

Quantitative Comparison of the Laboratory and Field Competitiveness of *Rhizobium leguminosarum* biovar *phaseoli*

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Rhizobium leguminosarum bv. *phaseoli* KIM5s outcompeted strain CE3 in bean (*Phaseolus vulgaris* L.) root nodulation when plants were grown at any of three field sites, each with a different soil type and indigenous population, or in the laboratory in a sterilized sand, a sterilized peat-vermiculite mixture, or a nonsterile field soil. A mathematical model describing nodulation competitiveness was empirically derived to evaluate the relative competitiveness of the two strains under these conditions. This model relates the proportional representation of the two strains in the inoculum to the proportional representation of nodules occupied by each strain or both strains and provides a measure of competitiveness, which is referred to as the competitiveness index. Statistical comparisons of competitiveness indices showed that the relative competitiveness of KIM5s and CE3 remained constant when the two strains were applied in a constant ratio over a range of inoculum concentrations, from 10^3 to 10^7 cells per seed, and when they were applied in various ratios to six *P. vulgaris* cultivars. Furthermore, the relative competitiveness of KIM5s and CE3 in the laboratory did not differ significantly from their relative competitiveness at the three field sites studied. Thus, a study of the basis for nodulation competitiveness of KIM5s and CE3 in the laboratory has the potential to provide an understanding of competitiveness both in the laboratory and in the field.

Rhizobial inoculants have the potential to reduce our dependence on nitrogenous fertilizers without sacrificing yields in the production of legumes. The success of inoculants requires that the inoculant strains be both highly effective in nitrogen fixation and highly competitive against the indigenous soil strains in nodule formation. Wild-type and mutant strains that are highly effective in fixing nitrogen have been identified (15, 33); however, their use in the field has often been unsuccessful because they do not compete well with the indigenous strains of rhizobia (6, 21). When highly effective inoculant strains are sufficiently competitive to nodulate a host in the presence of indigenous strains, inoculation can significantly increase the yield (33). In some fields, the indigenous strains may completely exclude the inoculant strains from the nodules of the host plant. For example, Kamicker and Brill (14) showed that of 543 nodules from Wisconsin soybean farms, none contained the commercial inoculant strains that were applied to the soybeans at planting. Thus, a major barrier to the successful use of inoculants is the inability of the inoculant strains to outcompete the indigenous strains of rhizobia in nodulation.

We define the relative nodulation competitiveness of two strains as the relationship between the proportional representation of the strains in the inoculum and the proportional representation of nodules occupied by each strain. A strain is a successful competitor against a second strain if the proportion of nodules it occupies is greater than its proportional representation in the inoculum.

Understanding the mechanisms involved in nodulation competitiveness may enable us to select or construct better inoculant strains. A genetic analysis directed toward elucidating these mechanisms requires two strains that exhibit a

reproducible and consistent relative competitiveness phenotype. Previous studies have shown that many factors can influence competitiveness. Biological factors such as bacteriophages (9) and epiphytic bacteria (11) can influence which strain predominates in the nodules. Environmental factors such as temperature (26), soil pH (8), and soil nitrate (17), as well as plant growth in the laboratory versus in the field (18) or at one field site versus another (16), can affect competitiveness. This variability means that it has been difficult to identify in the laboratory those genetic factors that contribute to competitiveness in the field.

To identify strains whose relative competitiveness is the same under a variety of conditions and that would therefore be appropriate for a genetic analysis of competitiveness, we must be able to measure competitiveness. Such a measure could be provided by a mathematical model that relates the proportional representation of a strain in the inoculum to the proportional representation of nodules occupied by that strain. Mathematical models have been presented that describe nodulation competitiveness (1, 32); however, they have not been used to evaluate the variability in the relative competitiveness of a pair of strains under a variety of conditions. Instead, these models have been used to compare either the competitiveness of various strains under a single set of conditions (1) or the competitiveness of particular strains relative to an indigenous population under a variety of conditions (32).

In this study, we have derived a mathematical model of competitiveness empirically and used it to evaluate the relative competitiveness of two *Rhizobium leguminosarum* bv. *phaseoli* strains in three plant growth matrices in the laboratory and at three field sites. We have demonstrated that the relative competitiveness of these two strains, KIM5s and CE3, does not differ significantly under these various laboratory and field conditions, which suggests that these

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TABLE 1. Summary of experiments

Plant growth conditions	Expt no.	Design ^a	Treatments (KIM5s:CE3)
Laboratory			
Sterilized sand	1	CRD (8/6)	10 ratios: 2,000:1, 1,000:1, 400:1, 200:1, 100:1, 40:1, 20:1, 2:1, 1:2.5, 1:5
Sterilized sand	2	CRD (8/6)	10 ratios: 15:1, 7:1, 3:1, 1.5:1, 1:1.4, 1:3, 1:7, 1:70, 1:350, 1:700
Sterilized Jiffy-Mix	3	CRD (8/6)	9 ratios: 750:1, 75:1, 8:1, 1.5:1, 1:1.3, 1:3, 1:12, 1:125, 1:1250
Unsterilized soil	4	CRD (9/6)	9 ratios: same as expt 3
Field			
Joy soil (1988)	5	RCB (8/2/6)	2 ratios: 7:1, 1:30
Colwood soil (1988)	6	RCB (8/2/6)	2 ratios: 7:1, 1:30
Plainfield sand (1988)	7	RCB (8/2/6)	14 ratios: 650:1, 65:1, 7:1, 1.6:1, 1:1.5, 1:3, 1:15, 1:30, 1:75, 1:150, 1:300, 1:1,500, 1:3,000
Plainfield sand (1988)	8	RCB (8/2/6)	9 concn ^b KIM5s: 1×10^9 , 1×10^8 , 6×10^7 , 1×10^7 , 1×10^6 , 1×10^5 , 1×10^4 , 1×10^3 , 6×10^2 8 concn ^b CE3: 2×10^9 , 2×10^8 , 2×10^7 , 2×10^6 , 2×10^5 , 2×10^4 , 2×10^3 , 2×10^2
Plainfield sand (1987)	9	RCB (4/8/4) ^c	1 ratio: 2:1
Plainfield sand (1987)	10	RCB (4/4/8) ^d	1 ratio: 1:11
Plainfield sand (1987)	11	RCB (4/2/6)	4 concn ^b KIM5s: 1×10^8 , 7×10^7 , 1×10^7 , 1×10^6 4 concn ^b CE3: 4×10^8 , 2×10^8 , 4×10^7 , 4×10^6

^a Experimental design: CRD, completely randomized design (no. of plants sampled for each treatment/no. of nodules taken per plant); RCB, randomized complete block design (no. of blocks/no. of plants sampled for each treatment in each block/no. of nodules taken per plant).

^b No. of cells per seed.

^c Two sets of four plants each were sampled per block.

^d Two sets of two plants each were sampled per block.

strains are suitable for a genetic study of nodulation competitiveness.

MATERIALS AND METHODS

Bacterial strains and plant cultivars. *R. leguminosarum* bv. *phaseoli* CE3 is a spontaneous streptomycin-resistant mutant of strain CFN42 (22). Strain KIM5s is a spontaneous spectinomycin-resistant mutant of KIM5 (13). Both mutants retained the symbiotic properties of their parent strains. Bacterial stock cultures were stored in 10% dimethyl sulfoxide at -80°C .

In experiments 1 to 8 and 11 (see Table 1), the common black bean (*Phaseolus vulgaris* L.) cultivar Puebla 152 was the host. In experiments 9 and 10, *P. vulgaris* cv. Puebla 152, ICA Pijao, Rio Tibagi, WBR22-03, WBR22-34, and WBR23-52 were the hosts. All cultivars were the generous gift of F. Bliss, Department of Pomology, University of California, Davis.

Plant growth conditions. In the laboratory, plants were grown under three sets of conditions: in an unsterilized sandy soil (Plainfield sand from Hancock, Wis.) in clay pots; in a sterilized mixture of sphagnum peat moss and vermiculite (Jiffy-Mix; Jiffy Products, Chicago, Ill.) in plastic cones (2.5 by 16 cm; Cone-tainers Nursery, Canby, Oreg.); and in sterilized sand and vermiculite (1:1 by volume) in modified Leonard jars (29). Matrices were sterilized by autoclaving. Plants in the untreated field soil were grown in a greenhouse and watered as needed. Plants in the sterilized matrices were grown in a growth chamber at 24°C with a 12-h photoperiod and watered with a nitrogen-free plant nutrient solution containing, per liter, 0.075 g of K_2SO_4 , 0.023 g of K_2HPO_4 , 0.137 g of KH_2PO_4 , 3.425 g of $\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$, 0.247 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.04 g of sodium iron EDTA, and 1 ml of micronutrient solution. The micronutrient solution contained (per liter) 0.15 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.44 g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.40 g of $\text{MnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02 g of $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24} \cdot \text{H}_2\text{O}$, and 1.43 g of H_3BO_3 . Seeds planted in the sterilized matrices were surface sterilized, immediately

before being planted, by being rinsed with ethanol for 30 s, 0.05% hypochlorite for 1 min, and sterile water for 1 min.

Field experiments in 1987 were all performed at the University of Wisconsin Experiment Station at Hancock, Wis., which has a sandy soil (Plainfield sand, mixed mesic, Typic Udipsamments, pH 6.2). Beans had not been grown at the site during the previous 2 years, but they had been grown and inoculated with a five-strain inoculant in 1984. Field experiments in 1988 were performed at three sites. The first site, at Hancock, Wis., had not been planted with beans for at least the previous 8 years. The second site, at the University of Wisconsin Experiment Station, Arlington, Wis., had a silty loam soil (Joy silt loam, mixed mesic, Typic Argiudolls, pH 6.8) that had not been planted with beans for the previous 10 years. The third site, in Madison, Wis., had a silty loam soil (Colwood silt loam, pH 7.4) that had been planted with beans 2 years before this study.

Experimental design. The treatments and designs for the experiments are summarized in Table 1. The experimental design for the laboratory experiments was a completely randomized design. For example, in experiment 1, each of the 10 treatments was applied to 8 plants, the 80 plants were completely randomized, and six nodules were removed from each plant. The proportion of nodules on a plant occupied by each strain was then determined as described below. Experiments 2 to 4 had designs similar to that of experiment 1. An additional experiment (not shown in Table 1) was performed which had the same design as experiment 1 and treatments consisting of four inoculum ratios of KIM5s and CE3 at various cell concentrations.

The experimental design for the field experiments was a randomized complete block design. For example, in experiment 7, the 14 treatments were randomized within each of eight blocks, each treatment was applied to two adjacent plants in each block, and six nodules were removed from each plant. The proportion of the 12 nodules from the two adjacent plants that was occupied by each strain was then determined. Experiments 5, 6, and 8 had designs similar to

that of experiment 7. In experiment 9, the inoculum was applied to two sets of four adjacent plants for each of six cultivars, the sets were completely randomized within each of eight blocks, and four nodules were removed from each plant. The proportion of the 16 nodules from the four adjacent plants that was occupied by each strain was determined. Experiment 10 had a similar design.

Assay for competitiveness. Inocula were prepared by growing each strain in yeast extract-mannitol (YM) broth (30) containing 5 µg of the appropriate antibiotic per ml. Cultures were grown with vigorous shaking on a rotary shaker at 28°C and then enumerated by dilution plating at the time of seed inoculation. Cultures of each strain were diluted in phosphate-buffered saline, and the strains were mixed in various proportions to prepare a range of inoculum ratios.

At the time of planting, 1 ml of inoculum containing 10^7 to 10^9 cells, unless otherwise stated, was applied directly to each seed before sowing. Plants were harvested 3 weeks (laboratory-grown plants) or 4 weeks (field-grown plants) after planting. Nodules were selected only from the crown region of each plant, since previous studies showed that bacteria applied to the seed are rarely found in nodules far from the crown (G. A. Beattie and J. Handelsman, unpublished data), perhaps owing to the limited ability of rhizobia to move in soil (10, 31). The nodules were frozen at -20°C in 20% glycerol (5) and were later thawed, sterilized, and plated as described previously (4). The nodule isolates were identified by growth on YM agar containing 250 µg of either streptomycin or spectinomycin per ml.

Serological identification of nodule isolates. Maintenance of bacterial antibiotic resistance during nodulation was measured by comparing nodule occupancy determined by antibiotic resistance with nodule occupancy determined by serology on the same nodules. KIM5-specific antiserum was raised by injecting 12-week-old New Zealand White rabbits at 10 subcutaneous sites with a total of 10^9 bacteria that had been washed with phosphate-buffered saline and suspended in an emulsion of 0.5 ml of phosphate-buffered saline and 0.5 ml of Freund complete adjuvant (Sigma Chemical Co., St. Louis, Mo.). Booster injections of bacteria suspended in Freund incomplete adjuvant were administered in the same manner at 4-week intervals until the serum titer was at least 1:1,000 as determined by enzyme-linked immunosorbent assay (20) with alkaline phosphatase-conjugated goat anti-rabbit immunoglobulin G antibodies and 4-methylumbelliferyl phosphate as the substrate. The rabbits were bled through the central ear vein.

CE3-specific antiserum was raised by injecting 8-week-old BALB/c mice intraperitoneally at 4-week intervals with 1.0 ml containing 10^7 bacteria, prepared as described above. When the serum titer reached 1:1,000, an intravenous injection of 0.1 ml containing 10^6 bacteria in phosphate-buffered saline was administered through the tail vein. Four days later, the mice were anesthetized with ether and bled through the orbital vein, and the serum titer was determined as above, except that alkaline phosphatase-conjugated goat anti-mouse immunoglobulin G antibodies were used.

Nodule occupancy was determined by plating nodule contents on a selective medium (YM containing either streptomycin or spectinomycin) and by enzyme-linked immunosorbent assay. Six nodules from each of 15 plants that had been inoculated with either KIM5s or CE3 in an untreated field soil were subjected to the double analysis.

Estimation of the indigenous populations. The most-probable-number technique (25) was used to estimate the indigenous *R. leguminosarum* bv. *phaseoli* populations at each

field site in 1988. A soil sample was taken from within each of the eight blocks in each experimental plot. Each sample was taken from a 3-in. (7.62-cm) core, the top of which was 1 in. (2.54 cm) below the soil surface, and a subsample was oven dried (110°C) for 24 h to determine the moisture content. Serial fourfold dilutions were prepared from each sample by using an equivalent of 1 g (dry weight) of soil. A 1-ml sample of each dilution was applied directly to the roots of pregerminated seeds in glass tubes (2.5 by 20 cm) that contained sterilized sand and vermiculite (1:1 by volume) and were closed with foam plugs (3). Two replicates were prepared for each dilution. Most-probable-number population estimates were made 18 days after inoculation.

RESULTS

Stability of antibiotic resistance markers during nodulation.

To determine whether the antibiotic resistance markers were maintained during nodulation, the nodule isolates from plants inoculated with either KIM5s or CE3 were identified by both antibiotic resistance and serology. On plants inoculated with KIM5s in an untreated field soil, KIM5s occupied $97 \pm 3\%$ (standard error) of the nodules on each plant based on identification by growth on spectinomycin and $90 \pm 5\%$ based on identification by serology. On plants inoculated with CE3 in an untreated field soil, CE3 occupied $64 \pm 9\%$ of the nodules on each plant based on identification by growth

TABLE 2. Percentage of nodules occupied by KIM5s and CE3 after coinoculation in various ratios

$I_{KIM5s}:I_{CE3}^a$	KIM5s:CE3:both ^b
Sterilized sand (expt 2)	
15:1.....	97:0:3
7:1.....	96:0:4
3:1.....	84:6:10
1.5:1.....	92:4:4
1:1.4.....	88:8:4
1:3.....	90:7:3
1:7.....	69:17:14
1:70.....	50:50:0
1:350.....	48:44:8
1:700.....	60:27:13
Unsterilized field soil (expt 4)	
750:1.....	78:0:0
75:1.....	89:0:0
8:1.....	83:0:6
1.5:1.....	75:4:5
1:1.3.....	79:2:5
1:3.....	70:11:11
1:12.....	78:13:4
1:125.....	49:26:4
1:1,250.....	11:60:7
Field—Plainfield sand (expt 7)	
650:1.....	67:2:1
65:1.....	66:0:1
7:1.....	74:1:2
1:1.5.....	75:1:5
1:3.....	60:6:11
1:15.....	47:24:12
1:75.....	37:24:7
1:150.....	44:12:9
1:300.....	20:31:9
1:1,500.....	9:31:1

^a Ratio of KIM5s to CE3 in inoculum.

^b Percentage of nodules occupied by KIM5s alone, CE3 alone, and both KIM5s and CE3.

TABLE 3. Percentage of nodules occupied by KIM5s and CE3 after coinoculation in a range of ratios and inoculum concentrations

$I_{KIM5s}:I_{CE3}^a$	Inoculum concn (cells/seed)	KIM5s:CE3:both ^b
8:1	10^7	$(80 \pm 8):(0 \pm 0):(20 \pm 8)$
	10^6	$(84 \pm 5):(4 \pm 4):(12 \pm 5)$
	10^5	$(85 \pm 5):(0 \pm 0):(15 \pm 5)$
	10^4	$(91 \pm 5):(0 \pm 0):(9 \pm 5)$
	10^3	$(83 \pm 7):(0 \pm 0):(17 \pm 7)$
1:1	10^7	$(75 \pm 7):(5 \pm 3):(20 \pm 7)$
	10^6	$(58 \pm 5):(6 \pm 3):(35 \pm 6)$
	10^5	$(66 \pm 9):(6 \pm 4):(28 \pm 6)$
	10^4	$(62 \pm 12):(8 \pm 4):(30 \pm 10)$
	10^3	$(76 \pm 11):(7 \pm 5):(17 \pm 7)$
1:4	10^9	$(53 \pm 6):(12 \pm 6):(35 \pm 8)$
	10^8	$(63 \pm 6):(10 \pm 4):(27 \pm 4)$
	10^7	$(61 \pm 19):(13 \pm 10):(26 \pm 16)$
	10^6	$(60 \pm 9):(11 \pm 4):(28 \pm 7)$
	10^5	$(56 \pm 10):(4 \pm 4):(40 \pm 10)$
	10^4	$(64 \pm 11):(14 \pm 7):(23 \pm 7)$
	10^3	$(57 \pm 10):(17 \pm 8):(26 \pm 10)$
1:13	10^7	$(54 \pm 11):(28 \pm 8):(18 \pm 6)$
	10^6	$(44 \pm 7):(24 \pm 7):(30 \pm 7)$
	10^5	$(52 \pm 6):(9 \pm 4):(39 \pm 4)$
	10^4	$(55 \pm 9):(21 \pm 5):(25 \pm 7)$
	10^3	$(50 \pm 15):(39 \pm 13):(11 \pm 4)$

^a Ratio of KIM5s to CE3 in inoculum.^b Percentage of six nodules per plant occupied by KIM5s alone, CE3 alone, and both KIM5s and CE3. Values represent the mean \pm standard error for eight plants.

on streptomycin and $76 \pm 5\%$ based on identification by serology. These results indicate that the antibiotic resistance markers in KIM5s and CE3 were maintained during nodulation.

Competition studies with KIM5s and CE3. In experiments in which KIM5s and CE3 were applied to plants in a range of ratios, KIM5s occupied the majority of the nodules unless it was outnumbered in the inoculum by CE3 by at least 70:1 in sterilized sand or Jiffy-Mix or by greater than 125:1 in unsterilized soil. Table 2 shows the percentage of nodules occupied by KIM5s and CE3 in experiments 2, 4, and 7; similar percentages were observed in experiments 1, 3, 5, and 6. In experiments in which KIM5s and CE3 were applied to plants in four ratios and over a range of total bacterial concentrations at each ratio, KIM5s occupied the majority

of nodules at every bacterial concentration tested, even when outnumbered 13:1 by CE3 at each concentration (Table 3). Thus, in these experiments, KIM5s was consistently more competitive than CE3.

Development of a quantitative model of competitiveness. To quantify the relative competitiveness of KIM5s and CE3, we developed a model that related the ratio of the cells of each strain in the inoculum ($I_{KIM5s}:I_{CE3}$) to the proportion of nodules occupied by each strain, P_{KIM5s} and P_{CE3} . Regression was the method of analysis, and several models were considered. All of the models involved regressing a function of the nodule occupancies of the strains on a function of the inoculum ratio (Table 4). In one model studied, the ratio of the proportion of nodules occupied by each strain, $P_{KIM5s}:P_{CE3}$, was regressed on the logarithm of the ratio of the two strains in the inoculum, $I_{KIM5s}:I_{CE3}$. Other models studied involved the arc sine square root or the logarithmic transformation of $P_{KIM5s}:P_{CE3}$ regressed on $\log(I_{KIM5s}:I_{CE3})$. In other variations, each of the five nodule occupancy terms P_{KIM5s} , P_{CE3} , $(P_{KIM5s} + P_{both}):(P_{CE3} + P_{both})$, $P_{KIM5s}:(P_{KIM5s} + P_{CE3} + P_{both})$, and $P_{CE3}:(P_{KIM5s} + P_{CE3} + P_{both})$, where P_{both} was the proportion of nodules occupied by both KIM5s and CE3, was substituted for $P_{KIM5s}:P_{CE3}$ and the same transformations were applied. In yet another model studied, each of the nodule occupancy terms was regressed on both the $\log(I_{KIM5s}:I_{CE3})$ and its square to consider a quadratic fit, or on the $\log(I_{KIM5s}:I_{CE3})$, its square, and its cube to consider a cubic fit. The data used for these regressions were from experiments 1 to 7. To apply the logarithmic transformation, values of P_{KIM5s} or P_{CE3} that were 0/ N , where N was the number of nodules sampled, were converted to $0.5/N$. Also, values of P_{KIM5s} or P_{CE3} that were N/N , or 1, were converted to $(N-0.5)/N$ (24).

In cubic regressions applied to the data from each experiment, the cubed term was not significant ($P > 0.05$) for any of the nodule occupancy terms considered with any transformation in any of the experiments. In quadratic regressions, the squared term was not significant ($P > 0.05$) for any of the nodule occupancy terms considered with any transformation in any experiment except experiment 4. These significance levels were determined by testing whether the coefficient for the appropriate term differed significantly from zero (7). Since the quadratic term was not consistently significant and did not greatly improve the fit in the models in which it was significant, we chose simple linear regression for the analysis.

To identify the nodule occupancy term and transformation that provided the best-fitting model, we performed linear regressions on the data from each experiment of experiments

TABLE 4. Coefficients from the linear regressions of various nodule occupancy terms on $\log(I_{KIM5s}:I_{CE3})$ for the data from experiments 1 to 7

Nodule occupancy term	T ^a	k ^b	$C_{KIM5s:CE3}^b$	R ^{2c}
$(P_{KIM5s} + P_{both}):(P_{CE3} + P_{both})$	L	0.23 ± 0.02	0.59 ± 0.03	42
$P_{KIM5s}:P_{CE3}$	L	0.24 ± 0.02	0.69 ± 0.03	38
$P_{CE3}:(P_{KIM5s} + P_{CE3} + P_{both})$	L	-0.15 ± 0.01	-0.90 ± 0.02	36
$P_{KIM5s}:(P_{KIM5s} + P_{CE3} + P_{both})$	L	0.09 ± 0.01	-0.21 ± 0.01	30
P_{CE3}	L	-0.13 ± 0.01	-0.98 ± 0.02	30
	A	-0.07 ± 0.01	0.38 ± 0.01	30
P_{KIM5s}	L	0.11 ± 0.01	-0.29 ± 0.02	31
	A	0.12 ± 0.01	0.94 ± 0.02	39

^a T, Transformation. L = Logarithm, A = arc sine of the square root.^b k and $C_{KIM5s:CE3}$ are, respectively, the slope and the intercept, with their respective standard errors, of the equation from the regression of the transformed nodule occupancy term on $\log(I_{KIM5s}:I_{CE3})$.^c R², Coefficient of determination.

TABLE 5. Coefficients from the linear regressions of $\log(P_{KIM5s} + P_{both}) : (P_{CE3} + P_{both})$ on $\log(I_{KIM5s} : I_{CE3})$

Plant growth conditions	Expt no.	k^a	$C_{KIM5s:CE3}^a$
Laboratory			
Sterilized sand	1	0.17 ± 0.03	$0.52 \pm 0.06a$
Sterilized sand	2	0.23 ± 0.05	$0.70 \pm 0.06b$
Sterilized Jiffy-Mix	3	0.28 ± 0.02	$0.61 \pm 0.04ab$
Unsterilized soil	4	0.34 ± 0.04	$0.81 \pm 0.06b$
Overall laboratory	1-4	0.19 ± 0.02	0.60 ± 0.03
Field			
Silt loam (Joy)	5	0.34 ± 0.06	$0.91 \pm 0.07b$
Silt loam (Colwood)	6	0.14 ± 0.11	$0.83 \pm 0.13b$
Loamy sand (Plainfield)	7	0.27 ± 0.03	$0.55 \pm 0.06a$
Overall field	5-7	0.28 ± 0.03	0.65 ± 0.05

^a k and $C_{KIM5s:CE3}$ are, respectively, the slope and the intercept of equation 1 with their respective standard errors. Values followed by the same letter do not differ significantly at $P = 0.05$.

1 to 7. The model involving $(P_{KIM5s} + P_{both}) : (P_{CE3} + P_{both})$ had the largest coefficient of determination (R^2) in five of the seven experiments and a fairly large coefficient of determination in each of the remaining two experiments. We also combined the data from experiments 1 to 7 into one large set. In linear regressions performed on this data set, the model involving $(P_{KIM5s} + P_{both}) : (P_{CE3} + P_{both})$ as the dependent variable had the largest coefficient of determination (Table 4). Thus, the model that most consistently fit with the data from these seven experiments was as follows:

$$\log\left(\frac{P_{KIM5s} + P_{both}}{P_{CE3} + P_{both}}\right) = C_{KIM5s:CE3} + k \cdot \log\left(\frac{I_{KIM5s}}{I_{CE3}}\right) \quad (1)$$

From this equation, $C_{KIM5s:CE3}$ can be defined as a competitiveness index; if $I_{KIM5s} = I_{CE3}$, a positive value of $C_{KIM5s:CE3}$ would indicate that KIM5s occupies a larger proportion of the nodules than CE3 and therefore that KIM5s is more competitive than CE3. Thus, for any ratios of strains applied in an experiment, equation 1 can be used to extrapolate the value of $C_{KIM5s:CE3}$ when $I_{KIM5s} = I_{CE3}$. Similar interpretation of this equation was presented by Amarger and Lobreau (1). The slope of the line, k , indicates the rate at which the nodule occupancy ratio changes as the inoculum ratio changes.

Application of the model to competition studies. The values of k and $C_{KIM5s:CE3}$ for experiments 1 to 7 are shown in Table 5. Under all plant growth conditions tested, $C_{KIM5s:CE3}$ was significantly greater than zero. These positive values demonstrate that KIM5s was consistently more competitive than CE3. To calculate competitiveness indices for the experiments involving only one inoculum ratio, the value of k from the regression performed on the data from experiments 1 to 7, $k = 0.226$ (Table 4), was assumed and the equation was applied to the raw data from each experiment. The values of $C_{KIM5s:CE3}$ calculated in this manner were also positive: $C_{KIM5s:CE3} = 1.11 \pm 0.13$ and 1.08 ± 0.36 for experiments 9 and 10, respectively.

Differences among the slopes and the competitiveness indices for the seven experiments were evaluated with SAS software (23) by partitioning the total variability in the experiments into portions resulting from the experiments

TABLE 6. Competitiveness of KIM5s and CE3 at various inoculum concentrations

Inoculum concn (cells/seed)	k^a	$C_{KIM5s:CE3}^a$
10^7	0.21 ± 0.12	0.54 ± 0.09
10^6	0.26 ± 0.07	0.44 ± 0.05
10^5	0.22 ± 0.07	0.47 ± 0.05
10^4	0.28 ± 0.08	0.56 ± 0.07
10^3	0.30 ± 0.11	0.51 ± 0.08

^a k and $C_{KIM5s:CE3}$ are, respectively, the slope and intercept of equation 1 with their respective standard errors.

(experiment effect), the inoculum ratios (treatment effect, treated as a continuous predictor variable), the inocula used in each experiment (experiment \times treatment effect), and, when present, the blocks in each field experiment (block effect). There was no evidence of differences among the blocks ($P = 0.54$), as determined by using the block effect. By using the experiment \times treatment effect to test for differences among the slopes for each experiment, we found that $P = 0.05$, which we regard as providing at most weak evidence for differences among the slopes when taking into account the degrees of freedom for error ($df = 262$). There was strong evidence of differences among the competitiveness indices of the various experiments ($P = 0.008$); these differences are shown in Table 5.

Although there were significant differences among the competitiveness indices in experiments 1 to 7, the differences were not related to laboratory versus field conditions for plant growth (Table 5). To test this relationship directly, we further partitioned the variability among the experiments into two components: a location effect (laboratory versus field), and a within-location effect due to the matrix used (matrix effect). Averaging over the matrices within each location, there was no evidence of a difference between the competitiveness indices in the laboratory and in the field ($P = 0.17$) as determined by using the location effect (7). A regression performed on the data set that resulted from combining the data from all the laboratory experiments gave $C_{KIM5s:CE3} = 0.60 \pm 0.03$. A regression performed on a similar data set for the field experiments gave $C_{KIM5s:CE3} = 0.65 \pm 0.05$ (Table 5).

Similar analyses showed that the competitiveness indices of KIM5s and CE3 did not differ significantly when the strains were applied at various inoculum concentrations ($P = 0.77$ [Table 6]) or on various host cultivars ($P = 0.34$ [Table 7]). In the results shown in Table 3, the percentage of nodules occupied by each strain did not vary over the range of concentrations tested for each ratio ($P > 0.1$) as determined by a one-way analysis of variance (24). This further

TABLE 7. Competitiveness of KIM5s and CE3 on various host cultivars^a

Cultivar	k^b	$C_{KIM5s:CE3}^b$
Puebla 152	0.06 ± 0.15	0.90 ± 0.12
ICA Pijao	0.20 ± 0.20	0.90 ± 0.15
Rio Tibagi	0.20 ± 0.15	0.95 ± 0.12
WBR22-03	0.45 ± 0.10	1.21 ± 0.08
WBR22-34	0.17 ± 0.18	0.85 ± 0.14
WBR23-52	0.52 ± 0.14	1.00 ± 0.11

^a Data from experiments 10 and 11.

^b k and $C_{KIM5s:CE3}$ are, respectively, the slope and intercept of equation 1 with their respective standard errors.

showed that the inoculum concentration does not have an effect on the relative competitiveness of KIM5s and CE3.

Competitiveness of KIM5s and CE3 against indigenous soil strains. In experiments 8 and 11, the relative competitiveness of KIM5s and CE3 was examined indirectly by testing each of them against an indigenous *R. leguminosarum* bv. *phaseoli* soil population. The raw data from the experiments showed that KIM5s was more competitive than CE3 against the indigenous *R. leguminosarum* bv. *phaseoli* populations present at the University of Wisconsin Experiment Station, Hancock, Wis., in 1987 and 1988 (data not shown). For example, in both years KIM5s occupied between $33 \pm 7\%$ and $68 \pm 5\%$ of the nodules, whereas CE3 occupied between $3 \pm 2\%$ and $17 \pm 8\%$ of the nodules, when the inoculum concentration was 10^5 to 10^6 cells per seed.

To develop a model to measure the competitiveness of a single strain against an indigenous population, we regressed $\log [P_A/(1-P_A)]$, the arc sine of the square root of P_A , and $\log (P_A)$ on $\log (I_A/I_S)$, where P_A was the proportion of nodules occupied by strain A, I_A was the number of cells applied to the seed, and I_S was the number of indigenous *R. leguminosarum* bv. *phaseoli* cells per gram of soil. We used data only from experiment 8, since the indigenous population was not enumerated for experiment 11. The fit with $\log [P_A/(1-P_A)]$ had the largest coefficient of determination with either KIM5s or CE3 as strain A. In a cubic regression, neither the square nor the cube of $\log (I_A/I_S)$ was significant ($P > 0.05$) (24) for either strain. Thus, the model that best fit the data from experiment 8 is as follows:

$$\log [P_A/(1 - P_A)] = C_{A:S} + k \cdot \log (I_A/I_S) \quad (2)$$

For KIM5s, $C_{KIM5s:S}$ was -0.35 ± 0.07 and k was 0.30 ± 0.03 . For CE3, $C_{CE3:S}$ was -0.91 ± 0.06 and k was 0.22 ± 0.03 . Thus, $C_{KIM5s:S}$ was greater than $C_{CE3:S}$ ($P < 0.0001$) for the population present in experiment 8. When this model was applied to the data from experiment 11 and values between 10^3 and 10^6 cells per g of soil were used as I_S , $C_{KIM5s:S}$ was significantly greater than $C_{CE3:S}$ ($P = 0.0006$) for every value of I_S used. Thus, for both experiments 8 and 11, a greater value of $C_{KIM5s:S}$ than $C_{CE3:S}$ indicates that KIM5s was more competitive than CE3 against both indigenous populations.

The indigenous *R. leguminosarum* bv. *phaseoli* populations were 3.0×10^3 cells per g of soil at the field site for experiment 5, 1.7×10^4 cells per g of soil for experiment 6, and 1.7×10^5 cells per g of soil for experiments 7 and 8. In the direct competitions between KIM5s and CE3 at these field sites, there was a correlation between a small indigenous *R. leguminosarum* bv. *phaseoli* population and a large value of $C_{KIM5s:CE3}$ (Table 5).

DISCUSSION

We define the relative nodulation competitiveness of two strains as the relationship between the proportional representation of the strains in the inoculum and the proportional representation of nodules occupied by each strain. The model

$$\log \left(\frac{P_A + P_{\text{both}}}{P_B + P_{\text{both}}} \right) = C_{A:B} + k \cdot \log \left(\frac{I_A}{I_B} \right) \quad (3)$$

provides a means of measuring the relative competitiveness of strains A and B. P_A and P_B are the proportion of nodules occupied by A and B, respectively; P_{both} is the proportion of nodules occupied by both A and B; $C_{A:B}$, the intercept of the

line, is the competitiveness index; k is the slope; and I_A and I_B are the concentrations of A and B, respectively, in the inoculum. A positive value of $C_{A:B}$ indicates that A would occupy a larger proportion of the nodules than B when $I_A = I_B$ and therefore that A is more competitive than B. Equation 3 thus provides a way to directly determine the relative competitiveness of A and B for any ratios of strains applied in an experiment. An indirect comparison of the relative competitiveness of A and B can be made by using the model presented in equation 2. If A and B each compete against the same population S, a value of $C_{A:S}$ greater than $C_{B:S}$ indicates that A is more competitive than B against the population S, which suggests that A is more competitive than B.

The mathematical model describing the relative competitiveness of two strains (equation 3) was derived from the competition experiments with KIM5s and CE3 and is similar to the model previously described by Amarger and Lobreau (1). In the Amarger and Lobreau model, the nodule occupancy term includes only the number of nodules occupied exclusively by each strain, whereas in our model the nodule occupancy term includes both the proportion of nodules occupied exclusively by one strain and the proportion of nodules occupied by more than one strain. The frequency of double occupancy with KIM5s and CE3 on the cultivars of *P. vulgaris* that we tested was high. Double occupancy by KIM5s and CE3 ranged from 0 to 16% in unsterilized field soil and from 0 to 40% in sterilized matrices on these cultivars (Tables 2 and 3; data not shown for other cultivars). Thus, our model was appropriate for this system, in which there was a high frequency of double nodule occupancy.

In direct competitions between KIM5s and CE3 (Tables 5 to 7), $C_{KIM5s:CE3}$ was positive under all conditions tested, thus indicating that KIM5s was consistently more competitive than CE3. Others have also shown that KIM5s is a highly competitive strain (13; D. F. Bezdicsek, personal communication). In competitions between each of KIM5s and CE3 and a reference *R. leguminosarum* bv. *phaseoli* soil population, S, $C_{KIM5s:S}$ was larger than $C_{CE3:S}$ for both populations tested and hence KIM5s was also more competitive than CE3 against the indigenous soil populations. The competitiveness indices representing the relative competitiveness of KIM5s and CE3 were not affected by inoculum concentration or by host cultivar (Tables 6 and 7). Thus, a genetic analysis of the competitiveness of KIM5s and CE3 performed on a single cultivar with one inoculum concentration should be relevant to the other cultivars and inoculum concentrations. Moreover, such an analysis should be relevant to the competitiveness of the two strains under a variety of conditions, including various soil types and with various indigenous populations.

Previous studies have identified factors that contribute to competitiveness. These factors include motility (2, 19), cell surface polysaccharides (12, 28), and bacteriocin production (27). However, the importance of these factors for competitiveness in the field has not been demonstrated. The quantitative evaluation presented in this paper showed that the relative competitiveness of KIM5s and CE3 as measured under a variety of laboratory conditions did not differ significantly from the competitiveness under a variety of field conditions. The consistency in their relative competitiveness distinguishes KIM5s and CE3 from many other *Rhizobium* strains, whose competitiveness varies with environmental conditions. This system is appropriate for a study of the mechanisms of competitiveness, since a genetic study of the nodulation competitiveness of KIM5s and CE3 per-

formed in the laboratory has the potential to identify genes that are important for nodulation competitiveness both in the laboratory and in the field.

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