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ORIGINAL ARTICLE

Physiological and molecular characterization of locally adapted *Rhizobium* strains of lentil (*Lens culinaris* Medik.) having restricted phage sensitivity

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Abstract Indigenous rhizobia were isolated from root nodules of lentil plants collected from various agro-climatic regions of India. These isolates together with four standard lentil *Rhizobium* strains were screened for sensitivity against eight phages. Four strains, USDA 2431, BHULR 104, BHULR 113 and BHULR 115 having restricted sensitivity to lytic phages LRP-1, LRP-4, LRP-13 and LRP-15 respectively, were characterized for both physiological and molecular characters. All strains had a generation time of between 3.8 and 5.6 h in yeast extract–mannitol (YM) broth, and colonies on YM agar plates showed an acidic reaction. Compared to other strains, strain USDA 2431 grew poorly when sucrose was the sole carbon source and showed maximum growth in arabinose-containing medium. The intrinsic antibiotic resistance level in all strains was tested against seven antibiotics and found to be high with ampicillin and kanamycin (50–60 μg ml^{-1}) but very low with neomycin (0.03 μg ml^{-1}). With the exception of strain BHULR 113, all strains expressed ex planta nitrogenase activity, with strain USDA 2431 showing the maximum activity (26.8 nmol C_2H_4 h^{-1} mg^{-1} protein) after 6 h of incubation. Genomic and phylogenetic relationships among the strains were examined by randomly amplified polymorphic DNA and 16S rRNA sequence analysis. Genetic distance varied from 0.09 to 0.23 among the strains, and the primer OPL-11 was found to be suitable for the discrimination of these strains. The 16S rRNA sequence analysis revealed 99–100% similarity with *Rhizobium leguminosarum* bv. *viciae.* These results clearly indicate that phage sensitivity is a useful marker for discriminating locally soil-adapted rhizobial strains forming effective nodules in lentil.

Keywords Lentil *Rhizobium* · Rhizobiophages · RAPD primer · 16S rRNA

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Introduction

Lentil (*Lens culinaris* Medik.) is one of the most rewarding *rabi* pulse crop in India. It is second only to chickpea in area cultivated (1.48 mha) and production (0.99 tonnes) (McNeil et al. 2007). Lentil improves soil fertility by fixing atmospheric nitrogen (36.8 kg ha^{-1} $year^{-1}$) in root nodules. The crop is generally nodulated by indigenous rhizobia and responds variably to *Rhizobium* inoculation (Subba Rao 1976; Bremer et al. 1990). The success has been reported to depend on the survival, colonization, and effectiveness of the inoculated rhizobial strains (Athar 1998; Shah et al. 1996).

The adaptation of rhizobia in soils to local environmental conditions is crucial for the evolution and distribution of biodiversity. Both biotic and abiotic factors exert a driving force on soil rhizobia populations which eventually determines the degree of nodulation. Among the many factors that affect the soil populations of rhizobia, parasitism and predation have been studied to a limited extent. Bacteriophages that parasitize on rhizobia occur widely throughout the soils of India, and they have been isolated from field soils containing the appropriate host bacteria (Dhar et al. 1979; Dhar and Ramkrishna 1987; Dhar et al. 1993; Appunu and Dhar 2006; Jaiswal and Dhar 2010). The phage typing system

has been used to characterize the strains of several rhizobial species (Staniewski 1970; Lesley 1982; Lindstrom et al. 1983; Appunu and Dhar 2006). Phages are thought to be responsible for the intensity and spatially varying selection pressure on their host and vice versa (Vos et al. 2009). Understanding the population structure of bacteria and bacteriophages is of particular importance because microbial diversity ultimately determines how the ecosystem functions and responds to environmental changes (Bell et al. 2005).

The growth of strains of rhizobial species has been reported to vary with respect to various carbon and nitrogen sources, serology, phage sensitivity, and intrinsic antibiotic resistance level (Vincent 1974; Yao et al. 2002). These parameters have been used in ecological studies to determine heterogeneity among rhizobia. The identification and selection of efficient *Rhizobium* strains of lentil from field-grown soils is a difficult task; there is a need for a dependable screening system of sufficient sensitivity such that each individual may be easily differentiated from others of diverse origin. Phages have been used as a stable marker for identification, enumeration, and the tracking of *R. galegae* strains in field soils after 5 years of inoculation (Lindstrom et al. 1990). Importantly, phages are a potential mechanism for the dissemination of genes within groups of related bacteria, especially if they survive long periods in the environment in the absence of a host or have a wide host range (Mendum et al. 2001). We have recently analysed the 16S rDNA sequence of symbiotically effective, phage-typed lentil rhizobial strains and found a close match with *R. leguminosarum* bv. *viciae* (Jaiswal and Dhar 2011). In the study reported here, we examined the genetic diversity of locally adapted lentil rhizobia based on their phage sensitivity pattern and evaluated the physiological and molecular characteristics of rhizobial strains showing restricted phage infectivity.

Materials and methods

Rhizobial strains

Nodules and soil samples were collected from fields under lentil cultivation in various agro-climatic regions (Azamgargh, Gazipur, Jaunpur, Mirzapur and Varanasi) of Uttar Pradesh, India. Soil samples were analysed for their physical and chemical properties. The collection sites and soil characteristics of the fields are documented in Table 1. Four nodules were randomly excised from each plant sample and surface sterilized with ethanol (70%) and mercuric chloride (0.1%). Rhizobial clones were isolated on yeast extract mannitol agar (YMA; Vincent 1970) using standard procedures. The production of acid or alkali by each rhizobial strain was determined by streaking bacterial cells on YMA plates containing bromothymol blue (BTB). Four standard lentil rhizobial strains (USDA 2426, USDA 2431, USDA 2432 and USDA 2433) kindly provided by Dr. Patrick Elia, USDA, Beltsville, MD, USA, were used. To confirm rhizobial purity, we inoculated lentil seedlings grown in pouches with indigenous and exotic strains by standard procedures (Jaiswal and Dhar 2011), subsequently selecting those with confirmed nodulating ability. Clonal cultures were stored on YEMA slopes in a culture room at 28 $\pm 1^0$ C.

Phage sensitivity test

Rhizobiophages assessed to be infective on lentil *Rhizobium* strains were isolated from nodules and rhizospheric soil samples collected from various fields as a single plaque using double agar layer plates. The morphology and general characteristics of four lytic phages have been reported recently (Jaiswal and Dhar 2010). In total, 12 phages parasitizing on specific lentil rhizobial strains were purified, of which eight

Table 1 Location and soil characteristics of lentil fields used for the isolation of rhizobia

Collection sites		Soil type	Previous crop in the field	Soil pH	Organic carbon (%)	Electrical conductivity (mmhos cm^{-1})	Total Nitrogen (%)
Name of the District	Location						
Varanasi	BHU Farm	Loam	Rice	8.0	0.41	0.22	0.043
	Chiraiganw	Loam	Rice	7.8	0.47	0.23	0.041
	Mughal sarai	Clay	Mung bean	7.6	0.48	0.50	0.042
Ghazipur	Saidpur	Sandy loam	Rice	7.6	0.51	0.34	0.053
	Nandganj	Loam	Mung bean	7.9	0.40	0.26	0.021
Mirzapur	RGS Campus Barkachha	Sandy loam	Maize	6.8	0.59	0.39	0.021
	Chunar	Clay	Fallow	7.1	0.55	0.24	0.052
Azamgarh	Nayabazar	Loam	Rice	7.2	0.38	0.29	0.049
	Doharighat	Loam	Fallow	7.1	0.36	0.34	0.050
Jaunpur	Badshahpur	Sandy loam	Rice	7.5	0.33	0.34	0.044

showing distinct lytic behavior with their host strain were used for the sensitivity test. These phage strains completely lysed the cells of their indicator rhizobial strains within 12–24 h of incubation in broth culture. The phage typing of rhizobial strains was performed on soft agar medium as described earlier (Appunu and Dhar 2006). Lytic reactions on the bacterial lawn at phage spotted drops were observed after 2–3 days of incubation. In total, 268 indigenous rhizobial isolates of lentil (190), fieldpea (46) and French bean (32) were screened for sensitivity against eight phages. Approximately 40% (105) of the total rhizobial isolates (268) and two (USDA 2426 and 2431) of four standard strains showed susceptibility to these phages. Among all of the phage-sensitive rhizobia, four strains, namely, USDA 2431, BHULR 104, BHULR 113 and BHULR 115, showing restricted sensitivity to these phages were selected for characterization.

Growth on synthetic medium

The growth of rhizobial strains was compared in synthetic medium (SM) (O'Gara and Shanmugam, 1976) containing different carbon (mannitol, glucose, arabinose, sorbitol and sucrose at a concentration of 1 gl^{-1}) and nitrogen sources [potassium nitrate (1 mM), sodium nitrite (0.5 mM), ammonium chloride (1 mM), ammonium sulphate (1 mM), and urea (1 mM)]. Growth in terms of optical density (at 660 nm) was compared after 4 days. The colony morphology of these strains on agar plates was compared in two media (YM and SM). The viable number of cells per unit volume of culture was estimated by the colony count method. In brief, 1.0 ml culture was withdrawn at regular growth intervals, suitably diluted in medium, and plated. Colony counts were made after 5–6 days of incubation and backcalculated for colony-forming unit (CFU) per millilitre broth taking the dilution factor into account.

Antibiotic sensitivity

Seven antibiotics (streptomycin, spectinomycin, neomycin, kanamycin, chloramphenicol, ampicilin, and rifampicin) procured from Sigma Chemical (St. Louis, MO) were used. The stock solution was prepared in sterile distilled water, with the exception of rifampicin (95% ethanol). Membrane-filtered solutions of all antibiotics were used in the sensitivity test.

Ex planta nitrogenase activity

The induction of nitrogenase activity in bacterial culture was determined by growing cells on agar slants using the CS-7 medium of Pagan et al. (1975). The agar slope was prepared in 15-ml assay tubes containing 7 ml medium. The cotton plug of each culture tube was replaced with a Subba Seal stopper, and 10% air was replaced with pure acetylene gas. The gases in each tube was analysed a 2-h intervals on a gas chromatograph (model 5765; Nucon, Balanagar Hyderabad, India) using a hydrogen flame detector. The soluble cell protein content of the bacterial culture was estimated by the method of Lowry et al. (1951).

DNA isolation, PCR amplification and purification of PCR products

The total genomic DNA of each rhizobial strain was extracted from cells grown in YM broth until the late log- phase (10^9 cells/ml) using the standard cetyltrimethyl-ammonium bromide protocol (Ausubel et al. 1994). The PCR reaction was carried out in a final reaction mixture volume of 25 μl containing 12.5 μl of PCR SYBR Green Master Mix (Fermentas, Thermo Fisher Scientific, Waltham, MA), 2 μl of each primer of 10 pM and 50 ng of purified template DNA. Ten randomly amplified polymorphic DNA (RAPD) oligonucleotide sequence primers were used for PCR-based fingerprinting, and the 16S rRNA gene was amplified with the 27f (5′-AGAGTTTGATCMTGGCTCAG-3′ and 1492r (5′-TACGGYTACCTTGTTACGACTT-3′) primers (Operon Technology, Alameda, CA) to amplify the random position of the whole genomic DNA and nearly full-length 16S rRNA gene, respectively. Amplification was performed using a thermo cycler (GeneAmp PCR System 2720; Applied Biosystems, Foster City, CA) with a standard temperature profile and following the procedures of Dooley et al. (1993) for RAPD primers; the 16S rRNA region was amplified and purified by the procedure of Jaiswal and Dhar (2011).

16S rRNA sequence determination and GenBank accession number

The amplified 16S rRNA region of all four *Rhizobium* strains was submitted for direct sequencing in an Applied Biosystems automated sequence analyzer (model ABI 3010). The generated rRNA sequences were confirmed and submitted to the GenBank database (www.ncbi.nlm.nih.gov/blast) (Table 2).

RAPD analysis

A dendrogram was constructed with RAPD primer-generated bands from the similarity matrix using the unweighted pair group method with arithmetic mean (UPGMA) in the Alpha-VIEW sofware program (Alpha Innotech, San Leandro SA).

Results and discussion

Indigenous rhizobia were collected from soils of fields in northern India that had been under long-term lentil cultivation. The field soils were loam or sandy loam with a pH that

Table 2 Growth characteristics of the phage-tagged strains of *Rhizobium leguminosarum* bv. *viciae*

Strain	Sensitive to the phage	Geographical origin	Colony morphology(3 days)	Generation time (h)	Ex-planta N_2-ase activity (nmol C_2H_4 h^{-1} mg^{-1} protein)		16S rRNA accession no.
					6 h	12 h	
USDA 2431	LRP-1	USA	3- to 3.5-mm highly mucilaginous, milky colonies	3.8	26.8±1.3	13.2±1.1	FJ997872
BHULR 104	LRP-4	India (25.20°N, 83.0°E)	2- to 2.5-mm round, opaque, gummy, raised colonies	5.0	6.3±0.8	0	GQ339913
BHULR 113	LRP-13	India (26.03°N, 83.13°E)	4- to 5-mm white, round, dome-shaped colonies	4.4	0	0	FJ997871
BHULR 115	LRP-15	India (25.46°N, 82.22°N)	1.5- to 3-mm milky, round, gummy, raised colonies	5.6	13.7±0.92	7.8±0.67	GQ240633

varied from 7.1 to 8.0 (Table 1). Colonies formed by the four rhizobial strains chosen for detailed analysis on agar plates varied from 1.5 mm (BHULR 115) to 5.0 mm (BHULR 113) in diameter after 4 days. Strain USDA 2431 produced highly mucilaginous and milky colonies, while all native isolates produced white, gummy and raised colonies. The growth of the rhizobial strains was compared in SM containing sodium glutamate as the nitrogen source and complex medium (YM). All strains grew well in complex medium (Fig. 1a). The generation time of the rhizobial strains in YEM broth varied between 3.8 h (USDA 2431) to 5.6 h (BHULR 115) (Table 2). All strains formed a yellow zone (acidic reaction) on YMA+BTB. All strains grew well on different carbon sources, but maximum growth occurred when sucrose was the carbon source, with the exception of USDA 2431, which showed maximum growth on arabinose. suggesting that the indigenous rhizobia possess disaccharide hydrolytic enzymes and an uptake system similar to those reported for the rapid growers (Glenn and Dilworth 1981). The growth rate of these rhizobial strains on different carbon and nitrogen sources varied in the synthetic medium, athough strain BHULR 113 displayed a nearly similar growth rate on glucose, arabinose and sorbitol (Fig. 1b). The growth of all strains was limited in medium containing inorganic nitrogen compared to that in medium containing sodium glutamate. The strains showed marked differences in growth rate when tested on potassium nitrate, with strain BHULR 113 showing its maximum growth rate on this nitrogen source and strain USDA 2431 showing its minimum. Two other strains had a similar growth pattern, but the magnitude of growth was lower than that of BHULR 113. The growth patterns of all strains on urea were similar to those on sodium glutamate (Fig. 1c), suggesting that nitrate reductase activity (NRA) was induced in strain BHULR 113, while it was downregulated in strain USDA 2431 However, all strains grew well in nitrite-containing medium, indicating functional nitrite reductase activity (NiRA). Manhart and Wong (1979) reported that strains of *R. leguminosarum* do not express NRA when grown in YM medium or minimal medium containing Na-glutamate because NR is an inducible enzyme and therefore only expressed in the presence of its substrate. Strains of *Rhizobium* and *Bradyrhizobium* have been reported to differ widely in terms of their NRA and NiRA (Arrese-Igor et al. 1989; Giannakis et al. 1988; Monza et al. 1992).

The intrinsic antibiotic resistance (IAR) level in all strains was higher to ampicilin and kanamycin (50–60 μg ml^{-1}) but very low to neomycin (0.03 μg ml^{-1}). The resistance level towards the other antibiotics tested (streptomycin, spectinomycin, rifampicin and chloramphenicol) was almost comparable (15–20 μg ml^{-1}). Similar variations in IAR have been reported earlier with strains of rapidly growing rhizobia (Beynon and Josey 1980; Eaglesham 1987); consequently, IAR was used as a distinctive marker for strain identification in these studies.

The induction of nitrogenase activity in these rhizobial strains was observed the under cell culture conditions. With the exception of strain BHULR 113, all strains expressed N_2-ase activity ex planta, with strain USDA 2431 showing the highest activity (26.8 nmol h^{-1} mg^{-1} protein). In vitro induction of nitrogenase activity has been demonstrated in earlier studies with strains of slow-growing *Rhizobium* spp. (McComb et al. 1975; Pagan et al. 1975; Kaneshiro et al. 1978). Only a few reports have documented the de-repression of nitrogenase activity under cell culture conditions in some fast-growing *R. leguminosarum* strains (Kurz and La Rue 1975; Stam et al. 1983; Maheshwari and Saraf 1994). However, in most assays ethylene production was

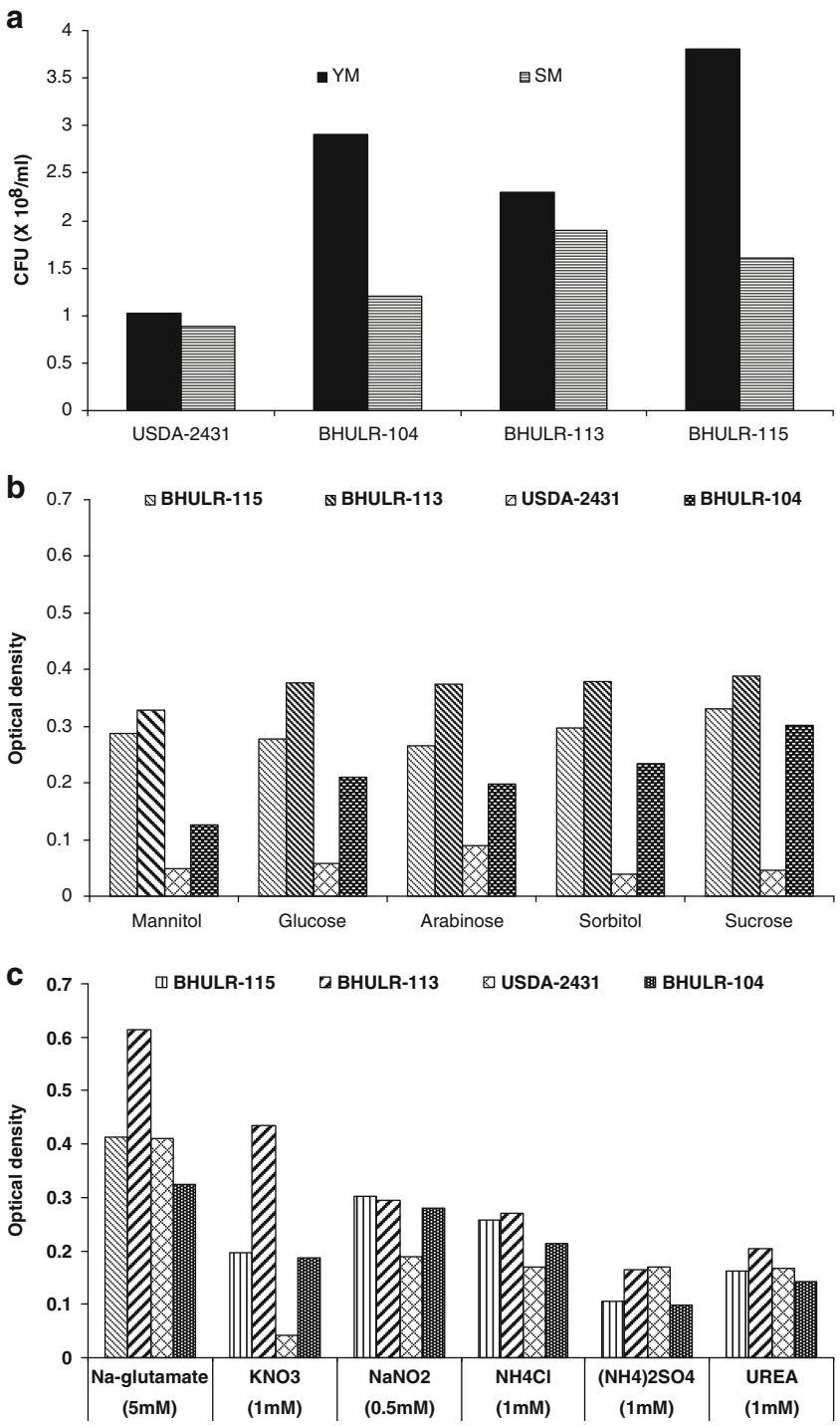

Fig. 1 **a** Comparative growth of the rhizobial strains in complex (*YM*) and defined (*SM*) media, **b** effect of different carbon sources on the growth of the rhizobial strains on SM (4 days), **c** effect of different nitrogen sources on the growth of rhizobial strains on SM (4 days)

reported after 72 h of incubation. In contrast, our lentil rhizobial strains expressed high acetylene reduction activity (26.8 nmol h^{-1} mg^{-1} protein) in cultures immediately after 6 h of incubation (Table 2).

Genomic and phylogenetic relationships were examined on the basis of RAPD primer and 16S rRNA sequence analysis. Of the oligonucleotide RAPD primers used, only one, OPL-11 (5′-ACGATGAGCC-3′) showed polymorphism and had reproducible results. Nine polymorphic bands were observed whose size varied between 500 to 2500 bp. The UPGMA dendrogram (Fig. 2) of RAPD analysis showed one cluster with three separate strains exhibiting substantial variation

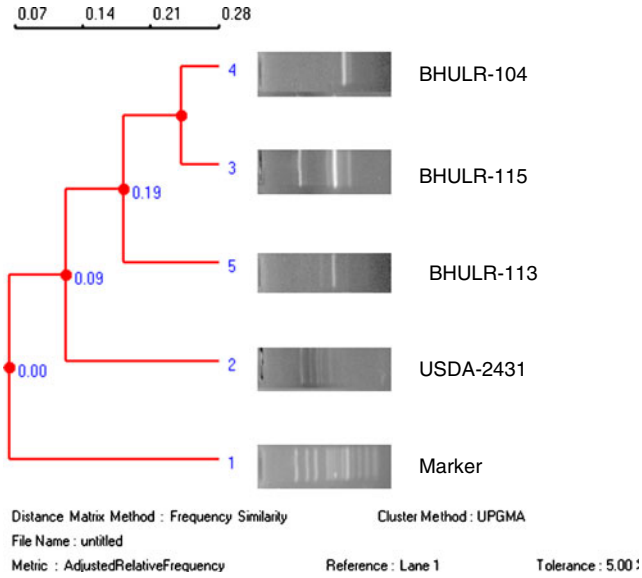

Fig. 2 Dendrogram constructed from randomly amplified polymorphic DNA (RAPD) data of primer OPL-11

(range in similarity coefficient: 0.07 to 0.28). These coefficients formed a single major cluster with two lineages, namely, cluster I, comprising two strains, BHULR 104 and BHULR 115, with a coefficient of genomic similarity of 0.23; strains BHULR 113 and USDA 2431 formed separate lineages. Lineage I and II showed a coefficient of genomic similarity rate 0.19 and 0.09, respectively, with cluster I. Dooley et al. (1993) also used a single RAPD primer as a potent tool for grouping rhizobial strains. Thus, PCR-based DNA fingerprinting does provide information on the possibility of different genotypes. The 16S rRNA sequence analysis of the studied strains revealed that they were closely related with strains of *R. leguminosarum* bv. *viciae* (Jaiswal and Dhar 2011).

Our studies indicate that nearly 40% of the 268 rhizobia isolated from different agro-climatic zone have sensitivity with 12 phages originated from the soil of same fields. Some isolates showed sensitivity with two, three and even four phages. Only four strains namely USDA 2431, BHULR 104, BHULR 113 and BHULR 115 exhibited only lytic activity with phage strain LRP-1, LRP-4, LRP-13 and LRP-15, and belong to different phage groups i.e. X, II, V and VI, respectively (Table 3). These strains also produced distinct type of plaques with respective phage strains i.e. large clear plaque with sharp margin by LRP-1 on USDA 2431, minute plaques with sharp margin by LRP-4 on BHULR 104, small plaques with hazy margin by LRP-13 on BHULR 113 and plaques having central hallow with hazy margin by LRP-15 on BHULR 115.This high degree of lytic specificity of phages suggest that they may impose very strong selection pressure among soil inhabiting rhizobia. Recently, Vos et al. (2009) have suggested that the biotic interaction, in addition to variation in physical environment plays a crucial role in the small-scale spatial structuring of microbial diversity in soil. The remaining 164 isolates which showed resistance to isolated phages may possibly present spacer sequences within the Clustered regularly interspaced short palindromic repeats (CRISPR) loci, which identity to viral genome confer specific resistance to viruses (Andersson and Banfield 2008; Sontheimer and Marrafini 2010). Thus, the obligate-killing parasites (phage) played a major role in rhizobial diversity prevailing in soils of lentil cultivated field.

Table 3 Phage-sensitivity pattern and distribution of indigenous *Rhizobium leguminosarum* isolates

Phage group	Pattern of lysis (+) with phage strain								Total number belongs	Sensitive number distributed to the rhizobial isolates from		
	LRP-16	LRP-4	LRP-13	LRP-20	LRP-1	LRP-15	LRP-21	LRP-24		Lentil (*Lens culinaris*)	Fieldpea (*Pisum sativum*)	French bean (*Phaseolus vulgaris*)
I	+	+		+					14	10	3	1
II		+					+		7	7	0	0
III				+				+	8	7	1	0
IV	+			+					12	2	10	0
V			+				+		4	4	0	0
VI						+			3	3	0	0
VII		+		+					21	9	8	4
VIII		+					+		19	15	1	3
IX				+				+	9	4	0	5
X					+	+			8	8	0	0
Total									105	69	23	13

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