strain ASR011 which indicates the predominance of this

Table 2: Existence of rhizobiophages in soils of soybean grown fields

Place	<i>Bradyrhizobium</i> strain (Phage population Pfu g <sup>-1</sup> soil)			
	USDA123	CB1809	ASR011	ISR076
Guna	-	-	1800	-
Bhopal	50	395	150	700
Ujjain	-	-	20	60
Indore	280	110	40	2700

particular strain in the region. Occurrence of phages active against soybean *Bradyrhizobium japonicum* strains have been reported in India (Appunu and Dhar, 2004) and other countries (Kowalski *et al.*, 1974; Hashem and Angle, 1988; Ali *et al.*, 1998; Hashem and Angle, 1990). Phages were detected in all the 4 places sampled, but phage titre varied considerably on the indicator strains suggesting that the composition of phage population changed between locations. This could be due to the effect of several biotic and abiotic factors. A significant correlation of rhizobiophage population with both soil water potential and soil type have been reported in case of *Rhizobium leguminosarum* bv. *trifolii* (Lawson *et al.*, 1987).

Distribution of phages in nodules and soil has been reported in soybean grown fields with different previous crops (Table 3). Usually, both nodules and soils from the same location contained phages against the same indicator strain. Considerable differences in titre and host range of the phages were observed from each field. The field with soybean after wheat or gram or sorghum contained phages infective against more strains than the fields with soybean after soybean. For individual sample the titre of phage in nodule and soil ranged from 0 to 4.5×10<sup>4</sup> Pfu g<sup>-1</sup> nodule and 0 to 2×10<sup>3</sup> Pfu g<sup>-1</sup> soil, respectively. The nodular occupancy of rhizobiophages, co-existence of sensitive and resistant forms of rhizobia in one nodule (Dhar *et al.*, 1993; Kowalski *et al.*, 1974) and susceptible *Rhizobium* strains protected against lytic action of phages (Barnet, 1979) have been reported.

The lytic activity and plaque characteristics of four phages were studied under ideal conditions. Cross infectivity test revealed that all phage strains were highly specific to their host strain. Plaques appeared on strain USDA123 within 36 h whereas on the strains after 2-4 days of incubation. All four phages exhibited high phage-titre ranging from 7.8×10<sup>8</sup> to 1.4×10<sup>10</sup> Pfu mL<sup>-1</sup> (Table 4).

Table 3: Distribution of phages in soybean Root Nodules (RN) and in the rhizosphere soil (RS) with different cropping systems

Place	Previous crop	Indicator <i>Bradyrhizobium</i> strain (Phage population Pfu g <sup>-1</sup> )							
		USDA123		CB1809		ASR011		ISR076	
		RN	RS	RN	RS	RN	RS	RN	RS
Guna	Soybean	-	-	-	-	7,800	170	-	-
Bhopal	Wheat	45,000	1,450	17,600	260	22,000	1,750	33,800	2,850
Ujjain	Sorghum	2,200	130	13,100	380	8,200	430	-	-
Indore	Gram	-	-	-	-	4,300	20	2,000	20

Table 4: Plaque characteristics of rhizobiophages on indicator strain

Phage strain	Host strain	Phage titre in broth* (Pfu mL <sup>-1</sup> )	Phage characteristics		
			Type	Central zone	Size (mm <sup>2</sup> )
SR1	USDA123	7.8×10 <sup>8</sup>	C	-	3.1
SR2	CB1809	5.7×10 <sup>9</sup>	C	-	2.2
SR3	ASR011	5.6×10 <sup>9</sup>	T	*	5.3
SR5	ISR076	1.4×10 <sup>10</sup>	T	+	19.3

Pfu-Plaque forming unit; C-Clear plaque with sharp margin; T-Turbid plaque with hazy margin; Plaque center halo (-); turbid (+), concentric ring (\*)

Table 5: Competitive ability on nodulation and symbiotic effectiveness of phage-marked *Bradyrhizobium* strains with soybean cultivar JS335

<i>Bradyrhizobium</i> strain	Nodule occupancy (%)	Total No. of nodules	Dry wt. of nodules (g)	N <sub>2</sub> -ase activity of nodules*	Total plant dry wt. (g)	N content of plant (%)
USDA123	100	20.19±2.13 <sup>c</sup>	0.10±0.02 <sup>c</sup>	5.01±0.41 <sup>c</sup>	4.95±0.17 <sup>c</sup>	1.7
ASR011	100	51.54±1.72 <sup>a</sup>	0.23±0.03 <sup>a</sup>	11.8±0.27 <sup>a</sup>	6.43±0.27 <sup>a</sup>	2.2
CB1809	100	23.00±2.13 <sup>c</sup>	0.10±0.01 <sup>c</sup>	5.51±0.41 <sup>c</sup>	4.82±0.17 <sup>c</sup>	1.7
ISR076	100	32.01±2.15 <sup>b</sup>	0.21±0.01 <sup>b</sup>	7.88±0.33 <sup>b</sup>	5.62±0.12 <sup>b</sup>	1.9
ASR011+USDA123	96.3±3.7	50.13±1.31 <sup>a</sup>	0.2±0.02 <sup>a</sup>	12.11±0.31 <sup>a</sup>	6.39±0.24 <sup>a</sup>	2.2
ASR011+CB1809	92.5±7.5	48.63±2.21 <sup>a</sup>	0.24±0.03 <sup>a</sup>	10.50±0.62 <sup>a</sup>	6.11±0.20 <sup>a</sup>	2.1
ASR011+ISR076	87.2±2.8	46.71±1.78 <sup>a</sup>	0.25±0.02 <sup>a</sup>	10.70±0.58 <sup>a</sup>	6.07±0.19 <sup>a</sup>	2.1
Control	0.0	0.0	0.0	0.0	4.55±0.18 <sup>c</sup>	1.6

\*Values are given in μmol C<sub>2</sub>H<sub>2</sub> reduced/h/plant; Data are means of five replicates±SE; Same letters are not significantly different at LSD p<0.05 level

Figure 1 depicts the different types and sizes of plaque were produced by four phages on their respective indicator strain. Phages SR1 and SR2 produced small plaques on their indicator strains and plaque size did not increase on prolonged incubation. These phages could be used as an indicator for the distribution of particular type of rhizobia present in soil. Rhizobiophages have been used to characterize the population of indigenous bradyrhizobia isolated soybean root nodules (Ali *et al.*, 1998).

Nodule occupancy and symbiotic effectiveness of phage marked *Bradyrhizobium* isolates on soybean cv JS335 have been presented (Table 5). A significant variation in nodule number, dry weight and N<sub>2</sub>-ase activity were found in the treatment inoculated with the strain ASR011. This result confirms our previous host-cultivar compatibility results (Appunu and Dhar *et al.*, 2006). So, we chose the ASR011 for nodule occupancy test in the combination of other strains (Table 5).

Nodule occupancy by ASR011 strain when inoculated with USDA123, CB1809 and ISR076 was 96.3, 92.5 and 87.2%, respectively. In comparison to control an increase of 8.79 to 41.31% in total plant dry weight was observed with strain inoculated treatments. Maximum enhancement in total plant dry weight (41.31%) was noticed with the strain ASR011, followed by ISR076

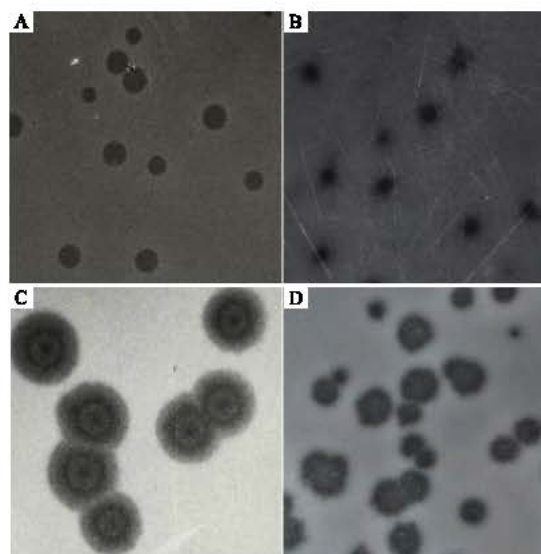


Fig. 1: Plaque morphology of phages infecting specific host strain. A-SR1 (USDA123), B-SR2 (CB1809), C-SR3 (ASR011), D-SR5 (ISR076)

(23.5%), USDA123 (8.79%) and CB1809 (5.93%). Percent increase of nitrogen content was also potentiated by the inoculation of strains. Highest nitrogen content (2.20%) per plant was estimated in strain ASR011 inoculated

plants, which was 37.50% higher than that of control plants. Symbiotic efficiency of strain ASR011 did not differ significantly when applied either singly or in combination with other strains. Further, native bradyrhizobial strains are symbiotically more effective on Indian cultivars of soybean than strains of foreign origin. Similar results have been observed in symbiotic compatibility (Shutsrirung *et al.*, 2002) and nodule occupancy (Botha *et al.*, 2004) by native rhizobia with soybean cultivars.

The present study, although on limited material suggests that native rhizobial populations differs for phage susceptibility, which in some cases may have an effect on the competitive success of inoculant strains. Rhizobiophages can also be used as stable biological marker for easy identification, enumeration and tracking of soybean bradyrhizobia in the nodule under laboratory and field conditions. This study also provides information about the ecological distribution of the phages and their host rhizobial strains in soybean grown fields.

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