Isolation and Characterization of Indigenous *Rhizobium leguminosarum* Bv. *viceae*, Nodulating *Lens culinaris* Medic, from Four Pakistani Soils

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Rhizobia strains (thirty-two) were isolated from indigenous populations of four soils from different agro-climatic conditions. These isolates were characterized on the basis of their effectiveness for N_2-fixation on lentil plants which were growing in Leonard jars containing sterilized sand. Competitiveness of these isolates with native populations of rhizobia, cross inoculation ability, utilization of carbon sources, intrinsic antibiotic resistance pattern (IAR) and total cell protein profile were also used as criteria for identification. It was found that a great diversity amongst these isolates exists. Not any single technique employed proved reliable for identification of rhizobial strains but these should be used as complementary to each other. The isolates showing high effectiveness and competitiveness fell in the same IAR group.

Isolation and selection of superior *Rhizobium* strains is a time consuming process. However, it can be highly justified and productive when the soil and climatic conditions vary from those under which the inoculant has been developed (11) or when the exotic *Rhizobium* strains fail in effective nodulation due to failure in coping with biological and abiotic factors in the new surroundings(1). The strains which are most competitive in nodule formation and persistent in a particular field environments are often those which emanate from similar milieu (2). Lentil which is very important pulse crop for Pakistan has very poor nodulation when grown in the central Punjab region (7,20) and it could be attributed to little indigenous soil populations, low rainfall and moisture contents, high air and soil temperatures (9,19,20). It was found that native population of *Rhizobium leguminosarum* bv. *viceae* which already exists in the soil declined significantly during the months of summer (20). Under these circumstances, it becomes indispensable to inoculate the lentil crop with highly efficient indigenous strains of *Rhizobium* to enhance nodulation and nitrogen fixation. The present study, was undertaken to isolate and characterize highly efficient *R. leguminosarum* strains from soil samples belonging to different climatic and environmental conditions and varied cropping pattern, to be used as inoculum for lentil to improve its nodulation, nitrogen fixation and yield.

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TABLE 1: Effectivenss of *Rhizobium leguminosaru* bv. *viceae* isolates in sterilized sand

Strains	Nodule number	Nodule dry wt.(mg)	ARA*	Strains	Nodule number	Nodule dry wt.(mg)	ARA*
		GROUP I			GROUP II		
Lc 1	12	8	104	Lc 3	7	10	50
Lc 2	16	18	198	Lc 4	8	9	63
Lc 6	18	15	205	Lc 5	9	8	64
Lc 8	12	13	130	Lc 7	8	6	64
Lc 12	18	21	399	Lc 9	6	9	54
Lc 14	11	13	260	Lc 11	7	7	63
Lc 16	7	9	135	Lc 13	7	8	96
Lc 20	9	12	156	Lc 15	11	11	99
Lc 22	12	14	168	Lc 17	15	6	48
Lc 24	12	13	156	Lc 18	5	6	42
Lc 26	15	19	399	Lc 19	14	5	15
Lc 27	10	13	130	Lc 21	7	7	56
Lc 30	14	17	293	Lc 23	9	8	64
Lc 32	10	9	103	Lc 25	9	10	90
Lc 33	12	10	190	Lc 28	8	6	48
				Lc 29	7	7	21
				Lc 31	8	10	80

*nmol plant^{-1} hr^{-1}

The values given in the columns are averages of 3 repeats and were compared with Duncan's MR test at P=0.05 LSD values are in brackets: Nodule number (3.7); Nodule wt. (3.9); ARA (29).

MATERIALS AND METHODS

Isolation and effectiveness of rhizobial strains: Nodules of lentil were collected from four soils of Pakistan by a plant infection technique using growth pouches (20). The nodule extract was streaked on yeast extract mannitol (YEM) agar plates having bromothymol blue and Congo red as indicator with pH 6.8 (17). The plates were incubated at 28±2°C for 3-5 days. Single colonies from these plates were picked and further purified by streaking on the same medium. The purified cultures were authenticated by their infectivity to their host plants grown in growth pouches containing N-free nutrient solution. Rhizobia were re-isolated from the nodules developed and finally pure cultures were maintained on YEM agar slants using techniques as described earlier (23). Cultures grown on YEM agar for three days at 25°C were examined for cell morphology and Gram reaction (23) by light microscopy. Effectiveness of the isolates for N_2-fixation was determined on lentil plants grown in Leonard jars containing sterilized sand and supplemented with N- free nutrient solution. In each jar four sterilized lentil seeds were sown and thinned to two. Each isolate was grown in YEM broth for 3-5 days and each jar was inoculated with 1 ml of the cultured broth of 0.9 optical density at 660 nm. An uninoculated control and one nitrogen control containing 12 mM N as NH_4NO_3 were also run. One month after the inoculation, the plants were harvested and nodulation was measured. The nodulated roots were also subjected to acetylene reduction assay (8) and the results were compared statistically by using Duncan's Multiple Range Test (4).

Cross inoculation experiment: The *R. leguminosarum* isolates were also studied for their cross inoculation ability. Sterilized seeds of different legume host plants, i.e., *Phaseolus vulgaris, Glycin max, Vigna radiata, Cicer arietinum, Medicago sativa, Trifolium alaxendrium and Pisum sativum,* were germinated on water agar plates and transferred to growth pouches. These plants were inoculated with isolates (1 ml/pouch) and after 30 days of inoculation, the presence or absence of the nodules was checked.

TABLE 2: Competitiveness of *Rhizobium leguminosaru* bv. *viceae* isolates in the presence of native population

Strains	Nodule number	Nodule dry wt.(mg)	ARA*	Strains	Nodule number	Nodule number	ARA* dry wt.(mg)
	-- per plant--				-- per plant--		
	Group I				Group II		
Lc 2	10	13	169	Lc 1	4	6	48
Lc 3	10	13	104	Lc 5	8	10	48
Lc 4	9	11	121	Lc 7	6	4	16
Lc 6	15	13	195	Lc 8	8	6	72
Lc 12	11	14	238	Lc 9	9	10	70
Lc 17	11	10	130	Lc 11	7	9	45
Lc 18	7	11	121	Lc 13	8	10	70
Lc 20	10	16	192	Lc 14	11	9	81
Lc 22	9	8	146	Lc 15	8	9	90
Lc 23	8	10	110	Lc 18	6	7	28
Lc 24	11	14	224	Lc 19	10	8	48
Lc 25	10	14	112	Lc 21	9	13	52
Lc 26	16	17	340	Lc 28	11	15	75
Lc 27	12	9	135	Lc 29	9	9	27
Lc 30	12	11	198	UN	8	9	63
Lc 31	10	13	234				
Lc 33	16	11	187				

*nmol plant^{-1} hr^{-1}

The values given in the columns are averages of 3 repeats and were compared with Duncan's MR test at P=0.05; LSD values are in brackets: Nodule number (2.5); Nodule wt. (4.3); ARA (0.32).

Competition experiment: The experiment was conducted in Leonard jars containing soil collected from NIAB [Sandy loam; pH 8; ECe 0.81 dSm^{-1}; CEC 18.9 Cmol kg^{-1}; organic C 0.82%; total N 0.06%; Olsen's P 9.4 ppm and extractable K 227 ppm]. The soil had very little native *Rhizobium* population (100 cells g^{-1} soil). Plants which were fifteen days old were inoculated with these isolates in triplicate. Nitrogen control (12 mM N as NH_4NO_3 which was replenished weekly) and uninoculated control were also run. After 30 days of inoculation, the plants were uprooted and studied for nodulation and nitrogen fixation efficiency by acetylene reduction assay (8). The results were compared by using Duncan's Multiple Range Test (4).

TABLE 3. Utilization of various carbon sources by *Rhizobium viceae* isolates

Strains		A	X	R	Gl	F	Ga	S	L	Mal	R	Man	D	Na	Y
						Group I									
Lc	1	++	++	++	++	++	++	++	++	++	++	++	-	++	++
Lc	2	++	++	++	++	+	++	++	++	+	++	++	-	++	++
Lc	3	++	-	++	++	+	++	++	++	++	++	++	-	++	++
Lc	4	++	++	++	++	++	++	++	++	+	+	++	-	+	++
Lc	5	++	++	++	++	++	++	++	++	++	++	++	-	+	++
Lc	8	++	++	++	++	++	++	++	++	++	++	++	-	+	++
Lc	9	++	++	++	++	++	++	++	++	+	++	++	-	+	++
LC	11	++	++	++	++	+	++	++	++	+	++	++	-	+	++
Lc	16	++	++	-	+	++	++	++	++	++	+	++	-	+	+
Lc	17	++	++	+	++	++	++	++	++	+	++	++	-	+	++
Lc	18	++	++	++	++	++	++	++	++	++	++	++	-	+	++
Lc	19	++	++	+	+	++	++	++	+	++	+	++	-	+	++
Lc	21	++	++	+	++	++	++	++	+	+	+	++	-	+	++
Lc	22	++	++	++	++	++	++	++	++	+	++	++	-	+	++
Lc	23	++	++	++	++	++	++	++	++	++	++	++	-	+	++
Lc	25	++	++	++	++	++	++	++	++	++	++	++	-	++	++
Lc	27	++	++	+	++	+	+	++	+	+	+	++	-	+	++
Lc	28	++	++	++	++	++	++	++	++	++	++	++	-	+	++
Lc	29	++	++	++	++	++	++	++	++	++	++	++	-	+	++
Lc	31	++	++	++	++	++	++	++	++	++	++	++	-	+	++
						Group II									
Lc	12	++	+	-	++	+	++	++	+	+	-	++	-	+	++
Lc	13	++	++	++	++	++	++	++	++	+	-	++	-	+	++
Lc	14	++	-	-	++	-	-	+	-	-	-	+	-	+	++
Lc	20	++	++	++	-	++	++	++	-	++	-	++	-	+	++
Lc	24	++	+	+	++	+	++	++	++	++	-	++	-	+	++
Lc	26	++	++	++	+	++	++	++	+	-	-	++	-	+	++
Lc	30	++	+	+	+	+	++	++	+	++	-	++	-	+	++
						Group III									
Lc	15	-	+	-	+	-	-	+	-	-	-	+	-	-	+
Lc	32	-	++	+	-	-	+	-	-	-	-	+	-	-	+
Lc	33	-	+	-	+	-	+	-	+	+	-	++	-	-	+
						Group IV									
Lc	7	++	-	-	-	-	-	-	-	-	-	++	-	+	++
						Group V									
Lc	6	++	++	++	++	+	++	++	++	++	++	++	+	++	++

(A) arabinose; (X) xylose; (R) ribose; (Gl) glucose; (F) fructose; (Ga) galactose; (S) sucrose; (L) lactose; (Mal) maltose; (R) raffinose; (Man) mannitol; (D) dulcitol; (Na) Na-gluconate and (Y) yeast extract;(-) showing no growth ;(+) showing moderate growth; (++) showing heavy growth.

Carbon utilization: These isolates were also tested for their capacity of utilizing different carbon sources. In HEPES-MES basal agar (AG) medium (3) arabinose, xylose, ribose, glucose, fructose, galactose, raffinose, sucrose, lactose, maltose, mannitol, dulcitol and Na-gluconate were used as alternative carbon

sources for yeast extract, Na-gluconate and arabinose to a final concentration of 1% (W/V). Each carbon source was filter sterilized by passing through membrane filters (0.22 μm size, Millipore Corp). The isolates were streaked on the plates in triplicate and after 3-5 days presence or absence of their growth was observed. A media without any carbon source was used as a positive control.

Intrinsic antibiotic resistance pattern (IAR): Resistance of the isolates to low concentrations of various antibiotics [Kanamycin (10 μg/ml), nalidixic acid (10 μg/ml), neomycin (2.5 μg/ml), chloramphenicol (12.5 μg/ml and 25 μg/ml), rifampicin (5 μg/ml and 10 μg/ml), streptomycin (2.5 μg/ml and 10 μg/ml) and tetracycline (4 μg/ml)] was determined (5,14) by streaking them on AG medium containing these antibiotics.

Total cell protein profile: Protein profile of the isolates was determined through SDS-polyacrylamide (12.5%) gel electrophoresis. The cells were prepared as described by Wright et al (24). The gel was run at a constant current of 50 mA. Cell extract (60 μl) was applied to 5 mm wide lane. When electrophoresis was complete, the gels were stained with coomassie blue, photographed and dried for storage.

RESULTS AND DISCUSSION:

Morphology: The reisolated cultures showed sufficient growth in three days and turned YEM agar media with bromothymol blue (BTB) to yellow color showing that they all were fast growers and acid producers (13). All the cultures were found to Gram negative rods. The stained cells often appeared banded under phase contrast microscope (23). The colonies produced were circular, convex with entire and smooth edges, glistening and translucent or white. All isolate produced gummy colonies.

Cross inoculation ability: None of the isolates cross nodulated *Phaseolus vulgaris, Glycin max, Vigna radiata, Cicer arietinum, Medicago sativa* and *Trifolium alaxendrium* except *Pisum sativum*. It has already been reported that bacteriogenic plasmid, carrying Tn5 from *Rhizobium leguminosarum* bv. *viceae*, when transferred to biovars *phaseoli* and *trifolii*, conferred the ability to nodulate *Pisum* (12). Neverthless, inability of these isolates to nodulate hosts other than *Pisum* and *Lens* confirmed that they were highly specific belonging to *R. leguminosarum* bv. *viceae* group. The biovar indicated for *R. leguminosarum* are based largely on host plant specificity (13).

Effectiveness and competitiveness of the isolates: Rhizobium leguminosarum bv. *viceae* isolates were further characterized on the basis of their acetylene reduction ability, number of nodule and nodule dry weight. These isolates were differentiated into two groups; one containing more effective and competitive isolates and the other relatively less effective and competitive. The results (Table 1) show that 15 isolates were highly effective and 17 were less effective. There was positive correlation (r=0.89) between nodule number and nodule weight but poor correlation was found to exist between nodule number and ARA (r=0.51) and nodule weight and ARA (0.48) in sterilized sand. Table 2 shows that 18 isolates were more competitive than the other 14 isolates. Isolates Lc 1, 8, 14 and 16 that had shown high effectiveness, could not compete the native population while the isolates Lc 3, 4, 17, 18, 23, 25, 27 and 31, relatively dull in effectiveness, showed higher competitiveness. It could be inferred from these results that the effectiveness status alone of the isolates in sterilized growth medium is not sufficient to grade the isolates but their competitive abilities in the soil or field conditions are more differentiative and reliable. The isolates showing high effectiveness and competitiveness emanated from all the four soils used for isolation. Obviously all the isolates from single locality were not equally efficient. Thus to select and use the highly effective and competitive strains as inoculum may prove its worth in increasing the yield of the crop.

TABLE 4. Resistance of *Rhizobium leguminosarum* bv. *viceae* isolates to low levels of different antibiotics

	μgml^{-1}									
	Ka	Na	Neo	Ch		Rif		Str		Tet
Strains	**10**	**10**	**2.5**	**12.5**	**25**	**5.0**	**10**	**2.5**	**10**	**4.0**
					Group I					
Lc 2	-	++	-	++	++	++	++	-	-	-
Lc 3	--	++	--	++	++	++	++	++	-	-
Lc 19	-	++	-	++	++	++	++	++	-	-
Lc 21	--	+	--	++	--	++	++	++	-	-
Lc 22	-	++	-	++	++	++	++	++	-	-
Lc 24	-	++	-	++	++	++	++	+	-	-
Lc 25	-	++	-	++	-	++	-	++	-	-
Lc 28	-	++	-	++	-	++	++	++	-	-
					Group II					
Lc 5	-	++	+	++	++	++	++	-	-	-
Lc 8	-	++	++	++	++	++	++	+	-	-
Lc 9	-	++	++	++	++	+	++	++	-	-
Lc 11	-	++	++	++	++	++	++	++	-	-
Lc 27	-	++	++	++	++	++	++	+	-	-
Lc 30	-	++	++	+	++	+	+	++	-	-
Lc 31	-	++	++	++	++	+	+	++	-	-
					Group III					
Lc 14	-	-	-	++	-	++	++	+	-	-
Lc 18	-	++	++	++	-	++	++	++	++	-
Lc 20	-	-	++	++	-	-	-	++	++	-
Lc 23	-	++	-	++	-	++	++	++	+	-
Lc 29	-	++	+	+	-	+	+	++	-	-
Lc 33	-	-	++	++	-	++	++	++	++	-
					Group IV					
Lc 1	-	++	+	-	-	++	++	+	-	-
Lc 4	-	++	-	-	-	++	++	++	-	-
Lc 13	-	+	-	-	-	++	++	++	-	-
Lc 17	-	++	-	-	-	++	++	++	+	-
Lc 32	-	++	++	-	-	+	+	++	++	-
					Group V					
Lc 6	-	-	++	++	++	++	++	++	-	+
Lc 12	-	++	+	++	++	-	-	++	-	+
Lc 16	-	++	-	++	++	++	++	++	+	+
Lc 26	-	++	++	++	++	++	++	++	-	+
					Group VI					
Lc 7	++	++	++	++	-	++	++	++	-	--
					Group VII					
Lc 15	--	--	--	--	--	--	--	+	--	--

(Ka) kanamycin; (Na) nalidixic acid; (Neo) neomycin; (Ch) chloramphenicol; (Rif) rifampicin; (Str) streptomycin (Tet) tetracyclin. (-) sensitive to the antobiotic; (+) resistant to the antibiotic and showing some growth; (++) resistant to the antibiotic and showing heavy growth.

Carbon utilization: The results showed (Table 3) that majority of the isolates utilized a wide range of carbon sources, in accordance to our earlier findings that fast growers can oxidize a wide range of carbon sources as a sole carbon source (10). Out of 32 *Rhizobium* isolates 20 were placed in group I, these isolates utilized all the carbon sources tested (pentoses, hexoses, disaccharides, trisaccharides, polysacharides, polyhydric alcohols) except dulcitol. Only Lc 3 and 13 could not utilize xylose and ribose, respectively. Eight isolates were enlisted in group II on the basis that they were not able to oxidize raffinose in addition to dulcitol, whereas Lc 15, 32 and 33 were quite different from all these isolates as they were unable to utilize arabinose, fructose, raffinose, dulcitol and Na-gluconate. Lc 7 showed growth only on ribose, mannitol and yeast extract while Lc 6 was the only isolate that oxidized all the carbon sources including dulcitol. The isolates grouped on their effectiveness and competitiveness did not retain their identity and similar status behaved differently to the carbon utilization. Many workers have used and recommended carbon sources utilization as a helpful tool to characterize the isolates (10,15,16,18).

Intrinsic antibiotic resistance pattern: Table 4 shows that the isolates which appeared similar due to their common carbon utilization pattern behaved differently to antibiotics. Lc 2, 3, 19, 21, 22, 24, 25 and 28 were similar in their IAR pattern and were sensitive to kanamycin, tetracycline, neomycin and streptomycin (10 μg/ml) and placed in group I. While Lc 5, 8, 9, 11, 27 and 31 were resistant to neomycin and, therefore, were placed in group II. Isolates which were grouped in III were sensitive to higher level of chloramphenicol while isolates in group IV were sensitive to both levels of chloramphenicol. Lc 6, 12, 16 and 26 were found to be distinctive as these were the only isolates resistant to tetracycline. It was very interesting that members of this group, especially Lc 26 gave the highest grain yield ha^{-1} in another field experiment (20). Lc 7 was the only isolate resistant to kanamycin while Lc 15 showed resistance only to a lower level of streptomycin. Therefore, both of these strains can be used as markers for identification. Thus, IAR proved a very helpful tool in further characterizing the otherwise similar looking isolates. Evidently the isolates cannot be reliably characterized only on their carbon utilization and that IAR must be employed as a supplementary tool (10,15,18,21,22).

Total cell protein profile: The isolates were analyzed by SDS-Polyacrylamide gel electrophoresis to observe if the grouping based on their effectiveness and competitiveness, carbon utilization and IAR extended to their protein profile. In this study no clear difference was detected in the total cell protein profiles of the *Rhizobium leguminosarum* isolates (figure not shown). Although workers have used protein profile to identify the isolates but all alone it was not a reliable method as sometimes it did not help (5,6,10).

Conclusions: Rhizobium leguminosarum bv. *viceae* are very specific strains and to maintain their population in the soil, continuous cultivation of lentil crop is very essential. That may be the main reason for very low indigenous population of mainly ineffective and less competitive strains of *R. leguminosarum* bv. *viceae*. So, the regular use of a good quality inoculum for lentil crop is essential and highly beneficial.

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