

Influence of elevation and applied nitrogen on rhizosphere colonization and competition for nodule occupancy by different rhizobial strains on field-grown soybean and common bean¹

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In the absence of indigenous rhizobial populations, the pattern of competition between inoculum strains for nodule occupancy is found to be a stable characteristic, independent of rhizosphere population size, nitrogen application, or elevation. Soybean (*Glycine max* (L.) Merrill) and common bean (*Phaseolus vulgaris* L.), inoculated with peat-based rhizobia, were grown with three nitrogen levels at 320- and 150-m sites along an elevational transect on the island of Maui, Hawaii. Rhizosphere soil of 8-day-old plants was examined by immunofluorescence for populations of three strains each of *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. *phaseoli*, which made up the inocula applied to the respective host legumes at planting. Nodules were examined for occupancy by specific strains at two sampling times. Site differences and nitrogen treatment had no significant effect on rhizosphere colonization or nodule occupancy by the three strains. The three inoculum strains colonized their respective host rhizosphere in approximately equal numbers. In soybean, strain TAL 102 (USDA 110) occupied most of the nodules, while strain TAL 379 (USDA 1366) was the least competitive in nodule formation in all treatments. In common bean, TAL 182 outcompeted the other two strains in nodule formation, while TAL 1797 (CIAT 899) occupied the least number of nodules. High elevation decreased nodulation by soybean more than that by common bean. Nitrogen application reduced nodule number and mass of both legumes more at the low-elevation than the high-elevation site; the reductions in nodule mass were more pronounced in common bean. The pattern of nodule occupancy remained stable regardless of the differences in nodule number and mass between elevations, N levels, and sampling times.

Key words: rhizobial ecology, rhizosphere colonization, interstrain competition, immunofluorescence, legumes.

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En l'absence de populations rhizobiennes indigènes, le profil de compétition entre des souches d'inoculum pour l'occupation des nodosités s'est révélée être une caractéristique stable, indépendante de la dimension des populations des rhizosphères, des applications d'azote ou des niveaux d'élevation des sites de culture. Du soja (*Glycine max* (L.) Merrill) et du haricot commun (*Phaseolus vulgaris* L.), inoculés par des rhizobiums établis dans de la tourbe, ont été cultivés en fonction de trois concentrations d'azote et dans des sites A des niveaux de 320 et 1050 m, le long d'un transect d'élevation sur file de Maui, Hawaï. Les rhizosphères de sols porteurs de plantes âgées de 8 jours ont fait l'objet d'examen par immunofluorescence, relativement aux populations de trois souches de *Bradyrhizobium japonicum* et de trois souches de *Rhizobium leguminosarum* bv. *phaseoli*, qui ont servi à inoculer les plantes-hôtes respectives lors des semencements. L'occupation des nodosités par les souches spécifiques a été examinée au cours de deux périodes d'échantillonnage. Les différences de sites et les traitements azotés n'ont pas eu d'effets significatifs sur la colonisation des rhizosphères par les trois souches, ni sur leur occupation des nodosités. Les trois souches d'inoculum ont colonisé les rhizosphères des hôtes respectifs dans des nombres approximativement égaux. Chez le soja, la souche TAL 102 (USDA 110) a occupé la majorité des nodosités, alors que la souche TAL 379 (USDA 1366) a été la moins compétitive pour la formation des nodosités dans tous les traitements. Chez le haricot, la souche TAL 182 a surpassé les deux autres souches pour la formation des nodosités, alors que la souche TAL 1797 (CIAT 899) a été la moins compétitive. Le niveau supérieur d'élevation a davantage réduit la formation de nodosités chez le soja que chez le haricot. Les applications d'azote ont réduit le nombre et la masse de nodosités chez les deux légumineuses, mais davantage dans le site de faible élévation; de plus, les réductions de masses de nodosités ont été plus prononcées chez le haricot. Le profil d'occupation des nodosités est demeuré stable, quelles qu'aient été les différences dans les nombres et les masses de nodosités entre les niveaux d'élevation, les concentrations en azote et les périodes d'échantillonnage.

Mots clés : écologie rhizobienne, colonisation des rhizosphères, compétitions intersouches, immunofluorescence, légumineuses.

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Introduction

Successful nodulation and nitrogen fixation by legumes inoculated with rhizobia depend on genetic and environmental factors. Once introduced, rhizobia must multiply in the rhizosphere

and infect their host plant to initiate the symbiotic processes. The genetic factors include the strain's competitiveness, its ability to survive in the soil, as well as its compatibility with the host (for reviews see Ham 1980; Dowling and Broughton 1986). Among the environmental factors, soil temperature, soil characteristics, indigenous rhizobial populations, and combined nitrogen have been reported to influence rhizobial ecology and the success of inoculation. However, evidence also indicates that the competition pattern by rhizobial strains for nodulation is a stable feature of indigenous populations (Demezas and Bottomley 1986; Kamicker and Brill 1986)

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or of multiple strains introduced as inoculum into soils devoid of the same species of rhizobia (George *et al.* 1987; Kossalak and Bohlool 1985; May and Bohlool 1983).

The symbiotic system, when beneficially established, operates at highest nitrogen-fixing efficiency in nitrogen-limiting systems, especially when other physiological requirements are adequately supplied. Combined nitrogen has been found to reduce nodulation and nitrogen fixation (Munns 1977). However, Semu *et al.* (1979) showed that nitrogen application of 50, 100, or 200 kg N · ha⁻¹ did not change the population size of *Bradyrhizobium japonicum* in a soybean field at three sites in 2 consecutive years. Evidence from studies in sand culture suggests that combined nitrogen can alter nodule occupancy of two strains on soybean (McNeil 1982). Gibson and Harper (1985) have also shown that different strains of *B. japonicum* have varied tolerances to external nitrogen application in their nodulation and nitrogen fixation characteristics.

The experiments described in this paper were designed to evaluate the effect of soil type, soil temperature due to elevation, and applied nitrogen on interstrain competition for rhizosphere colonization and nodule occupancy in field-grown soybean and common bean.

Materials and methods

Experimental design

The treatments were elevations of 320 and 1050 m; inoculated and uninoculated soybean, *Glycine max* (L.) Merrill, and common bean, *Phaseolus vulgaris* (L.); and nitrogen levels of 0, 40, and 300 kg N · ha⁻¹. The experimental design was a randomized complete block with three replications at each elevation. At each site, the treatments were arranged in a split-split plot with soybean and common bean assigned to main plots, inoculation to subplots, and the three levels of nitrogen to sub-sub plots.

Site characteristics and amendments

Two fields sites at elevations of 320 and 1050 m were selected on the island of Maui, Hawaii, and are referred to herein as Kuiaha and Olinda, respectively. The average soil/air temperatures during the experiment were 25/24°C at the Kuiaha site and 22/20°C at the Olinda site. The soil at the Kuiaha site was a clayey, ferritic, isohyperthermic Humoxic Tropohumult and that at the Olinda site was a medial over loamy-skeletal, mixed, isomesic Entic Dystrandept. The sites were determined to be free of indigenous rhizobia for soybean and common bean by the most probable number method (Wooster *et al.* 1988). Soil at both sites was amended before the start of the experiment to minimize differences in soil environment by equalizing pH and applying nutrients except N in recommended amounts (George *et al.* 1987). Soil pH at the two sites was adjusted between 5.5 and 6.0 by incorporating lime 4 weeks before planting. Nutrients added (kilograms per hectare) were 600 P; 300 K; 75 Mg; 25 Zn; 3 B; and 2 Mo. Extractable N (KCl) at planting was 0.05 and 0.10 g N · kg soil⁻¹ at the 320- and 1050-m site, respectively.

Inoculum strains

Bradyrhizobium japonicum strains TAL 102, TAL 377, and TAL 379 (USDA strains 110, 138, 136b, respectively) and *Rhizobium leguminosarum* bv. *phaseoli* strains TAL 182, TAL 1383, and TAL 1797 (NifTAL original, CIAT 632, and CIAT 899, respectively) were obtained from the NifTAL Project germ-plasm collection, Paia, Hawaii. All strains were serologically distinct.

Seed sources and inoculation method

Seeds of *Glycine max* (L.) Merrill 'Clark' (nodulating and nonnodulating isolines; USDA, Beltsville, MD) and *Phaseolus vulgaris* (L.) 'Brazil' (CIAT, Columbia) were increased in number at NifTAL for use in this experiment. Strains were checked for purity on yeast extract mannitol (YEM) agar containing either Congo red or bromothymol blue. Immunofluorescence (Schmidt *et al.* 1968) was used to verify the identity of each strain. Single-strain inoculants were prepared in

sterilized peat according to the protocol described by Somasegaran and Hoben (1985) and enumerated by spread plate method on YEM agar medium (Vincent 1970). Multistrain inoculants for soybean and common bean were prepared by mixing proportionate amounts of each peat culture to provide an equal number of each of the three strains in the final product.

Seeds were coated with gum arabic (Somasegaran and Hoben 1985) before adding the peat inoculum. The inoculated seeds were stored at 4°C and planted within 4 h.

Planting and nitrogen application

The two sites were planted within 7 days of each other. Seeds were sown in rows 60 cm apart in plots 9.5 x 3.0 m to attain a final plant population of 400 000 plants · ha⁻¹. The nonnodulating isolate of 'Clark' soybean and uninoculated common bean served as controls in the experiment. A drip-irrigation system was used to maintain soil moisture at approximately 3.0 x 10⁻⁴ Pa throughout the experiment. Nitrogen was applied at planting as (NH₄)₂SO₄ solution, uniformly sprayed onto the soil at rates of 3, 40, and 300 kg N · ha⁻¹.

Sampling of rhizosphere soil

Eight days after planting, 8 to 12 seedlings per replicate treatment were sampled at random for rhizosphere soil. Nonrhizosphere soil was removed from the root systems by gently shaking the loosely adhering soil off the root surface. Large soil aggregates that adhered to the root surfaces were then removed by hand. Rhizosphere soil was considered as soil which remained closely associated with the root after the shaking. The roots were kept in plastic bags and conveyed to the laboratory for recovery of rhizobia from the rhizosphere.

Recovery and treatment of rhizosphere soil

The entire contents of replicate plastic bags were treated for the recovery of inner rhizosphere soil by the procedure of Kingsley and Bohlool (1981), using the flocculating mixture of Demezas and Bottomley (1986). To calculate the dry weight of the rhizosphere soil, the remaining dispersed soil mixture was carefully washed into preweighed aluminum pans and dried at 100°C for 3 days. The weight was corrected for the amounts of solutes and the flocculating agent in the mixture.

Nodule sampling

Nodules were harvested from 8 to 12 roots collected at random from each inoculated plot at 21 days after planting (early) and at 50% flowering (late). After determining nodule number, nodules were oven-dried at 60°C for 48 h and weighed. A subsample of 30 nodules were then selected at random per inoculated plot and smears were prepared for identification of strains later.

Strain identification

Rhizosphere filter samples and nodule smears were treated with strain-specific fluorescent-antibody as described by Kingsley and Bohlool (1981) and Schmidt *et al.* (1968), respectively, using hydrolyzed gelatin-rhodamine conjugate to reduce nonspecific staining (Bohlool and Schmidt 1968). Fluorescent antibody treated rhizobia were observed with a Zeiss standard microscope 13 equipped with an Osram HBO 50-W mercury-vapor light source. Thirty microscopic fields were examined and counted per filter and cell populations were converted to cells per gram of oven-dried rhizosphere soil (Schmidt 1974). The recovery of added rhizobia (Kingsley and Bohlool 1981) (percent recovery ± standard deviation) was 67 ± 17 and 75 ± 21 % for *B. japonicum* and 61 ± 14 and 69 ± 9 % for *R. phaseoli* from the Kuiaha and Olinda soils, respectively. In the case of nodules, smears reacting with fluorescent antibodies of more than one strain were counted positive for each of the strains.

Statistical analysis

Analyses of variance were performed separately for legume species and sampling time. Data for each legume from individual sites were combined and subjected to analysis of variance (McIntosh 1983). Percent nodule occupancy data were transformed to arc sine and rhizosphere counts were transformed to log₁₀ before analysis of variance. When treatment effects or interactions were not significant, data were averaged for presentation in tables.

TABLE 1. summary of statistical significance for rhizosphere population (\log_{10}) and percent nodule occupancy by inoculum strains of *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. *phaseoli* in soils devoid of host rhizobia at two elevations at different applied N levels*

Source of variation	df	Rhizosphere population		% Nodule occupancy			
		<i>B. japonicum</i>	<i>R. leguminosarum</i> bv. <i>phaseoli</i>	<i>B. japonicum</i>		<i>R. leguminosarum</i> bv. <i>phaseoli</i>	
				Early†	Late‡	Early	Late
Elevation E	1	ns	**	ns	ns	ns	ns
Error A							
Block B \times (E)	4						
Nitrogen N	2	ns	ns	ns	ns	ns	ns
E \times N	2	ns	ns	*	ns	ns	ns
Error B							
E \times N \times B (E)	8						
Strain S	2	**	ns	***	***	***	***
E \times S	2	ns	**	ns	ns	ns	ns
N \times S	4	ns	ns	ns	ns	ns	ns
E \times N \times S	4	ns	ns	ns	ns	ns	ns
Error C							
(N) \times (E) \times B \times S	24						
CV (%)		2	1	17	26	22	16

NOTE: *, **, and ***, significant at the 0.05, 0.01, and 0.001 probability levels, respectively. ns, not significant. (N), (E) designate three N treatments and two elevations, respectively. All others are df.

*Analysis of variance was performed separately for *Rhizobium* sp. and sampling time.

†Early sampling was at 21 days after planting.

‡Late sampling was at flowering, which was 35 and 51 days after planting of soybean and 41 and 55 days after planting of common bean at 320- and 1050-m elevations, respectively.

Results and discussion

We studied rhizosphere colonization and interstrain competition for nodule occupancy by three inoculum strains each of soybean and common bean across differences in soil temperature, soil type, and applied N levels in the field. We generated the treatment differences by planting soybean and common bean in soils devoid of indigenous rhizobia at sites differing in soil type and temperature in an elevational transect and applying N at different rates. The effects of variables other than the treatment were minimized by equalizing pH between sites, planting different sites within a relatively short period of time, and by supplying adequate amounts of nutrients and water.

The results of this study showed that inoculum strains of both soybean and common bean colonized their respective host rhizosphere in equal numbers, but they differed significantly in their competitive ability in forming nodules. The competition pattern (the proportion of nodules formed by each of the three strains) was a stable character and was not related to rhizosphere colonization, environmental variables like soil type, soil temperature, and applied N, sampling time of nodules, and the number and mass of nodules.

The summary of statistical significance presented in Table 1 indicates that treatment effects were significantly different only in few instances. The most striking difference was in the competitive ability of the inoculum strains, reflected in their nodule occupancy. The few significant differences in rhizosphere populations observed between elevations and strains may have no biological significance, since the population of individual strains of both *B. japonicum* and *R. leguminosarum* bv. *phaseoli* varied within the same logarithmic (\log_{10}) scale (Table 2). The results also show that N application, even at the highest level, did not influence the colonization of the rhizosphere by

strains of either legume, a finding in agreement with that of Semu et al. (1979). Thus, the strains could be considered equally successful in host rhizosphere colonization despite differences in site environment and N level. In contrast, the percent of nodules occupied by each of three strains was significantly different, with the pattern being remarkably similar between elevations, N treatment, and sampling times for both legumes. Lack of correlation between rhizosphere populations and competitive success of rhizobial strains has been reported previously (Bushby 1984; Moawad and Bohlool 1984; Moawad et al. 1984; Robert and Schmidt 1983).

In soybean, TAL 102 was consistently a better competitor than TAL 377 and TAL 379 irrespective of site differences and rate of nitrogen application, occupying 68 to 78% of the nodules. George et al. (1987) also observed such dominance of TAL 102 in nodules of soybean from five maturity groups along the same elevational transect, thus establishing that site differences, soil type, and host genotype did not alter the competitive patterns of TAL 102, TAL 377, and TAL 379 in nodulation of soybean. In common bean, TAL 182 was the best competitor compared with TAL 1383 and TAL 1797, occupying a similarly high proportion of nodules as soybean. This strain has also been shown to be highly effective on several cultivars of common bean (Pacovsky et al. 1981). Abaidoo and van Kessel (1989) recently observed the same pattern of competitiveness for these strains when common bean was intercropped with maize.

Our treatments of elevation and N levels generated substantial differences in nodule number and weight in both legumes (Tables 3, 4, and 5). The higher elevation site, with a mean air temperature of 20°C, markedly decreased nodulation by both legumes, the effect being most pronounced on nodule mass of soybean. Similar results have been reported earlier by George

TABLE 2. Rhizosphere population (\log_{10}) and nodule occupancy (%) by inoculum strains of *Bradyrhizobium japonicum* and *Rhizobium leguminosarum* bv. *phaseoli* in soils devoid of host rhizobia at two elevations and different applied N levels*

Strain	Rhizosphere population†			% nodule occupancy	
	320 m	1050 m	Mean	Early‡	Late§
<i>B. japonicum</i>					
TAL 102	—	—	5.8(0.11)	68(6)	78(12)
TAL 377	—	—	5.7(0.08)	33(7)	25(8)
TAL 379	—	—	5.8(0.11)	37(6)	26(5)
<i>R. leguminosarum</i> bv. <i>phaseoli</i>					
TAL 182	5.7(0.07)	5.9(0.05)	—	68(7)	78(7)
TAL 1383	5.7(0.11)	5.8(0.06)	—	27(8)	28(6)
TAL 1797	5.8(0.10)	5.8(0.08)	—	6(2)	5(2)

NOTE: Values in parentheses are standard deviations.

*Means were averaged over elevations and nitrogen levels when their effects were not significantly different and unless significant interactions were encountered.

†Determined 8 days after seed inoculation.

‡Early sampling was at 21 days after planting.

§Late sampling was at flowering, which was 35 and 51 days after planting of soybean and 41 and 55 days after planting of common bean at 320- and 1050-m elevations, respectively.

TABLE 3. Summary of statistical significance for nodule numbers and nodule weight of soybean and common bean inoculated with rhizobia in soils devoid of host rhizobia at two elevations at different applied N levels*

Source of variation	df	Nodule number				Nodule weight	
		Soybean		Common bean		Soybean	Common bean
		Early†	Late‡	Early	Late		
Elevation E	1	*	*	**	*	***	*
Error A							
Block B × (E)	4						
Nitrogen N	2	**	***	**	***	***	***
E × N	2	*	ns	ns	***	***	***
Error B							
(E) × B × N	8						
CV (%)		19	16	24	7	8	14

NOTE: *, **, and ***, significant at the 0.05, 0.01, and 0.001 probability levels, respectively. ns, not significant. (N), (E) designate three N treatments and two elevations, respectively. All others are df.

*Analysis of variance was performed separately for *Rhizobium* sp. and sampling time.

†Early sampling was at 21 days after planting.

‡Late sampling was at flowering, which was 35 and 51 days after planting of soybean and 41 and 55 days after planting of common bean at 320- and 1050-m elevations, respectively.

TABLE 4. Effect of elevation and nitrogen application on nodule numbers (per plant) by soybean and common bean

Nitrogen applied (kg N · ha ⁻¹)	Soybean				Common bean			
	Early*		Late†		Early		Late	
	320 m	1050 m	320 m	1050 m	320 m	1050 m	320 m	1050 m
3	29(3)	15(3)	46(10)	28(9)	49(7)	25(7)	120(13)	56(11)
40	18(2)	17(2)	36(4)	20(6)	44(10)	22(3)	87(13)	67(12)
300	9(3)	14(4)	24(1)	18(3)	19(4)	13(4)	45(13)	42(7)

NOTE: Values in parentheses are standard deviation.

*Early sampling was at 21 days after planting.

†Late sampling was at flowering, which was 35 and 51 days after planting of soybean and 41 and 55 days after planting of common bean at 320- and 1050-m elevations, respectively.

TABLE 5. Effect of nitrogen application on nodule mass (mg/plant) of soybean and common bean at flowering stage

Nitrogen applied (kg N · ha ⁻¹)	Soybean		Common bean	
	320 m	1050 m	320 m	1050 m
3	188(21) ⁺	84(1)	170(22)	101(22)
40	150(13)	50(15)	116(19)	73(17)
300	66(6)	41(6)	9(2)	28(5)

NOTE: Values in parentheses are standard deviation.

et al. (1987) for five soybean cultivars grown in the same elevational transect. This study indicates that nodulation in common bean is also sensitive to low temperature.

The effect of applied nitrogen on legume-*Rhizobium* symbiosis is unequivocal. Nitrogen application resulted in reduced nodulation in soybean and common bean at both sampling times at both elevations. While N application reduced nodule number and mass by both legumes, the effect was more pronounced at the low-elevation than the high-elevation site. This may indicate that these legumes responded more readily to N application at the warmer site compared with the cool high-elevation site. At both elevations, nodule mass of common bean was greatly reduced by N application compared with soybean, indicating a greater response to N application by common bean.

Despite the substantial differences in nodule numbers between sites and N levels, the proportion of nodules formed by each strain remained stable. Moreover, the increase in nodule number with time also had no effect on the pattern of nodule occupancy. As reported earlier (George *et al.* 1987), the competition pattern seems to have no relationship with the number of nodules eventually formed by the plant.

Results in this paper suggest that despite the pronounced effect of elevation and nitrogen application on nodulation, the competitiveness of individual strains to form nodules on their respective hosts remained unaltered. Moreover, the population size of the strains in the rhizosphere had no influence on the proportion of nodules formed by each strain. Our results may have particular significance in strain selection for superior competitiveness. Under the experimental conditions we employed and with the strains used in this study, the outcome of interstrain competition was mostly determined by the genetic traits of the strains and their compatibility with the host.

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