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Competition between inoculated and indigenous *Rhizobium/Bradyrhizobium* spp. strains for nodulation of grain and fodder legumes in Pakistan

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Summary. The competitive ability of inoculated and indigenous *Rhizobium/Bradyrhizobium* spp. to nodulate and fix N_2 in grain legumes (*Glycine max*, *Vigna unguiculata*, *Phaseolus vulgaris*) and fodder legumes (*Vicia sativa*, *Medicago sativa*, and *Trifolium subterraneum*) was studied in pots with two local soils collected from two different fields on the basis of cropping history. The native population was estimated by a most-probable-number plant infectivity test in growth pouches and culture tubes. The indigenous rhizobial/bradyrhizobial population ranged from 3 to 2×10^4 and 0 to 4.4×10^3 cells g^{-1} in the two soils (the first with, the second without a history of legume cropping). Inoculated *G. max*, *P. vulgaris*, and *T. subterraneum* plants had significantly more nodules with a greater nodule mass than uninoculated plants, but N_2 fixation was increased only in *G. max* and *P. vulgaris*. A significant response to inoculation was observed in the grain legume *P. vulgaris* in the soil not previously used to grow legumes, even in the presence of higher indigenous population ($>10^3$ cells g^{-1} soil of *Rhizobium leguminosarum* bv *phaseoli*). No difference in yield was observed with the fodder legumes in response to inoculation, even with the indigenous *Rhizobium* sp. as low as <14 cells g^{-1} soil and although the number and weight of nodules were significantly increased by the inoculation in *T. subterraneum*. Overall recovery of the inoculated strains was 38–100%, as determined by a fluorescent antibody technique. In general, the inoculation increased N_2 fixation only in 3 out of 12 legume species-soil combinations in the presence of an indigenous population of rhizobial/bradyrhizobial strains.

Key words: *Bradyrhizobium* – Competition – Indigenous – Inoculated – Nodulation – *Rhizobium*

The legume-*Rhizobium* sp. symbiotic partnership is the most promising plant-bacterium association so far known. It is being exploited in many countries to increase crop yields, with beneficial strains being introduced to ensure effective nodulation (Thompson 1980; Moxley et al. 1986; Danso and Owiredu 1988; Kucey 1989). In Pakistan, legumes are grown on 2 million hectares, which constitutes about 10% of the total 20.9 million ha of land under cultivation (Anon 1989). The main leguminous pulse crops are chickpeas, mungbeans, blackgrams, lentils, peas, and recently, introduced soybeans, while alfalfa, clover, sesbania, vetch, and ipil-ipil are widely grown as fodder and green-manure crops. Except for chickpeas and a few fodder crops, nodulation of these crops is either poor or completely absent (Hafeez et al. 1987). Inoculated *Rhizobium* spp. strains often fail to compete with indigenous soil rhizobia and do not increase nodulation (Bromfield et al. 1986; Singleton and Tavares 1986). The successful use of a rhizobial/bradyrhizobial inoculant requires knowledge of the indigenous rhizobial/bradyrhizobial populations in terms of number, effectiveness, and competitive ability (Bromfield and Jones 1980; Singleton and Tavares 1986; Thurman and Bromfield 1988). Further, rhizobia/bradyrhizobia strains differ in their ability to survive and nodulate different hosts/cultivars under adverse soil conditions (Gaur and Lowther 1982; Hafeez et al. 1988, 1991). However, for Pakistani soils, no information is available on the effect of the soil environment on the competitive ability of rhizobia.

The present study was therefore designed to investigate the competitiveness of indigenous and inoculated *Rhizobium/Bradyrhizobium* spp. strains in nodulating six leguminous crops at two locations, in order to achieve maximum benefits and to establish the need for inoculation.

Materials and methods

The experiment was conducted in pots using soil collected from two fields of NIAB (located in a semiarid region of central Punjab, Pakistan) selected on the basis of their cropping history. In soil 1, mungbeans, blackgrams, cowpeas, chickpeas and lentils had been grown in

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the past 6 years, while no leguminous crop at all had been grown in soil 2. Both soils belong to the Hafizabad series and are non-saline. The main soil properties are given in Table 1.

Indigenous population

The population density of indigenous *Rhizobium/Bradyrhizobium* spp. in the soils was determined by a plant infection technique (Vincent 1970) using growth pouches containing an N-free nutrient solution or in N-free nutrient agar slants with various hosts (Table 2). The plants were grown in a growth room at 27±2°C with a 15-h photoperiod and a photon flux density of 392 μmoles m^{-2} S^{-1}. The seeds of all legumes tested were surface-sterilized with 1% NaOCl for 2–5 min, except *M. atropurpureum*, which was scarified with concentrated H_2SO_4 for 5 min followed by repeated rinsing with sterile water.

Soil samples were collected in each field by taking 25 cores (diameter 2.5 cm) to 20 cm depth in a grid pattern covering the entire field site after removing debris and the top 1 cm of soil. The pooled sample was thoroughly mixed and a subsamples of 1 kg soil were taken. The subsamples were oven-dried (at 100°C) to determine the moisture content and then the soil was diluted on a dry weight basis.

Serial fourfold dilutions (4^{-1} to 4^{-8}) were prepared and 1 ml of each dilution was applied directly to the roots of the test legumes, with four replicates for each dilution. Every 5th pouch and tube was kept as a control. Nodule observations were made daily and data were collected after 30 days of planting. Positive results were compared with a standard most probable number table (Vincent 1970) and 95% confidence limits were applied to estimate the size of the rhizobial/bradyrhizobial population.

Competitive plant-growth response

The competitive experiment in nodulation between the native and the inoculated strains was carried out in pots under natural light and temperature conditions. Three grain legumes (*G. max*, *V. unguiculata*, and *P. vulgaris*) and three fodder legumes (*Vicia sativa*, *M. sativa* and *T. subterraneum* were used as host species. Soil was collected from six locations in each field by mining the soil up to a depth of 20 cm, after removing litter and the top 1 cm of soil; the soil was air-dried and sieved (0.5 cm). Each pot was filled with 4 kg soil (oven-dry weight at 100°C) and fertilizer was added at a concentration of (mg kg^{-1} soil) 200 P, 252 K, 50 Mg, 66 S, 1.3 Zn, 0.1 Mo, 1.2 Mn, 3.75 Fe, 0.38 Cu, 0.08 Co, and 0.86 B. The experiment was performed in a completely randomized block design with four replicates and three treatments (uninoculated, inoculated, and N control). N was applied at 100 and 40–80 mg N kg^{-1} soil to the grain and fodder legumes, respectively. The pots were watered to field capacity and the soil was allowed to equilibrate for 2–3 days before planting. The host cultivars, bacterial strains, and their fluorescent antibodies were obtained from NifTAL Project Paia, Hawaii, USA, except *V. unguiculata* cv. AC 58 and *P. vulgaris* cv. A 429, which were obtained from Mutation Breeding Division, NIAB, Faisalabad, and National Agriculture Research Center, Islamabad, Pakistan, respectively. For *G. max* and *P. vulgaris*, two cultivars were used, each in soil from a different field. The seeds were inoculated with a peat-based multistrain (serologically distinct strains) inoculum, using equal numbers of each strain, except for *M. sativa*, in which a single-strain inoculum was used, as other inoculant strains had shown cross-reactions with strain TAL 380. The number of viable rhizobial cells was 10^4 and 10^7 per seed at the time of sowing for small and large seeds, respectively. The plants were thinned to 3 large-seeded and 10 small-seeded legumes per pot. The surface of the pots was covered with gravel to prevent cross-contamination between treatments. The plants were harvested 6–8 weeks after planting and nodulation data were recorded. The dry weight and total N were determined in shoots (Bremner 1965). Nodule occupancy (strain recovery) was studied by the method of Schmidt et al. (1968), by randomly taking 25 nodules per replicate of each treatment with the respective fluorescent antibodies of the inoculated strains.

Results and discussion

The response by inoculated rhizobial/bradyrhizobial strains in the presence of an indigenous rhizobial population was studied with six legume hosts in two soils. The indigenous population ranged from 3 to 2×10^4 and 0 to 4.4×10^3 cells g^{-1} soil in the soils with and without a legume cropping history, respectively (Table 2). The estimates of the indigenous population in these soils are the first to be obtained in this region. There are a few reports on inoculation responses on different legumes (Idris et al. 1986; Hafeez et al. 1987), but these authors did not take the indigenous population into account.

The response to inoculation varied among legume species and between soils (Tables 3 and 4). The nodulation response was most frequent in the soil where no previous leguminous crop had been grown, indicating that this soil was more receptive to introduced inoculant strains. Inoculation significantly increased nodule numbers in 6 cases (legume species-soil combinations) and nodule weights in 7 out of 12 cases (Table 3). In general, the increased numbers and weights of nodules led to a significant increase in N_2 fixation only in 25% of the legume species-soil combinations. *G. max* responded significantly to inoculation in both soils, with a 34–89% increase in N concentrations in the inoculated plants compared with the uninoculated plants. The indigenous population was extremely low (0–3 cells g^{-1} soil) in both soils, as *G. max* had not previously been grown in either soil. It has been reported that in tropical areas, where adverse soil conditions tend to prevail, substantially lower numbers of native *B. japonicum*, compared with the high rhizobial numbers in an inoculant, can provide effective nodulation on legumes (Singleton and Tavares 1986; Owiredu and Danso 1988; Thies et al. 1991). In the present study, inoculation significantly increased the number and dry weight of nodules on *P. vulgaris* in soil 2 (no previous legume crop) in the presence of a natural population of 4400 cell g^{-1} soil, but there was only a non-significant increase in nodulation in the other soil, which had a native population of 2200 cells g^{-1} soil (Tables 3,

Table 1. Some physicochemical properties of soils used

	Soil type	pH (H_2O)	CEC (cmol kg^{-1})	Organic C (%)	Total N (%)	NO_3^--N (mg kg^{-1})	NH_4^+-N (mg kg^{-1})	Mineralizable N (mg kg^{-1})	Annual rainfall (mm)
Soil 1	Sandy loam	7.6	9.4	0.60	0.06	18.1	2.8	1.6	310.9±48.9
Soil 2	Loam	7.8	10.0	0.54	0.06	15.1	1.5	1.2	

CEC, cation exchange capacity

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Table 2. Population of indigenous *Rhizobium/Bradyrhizobium* spp. in two soils

Host species	Estimate (cells g^{-1} soil)		Confidence limits (95%)	
	Soil 1	Soil 2	Soil 1	Soil 2
Glycine max	0.3	0.0	0.1 – 0.8	0
Phaseolus vulgaris	220.0	440.0	81 – 594	163 – 1188
Macroptilium atropurpureum	2000.0	52.0	740 – 5400	19 – 140
Vicia sativa	1.3	0.9	0.48 – 3.5	0.3 – 2.4
Medicago sativa	1.3	14.0	0.48 – 3.5	5.2 – 37.8
Trifolium subterraneum	14.0	1.8	5.2 – 37.8	0.7 – 4.9

Most probable number counts X 10; 95% confidence limits approximately between estimate divided by 2.7 and estimate multiplied by 2.7

4). A significant increase was observed only in the number of nodules in *V. unguiculata* in soil 2 in the presence of a native bradyrhizobial population of 520 cell g^{-1} (Table 3). *V. unguiculata* and *P. vulgaris* produced 2- and 15-fold more nodules in soil 2, respectively, in response to inoculation, but without a significant increase in total N in *V. unguiculata* (Tables 3, 4). Kucey (1989) reported that plants in soils containing less than 8×10^3 *R. leguminosarum* bv. *phaseoli* g^{-1} soil and with low levels of mineral N showed increased levels of plant N and dry matter accumulation in response to rhizobial inoculation. In our study significantly increased N concentrations (25 – 97%) were observed in *P. vulgaris* and *V. unguiculata* where N fertilizer was applied rather than inoculation (Table 4), indicating that the NifTAL strains (recommended as highly effective) formed inefficient symbiosis with their hosts in our soils, so that insufficient N_2 was fixed to allow the maximum yield.

Among the fodder legumes *T. subterraneum* responded significantly to the inoculation, with increased nodulation in both soils (Table 3). A non-significant inoculation response was obtained with *M. sativa* and *Vicia sativa* (Tables 3, 4), even though the indigenous population was not so high (9 – 140 cells g^{-1} soil). In contrast to the grain legumes, neither inoculation nor the application of N fertilizer led to a significant increase in the total N concentration of fodder legumes (Table 4). These results support the findings of other authors (Singleton and Tavares 1986; Thies et al. 1991) that relatively small indigenous populations of rhizobia are sufficient to meet the host N demand as long as there are some effective strains in the population.

Table 3. Comparison of nodulation in grain and fodder legumes by competition between inoculated and indigenous rhizobial/bradyrhizobial strains

Host species/cultivars	Soil 1				Soil 2			
	Number ($plant^{-1}$)		Dry weight (mg $plant^{-1}$)		Number ($plant^{-1}$)		Dry weight (mg $plant^{-1}$)	
	INO	UINO	INO	UINO	INO	UINO	INO	UINO
G. max cv. Lee (cv. William*)	18.2a	0.2b	43.1a	0.3b	54.0a	0b	220.0a	0b
P. vulgaris cv. Bush Bountiful (A429*)	1.8a	0a	1.5a	0a	91.7a	6.2b	158.0a	16.6b
V. unguiculata cv. AC58	24.6a	21.5ab	65.0a	72.5a	47.1a	21.0b	65.5a	55.7a
Vi. sativa	22.4a	16.3a	89.2a	52.9b	24.7a	16.7a	13.7a	6.8ab
M. sativa cv. Florida 77	8.7a	7.3a	1.7a	1.8a	15.3a	11.6a	7.5a	4.2b
T. subterraneum cv. Mt. Baker	27.1a	9.1b	10.6a	2.4b	19.2a	7.5b	8.1a	2.7b

Means of four replicates. Numbers between INO and UINO columns followed by the same letter are not significantly different at $P = 0.01$ by Duncan's Multiple Range Test; INO, Inoculated; UINO, Uninoculated; *G., Glycine; P, Phaseolus; V., Vigna; Vi., Vicia; M, Medicago;* T., Trifolium; * cultivars used only in soil 2

Table 4. Comparison of inoculated and indigenous rhizobial/bradyrhizobial strains on dry matter and total N concentration in grain and fodder legumes

Host species	Soil 1						Soil 2					
	Dry matter (g $plant^{-1}$)			Total N (mg $plant^{-1}$)			Dry matter (g $plant^{-1}$)			Total N (mg $plant^{-1}$)		
	INO	UINO	NC	INO	UNIO	NC	INO	UINO	NC	INO	UINO	NC
G. max	2.1a	2.1a	2.2a	51a	38b	54a	3.0a	2.7a	2.5a	72a	38b	90a
P. vulgaris	1.6b	1.9b	2.4a	53b	55b	91a	1.9a	1.8a	2.1a	48b	26c	67a
V. unguiculata	1.2a	1.0a	1.8a	34b	27b	67a	1.4a	1.3a	1.6a	37ab	31b	69a
V. sativa	0.7a	0.7a	0.7a	24a	22a	20a	0.8a	0.7a	0.7a	21a	21a	24a
M. sativa	0.3a	0.4a	0.3a	16a	15a	18a	0.5a	0.5a	0.5a	13a	11a	18a
T. subterraneum	0.5a	0.4a	0.5a	18a	14a	13a	0.3a	0.3a	0.3a	17a	15a	17a

NC, N control; for other explanations, see footnotes to Table 3

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Table 5. Relative abilities of inoculated strains to nodulate grain and fodder legumes in two soils

G. max			*P. vulgaris*			*T. subterraneum*			*V. sativa*			*V. unguiculatu*			*M. sativa*		
Strain number (TAL)	Nodule occupancy (%)		Strain number (TAL)	Nodule occupancy (%)		Strain number (TAL)	Nodule occupancy (%)		Strain number (TAL)	Nodule occupancy (%)		Strain number (TAL)	Nodule occupancy (%)		Strain number (TAL)	Nodule occupancy (%)	
	Soil 1	Soil 2		Soil 1	Soil 2		Soil 1	Soil 2		Soil 1	Soil 2		Soil 1	Soil 2		Soil 1	Soil 2
107	10	8	182	ND	22	1826	0	ND	1397	30	97	169	38	44	380	100	100
377	0	1	1797	ND	19	1827	0	ND	1399	0	0	658	0	0			
379	4	3	1383	ND	0	1828	0	ND	1397+1399	70	0						
102+377	34	33	182+1797	ND	49	1826+1827	50	ND									
102+379	9	8	182+1383	ND	0	1826+1828	0	ND									
377+379	11	9	1383+1797	ND	0	1827+1828	0	ND									
102+377 +379	32	38	182+1383 +1797	ND	3	1826+1827 +1826	30	ND									

ND, not detected; for other explanations, see footnotes to Table 3

Despite some increases in nodulation due to inoculation, no significant increases in plant dry matter were observed for any of the legume-soil combinations (Table 4). This suggests that under some soil conditions, factors other than soil N are the main constraints on plant growth (Owiredu and Danso 1988; Weiser et al. 1990).

Strain identification in nodules from the inoculated treatments indicated the pattern of competition between the inoculated and indigenous strains (Table 5). Nodule occupancy by inoculant strains ranged from 38 to 100%. Thies et al. (1991) reported that nodule occupancy by inoculant strains ranged from 7 to 100% and was inversely related to the number of indigenous rhizobia. In the present study, the inoculant strains formed nodules on all or almost all the inoculated plants. The exception was the cowpea, where only 38–44% of the nodules were partly occupied by TAL 169 in both soils, indicating that this strain is a poor competitor against a large indigenous population. Another cowpea bradyrhizobial inoculant strain, TAL 658, completely failed to compete with the indigenous rhizobia for nodule occupancy in this region. Nodule occupancy on *P. vulgaris*, however, indicated better competition against a moderately high native population (4400 cells of *R. leguminosarum* bv. *phaseoli* g^{-1} soil 2). The mixed strain inoculation used in this study showed vast differences in the competitiveness of individual strains for nodule occupancy. Single-, double- and triple-strain occupancy of nodules was high in *G. max*, *P. vulgaris*, *T. subterraneum* and *Vicia sativa* (Table 5), indicating that the strains inoculated were relatively competitive in this respect. A heterogenous nodule occupancy by inoculant strains has also been reported by Beynon and Josey (1981), Gaur and Lowther (1982), Danso and Owiredu (1988), and Owiredu and Danso (1988). The results of the present study support the findings of Thies et al. (1991) and indicate that the inoculant strains were, in general, very successful in competing with indigenous rhizobia for occupancy. However, there was commonly no yield response, even when the inoculant rhizobia occupied the majority of nodules.

This study examined the population of soil rhizobia on six leguminous crops representing various cross-inoculant groups, and varied responses to inoculation with selected rhizobia, were observed. In general, the inoculation led to an increased N_2 fixation, with 2.5 times more nodules in the inoculated than the uninoculated plants (Singleton and Tavares 1986). Our data clearly demonstrate that low native rhizobial/bradyrhizobial populations are not the only criterion for success with inoculation. In addition, the host cultivar, the soil type and environmental factors may also affect the establishment of active N_2-fixing symbioses. Thus the inoculated plants of *G. max*, *P. vulgaris* and *T. subterraneum* had 2.5 times more nodules than the uninoculated plants, and yet N_2 fixation increased only in *G. max* and *P. vulgaris*. Moreover, some plants (*P. vulgaris*) showed a significant response to the inoculation, even in the presence of higher native rhizobial population (10^3 cells g^{-1} soil), and even though no difference in nodulation, total N, or dry matter yield was observed in *M. sativa* and *Vicia sativa* in the presence of native rhizobia at less then 14 cells g^{-1} soil.

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