Competitive Ability and Efficiency in Nodule Formation of Strains of *Bradyrhizobium japonicum*†

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In the American Midwest, superior N₂-fixing inoculant strains of *Bradyrhizobium japonicum* consistently fail to produce the majority of nodules on the roots of field-grown soybean. Poor nodulation by inoculant strains is partly due to their inability to stay abreast of the expanding soybean root system in numbers sufficient for them to be competitive with indigenous bradyrhizobia. However, certain strains are noncompetitive even when numerical dominance is not a factor. In this study, we tested the hypothesis that the nodule occupancy achieved by strains is related to their nodule-forming efficiency. The nodulation characteristics and competitiveness of nine strains of *B. japonicum* were compared at both 20 and 30°C. The root tip marking technique was used, with the nodule-forming efficiency of each strain estimated from the average position of the uppermost nodule and the number of nodules formed above the root tip mark. The competitiveness of the nine strains relative to *B. japonicum* USDA 110 was determined by using immunofluorescence to identify nodule occupants. The strains differed significantly in competitiveness with USDA 110 and in nodulation characteristics, strains that were poor competitors usually proving to be inferior in both the average position of the uppermost root node and the number of nodules formed above the root tip mark. Thus, competitiveness was correlated with both the average position of the uppermost nodule (*r* = 0.5; *P* = 0.036) and the number of nodules formed above the root tip mark (*r* = 0.64; *P* = 0.005), while the position of the uppermost nodule was also correlated to the percentage of plants nodulated above the root tip mark (*r* = 0.81; *P* < 0.001) and the percentage of plants nodulated on the taproot (*r* = 0.67; *P* = 0.002).

The ability of certain strains of *Rhizobium* and *Bradyrhizobium* to dominate nodulation in a multistrain environment has been termed competitiveness and has been documented for strains of *Bradyrhizobium japonicum* (7, 12, 13, 16), *Rhizobium meliloti* (20), and *R. leguminosarum* biovars *phaseoli* (24a), trifolii (8), and vicieae (1). While the basis for strain competitiveness is unknown, recent reports suggest that the apparent noncompetitiveness of inoculant strains under field conditions is due, at least in part, to their inability to keep abreast of the expanding root system (21, 29). However, these studies do not account for the competitive success of certain rhizobial strains in situations in which numerical dominance is not considered to be a factor (23).

Studies by Bhuvaneshwari et al. (3, 5) with plants on which the root tip position at the time of inoculation was marked have shown that the infectible region in soybean is restricted to the preemergent root hair zone immediately above the root tip, with cells in this region open to infection for only 4 to 6 h (5). Given such developmental constraints, one explanation for competitive ability might be that competitive strains are more efficient in nodule formation, either needing few cells to initiate infection or completing the complex sequence of infection events with minimal delay. This has been briefly considered by both Dowling and Broughton (9) and Hodgson and Stacey (15), while a positive relationship between traits indicative of efficient nodule formation and strain competitiveness has been reported for *R. meliloti* (14), *R. leguminosarum* biovar *trifolii* (27), and *R. leguminosarum* biovar *phaseoli* (24a). The present study examines the relationship between efficiency in nodule formation, estimated by both the average position of the uppermost nodule relative to the root tip mark (RTM) and the number of nodules produced above the RTM, and strain competitiveness in *B. japonicum*.

**MATERIALS AND METHODS**

**Cultures.** *B. japonicum* USDA 38, USDA 66A, USDA 110, and USDA 135 were obtained from H. H. Keyser, U.S. Department of Agriculture, Beltsville, Md.; strains INPA 11 and INPA 37 were from L. A. de Oliveira, INPA, Manaus, Brazil; strain CIAT 51 (synonym 5006) was from G. Ocampo, CIAT, Cali, Colombia; strain CR 3425 was from the culture collection of the Rothamsted (United Kingdom) experiment station; strain CB 1809 was from R. A. Date, Commonwealth Scientific and Industrial Research Organisation, St. Lucia, Australia; and strain UMR 161 was from nodules collected in China. All strains were maintained on yeast extract-manitnol [11] agar.

**Competition studies.** Three-liter pots were filled with sand and vermiculite (9:1 by volume), placed in paper bags, and autoclaved for 2 h at 121°C. Seeds of soybean (*Glycine max* [L.] Merr.), cultivar Fiskeby-V, were surface sterilized by immersion in 95% ethanol (30s) followed by 5.25% sodium hypochlorite (5 min) and then washed with six changes of sterile deionized water. Five seeds were planted per pot. For liquid inoculants, each of the test strains was grown to early log phase in yeast extract-manitnol broth (48 h for all strains save USDA 135, which required 72 h), and then total cell count was estimated by optical density at 620 nm and adjusted to 10⁷ cells ml⁻¹ with phosphate-buffered saline. Each strain was then mixed 1:1 with the standard competitor strain USDA 110 and applied to soil at the rate of 10⁴ cells of each organism g of sand-vermiculite⁻¹. Each treatment was replicated four times. To determine viable cell counts and...
the actual ratio of test strain/USDA 110 in the inoculant, all counts were verified by serial dilution and the plating of aliquots on yeast extract-mannitol agar. Duplicate experiments were conducted at 20 and 30°C in identical but separate Convirion model E15 growth chambers. A photoperiod of 14 h and a light intensity of 600 μmol of photons m⁻² s⁻¹ were used. Pots were watered with a plant nutrient solution modified from those of Summerfield et al. (26) and Smith et al. (28) which contained the following (in milligrams per liter): Ca(HPO₄)₂ • H₂O, 100; Ca(NO₃)₂ • 4H₂O, 58; CaSO₄ • 2H₂O, 200; MgSO₄ • 7H₂O, 250; K₂HPO₄ • 3H₂O, 100; NaFeEDTA, 25; K₂S, 270; KCl, 5.58; H₂BO₃, 5; MnSO₄ • H₂O, 2.5; ZnSO₄ • 7H₂O, 0.54; NH₄NO₃, 24; H₂O, 0.54; CuSO₄ • 5H₂O, 0.5; H₂SO₄, 3.95; pH 6.0. Plants were thinned to three plants per pot at 10 and 14 days after planting and harvested at 21 and 35 days after planting for the 30 and 20°C experiments, respectively.

For determination of nodule occupancy, spherical non-lobed taproot nodules were surface sterilized in 5.25% sodium hypochlorite, washed five times with sterile deionized water, crushed, and then smeared onto duplicate microscopy slides. Gelatin-rhodamine isothiocyanate conjugate was applied to suppress nonspecific staining (6); then the slides were incubated at room temperature to near dryness, and stain-specific fluorescent antibodies (FAs), prepared by the procedures of Belser and Schmidt (2), were applied. For strains INPA 11, USDA 66A, CB 1809, and RCR 3425, for which FAs were not available, only a single smear was prepared and treated with USDA 110 FA. Stained smears were viewed with a Nikon Labophot microscope outfitted for epifluorescence, using a Nikon glycerin immersion (×100) objective with the iris fully open. Under these conditions nonreactive cells were clearly discernible and ranged in color from dull to bright orange, allowing for nodule occupancy determinations of the strains for which FAs were not available. This avoided the need to view nodule squashes under phase contrast (22), but in blind tests gave essentially 100% agreement with results obtained when FAs for both strains were used. Five percent or more orange cells in the presence of green-fluorescing cells was taken as evidence of dual occupancy (22).

**Nodulation characteristics.** The RTM methodology of Bhanuveswari et al. (3, 5) was used. Total cell counts of early log-phase cultures were estimated by optical density (A₄₂₀) and then verified by serial dilution and the plating of aliquots on yeast extract-mannitol agar. Cell preparations were diluted into plant nutrient solution, deliberately varying the concentration of the inoculant dose so as to bracket 10⁶ cells ml⁻¹. Seeds of soybean cultivar Fiskeby V were surface sterilized as described above, germinated on water agar, transferred to growth pouches (three seedlings per pouch), and equilibrated for 24 h at 30°C. Growth pouches were moistened with plant nutrient solution and autoclaved prior to seedling transplant. Each seedling was then inoculated with 1 ml of one of the 10 strains of *B. japonicum*. Seedlings were kept in the dark for 24 h after inoculation. The RTM studies were also conducted at 20 and 30°C, using a photoperiod and light intensity identical to that used in the competition study. All inoculants were preequilibrated for 40 min at the appropriate temperature. Seedlings and pouches in the 20°C experiment were maintained at 20°C for 2 h prior to inoculation. Pouches were kept moist with sterile deionized water, and plants were harvested 10 and 13 days after inoculation for the 30 and 20°C treatments, respectively. A minimum of 120 seedlings were scored for each strain-temperature combination.

**RESULTS**

**Competition experiments.** Nodule occupancy, determined as the number of nodules occupied by the test strain as a percentage of total nodules, varied from 0 to 79% at 20°C and

<table>
<thead>
<tr>
<th>Temp and strain</th>
<th>% Single occupancy</th>
<th>% Double occupancy</th>
<th>Competitive index*</th>
<th>Position of uppermost nodule (mm from RTM)</th>
<th>No. of nodules above RTM</th>
<th>% Plants nodulated above RTM</th>
</tr>
</thead>
<tbody>
<tr>
<td>20°C</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>USDA 135</td>
<td>0 a</td>
<td>0 a</td>
<td>0 a</td>
<td>3 abcd</td>
<td>0.6 a</td>
<td>55</td>
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<td>0 a</td>
<td>0 a</td>
<td>0 a</td>
<td>−6 a</td>
<td>0.5 a</td>
<td>38</td>
</tr>
<tr>
<td>USDA 38</td>
<td>0 a</td>
<td>31 b</td>
<td>0 a</td>
<td>−6 a</td>
<td>0.5 a</td>
<td>51</td>
</tr>
<tr>
<td>CIAT 51</td>
<td>2 ab</td>
<td>4 a</td>
<td>0.07 a</td>
<td>1 abc</td>
<td>0.5 a</td>
<td>33</td>
</tr>
<tr>
<td>INPA 11</td>
<td>2 ab</td>
<td>43 b</td>
<td>0.09 ab</td>
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<td>14</td>
</tr>
<tr>
<td>RCR 3425</td>
<td>8 bc</td>
<td>32 b</td>
<td>0.30 c</td>
<td>7 bcd</td>
<td>1.9 b</td>
<td>95</td>
</tr>
<tr>
<td>USDA 66A</td>
<td>13 c</td>
<td>31 b</td>
<td>0.32 c</td>
<td>−3 ab</td>
<td>0.7 a</td>
<td>47</td>
</tr>
<tr>
<td>CB 1809</td>
<td>15 c</td>
<td>34 b</td>
<td>0.29 bc</td>
<td>9 cd</td>
<td>2.4 bc</td>
<td>88</td>
</tr>
<tr>
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<td>37 d</td>
<td>45 b</td>
<td>1.10 d</td>
<td>9 cd</td>
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<td>93</td>
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<td>−12 b</td>
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<td>0 cd</td>
<td>1.7 bc</td>
<td>65</td>
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<tr>
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<td>6 d</td>
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<td>8 d</td>
<td></td>
<td></td>
<td>2.3 d</td>
<td>82</td>
</tr>
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</table>

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*Values followed by the same letter are not significantly different, least significant difference of 0.05.
*Defined as number of singly occupied nodules formed by the strain as a proportion of all nodules formed, divided by the proportional representation of viable cells of that strain in the inoculant mixture. i.e., For CB 1809 at 20°C, the ratio of viable cells of CB 1809/USDA 110 in the inoculant was 0.51. Competitive index = 0.15 + 0.51 = 0.29.
from 0 to 71% at 30°C (Table 1). Regardless of temperature, USDA 38, USDA 135, and CIAT 51 appeared poorly competitive, while INPA 11, RCR 3425, USDA 66A, CB 1809, and UMR 161 showed some competitive ability with USDA 110, producing 8 to 50% of the singly occupied nodules. Dual occupancy of nodules ranged from 0 to 45% at 20°C and from 0 to 46% at 30°C. Most of the nodules occupied by strains INPA 11, CIAT 51, USDA 38, and RCR 3425 also contained USDA 110. For INPA 37 and USDA 66A, both single- and double-strain occupancy rates increased with temperature, suggesting that these strains were more competitive at 30°C than at 20°C. UMR 161 was competitive at both temperatures, singly occupying 37 and 50% of total nodules at 20 and 30°C, respectively. This was approximately twice that of USDA 110 at each temperature.

Inoculant ratios between the test strains and USDA 110 were not all 1:1. To account for this, a competitive index was calculated (Table 1) in which the proportion of singly occupied nodules was divided by the actual representation of each strain in the inoculant mixture. The competitive index values for each strain are shown in Table 1, with a value of 1.0 being considered competitive under the conditions of this experiment. By this criterion, only UMR 161 could be considered competitive with USDA 110. Competitive index values roughly paralleled nodule occupancy data.

Nodulation characteristics. The average number of nodules formed above the RTM ranged from 0.2 to 3.7 at 20°C and from 0.1 to 3.4 at 30°C (Table 1). Temperature did not influence this trait except for strains INPA 37 and CIAT 51, for which the number of nodules formed above the RTM was significantly greater at 30°C (least significant difference, 0.05). Regardless of temperature, strains INPA 11, USDA 38, and USDA 135 averaged less than one nodule above the RTM, whereas strains USDA 110, CB 1809, and UMR 161 averaged at least two nodules above the RTM.

The mean position of the uppermost nodule at 20°C ranged from −6 mm for strain USDA 66A to +13 mm for USDA 110 (Table 1). At 30°C this value varied between −31 (INPA 11) and +11 (CIAT 51) mm. At both temperatures the uppermost nodule positions of USDA 38 and USDA 66A were at or below the RTM, whereas those of USDA 110, UMR 161, CB 1809, and RCR 3425 were consistently above this mark. Uppermost nodule position was found to be correlated with the number of nodules above the RTM (\( r = 0.63; P = 0.005 \)), with the percentage of plants nodulated above the RTM (\( r = 0.81; P = 0.001 \)), and with the percentage of plants nodulated on the taproot (\( r = 0.67; P = 0.002 \)).

Strain competitive index was correlated with the number of nodules produced above the RTM (\( r = 0.64; P = 0.005 \)) and with the average position of the uppermost nodule (\( r = 0.5; P = 0.036 \)).

The nodulation profiles for three representative strains are shown in Fig. 1. That for UMR 161 (Fig. 1A and B) was skewed toward the RTM and is similar to histograms reported previously for other wild-type strains of B. japonicum (4, 19), although clustering of nodules above the RTM was greater at 30°C. Similar distributions were obtained with USDA 110 and CB 1809. The nodulation profile for INPA 37 (Fig. 1C and D) was similar to those obtained with USDA 66A, CIAT 51, and RCR 3425. In each case nodulation was scattered along the taproot at 20°C, but was normally clustered within about 20 mm of the RTM at 30°C. Nodule distribution of INPA 11 (Fig. 1E and F), USDA 135, and USDA 38 was poor and sporadic at both temperatures.

![Relative Position from Root-Tip Mark (mm)](http://aem.asm.org/)

**FIG. 1.** Nodulation profiles at 20 and 30°C of representative strains of B. japonicum inoculated onto the roots of soybean cultivar Fiskeby V. Nodule location frequency is shown in 2-mm increments measured relative to the RTM made on the face of the plastic growth pouch immediately following inoculation. Root growth is from left to right, with positive values referring to positions above the RTM; \( n = 47 \) to 52 seedlings.

**DISCUSSION**

Temperature has been shown previously to affect nodulation (18, 24) and competitiveness (17, 32) and was found to affect both traits in the present study. For example, the competitiveness (percent single occupancy) of strains INPA 37, UMR 161, and CB 1809 was clearly increased at 30°C, was at the expense of the competitor strain USDA 110, and was paralleled by slightly enhanced nodulation profiles with UMR 161 and CB 1809, and a more dramatic increase in that of INPA 37. By contrast, the competitiveness of CIAT 51 and RCR 3425 was not affected by temperature, though their nodulation profiles were clearly enhanced at 30°C.

Fernandez-Flouret and Cleyet-Marel (10) used the RTM procedure to study the nodulation characteristics of two strains of B. japonicum that differed in competitiveness and concluded that the strains did not differ in their ability to initiate infection and promote nodulation. However, it is evident from their data that, when similar inoculum doses are compared, the competitive strain consistently formed more nodules above the RTM. Similarly, Smith and Wollum (25) compared the nodulation characteristics of six strains of B. japonicum that varied in competitive ability with USDA 110, reporting that nodulation efficiency was unrelated to competitive ability. However, when the number of nodules formed above the RTM by each of their strains (approximat-
ed from the reported figure) is regressed against the proportion of nodules formed by each in competition with USDA 110, the correlation value obtained ($r = 0.74$) is similar to that found in the present study. In both of these studies, the apparent relationship between competitiveness and nodule-forming efficiency did not hold at inoculum levels of $>10^5$ to $10^6$ cells plant$^{-1}$. Inoculum concentrations of $>10^4$ to $10^6$ cells plant$^{-1}$ are perhaps inappropriate since by sheer numbers they may mask the inferior nodule response of a strain. Soils previously cropped to soybeans contain, on average, approximately $10^9$ soybean rhizobia g of soil$^{-1}$ (31), although instances of as many as $10^9$ soybean rhizobia g of soil$^{-1}$ have been reported (30).

In contrast to the above reports, studies with clover (27) and bean (24a) have related competitiveness to the number of nodules formed above the RTM. In the present study, the parameters used to describe nodule by the different strains were indicators of efficient nodule formation. The relationship between these parameters and competitiveness was not always incremental, nor did nodule above the RTM guarantee competitiveness. The extreme competitiveness of USDA 110 undoubtedly contributed to this. Further, the correlation between competitive index and parameters indicating nodule formation was not strong. Competition data will have a lower limit (0% occupancy), yet uppermost nodule position for the noncompetitive strains used in this study ranged from -31 to +3 mm from the RTM. This must weaken the correlation, but is still consistent with a model in which poorly efficient strains are less efficient in nodule formation. At 20°C nodulation characteristics of USDA 110 were clearly superior to those of the other strains, and, with the exception of UMR 161, this strain dominated in paired nodulation tests. Overall nodule occupancy by the test strains increased at the higher temperature, and nodulation characteristics were enhanced. By contrast, the nodulation achieved by USDA 110 declined at 30°C, perhaps contributing to the better performance of the test strains.

Hodgson and Stacey (15) suggested that microcolonies of rhizobia in soil randomly come in contact with the growing root and so are favored to initiate nodule formation. Given the time constraints on subsequent infection, and probable physical barriers against movement toward the root, a physiologically efficient infection response might enhance the probability of successful nodulation. Strains that can quickly and efficiently respond to host signals, initiate, and sustain an infection attempt might then dominate nodulation and appear highly competitive. In this study, we have used nodulation above the RTM as an indicator of efficiency in nodule formation. It is not clear how much difference in nodulation efficiency is required for a strain to dominate in nodule occupancy. Strains such as INPA 11, USDA 38, and USDA 135 were clearly less efficient in nodule formation and were poor competitors. Those strains that formed at least two nodules above the RTM were significantly more competitive, although CIAT 51 was clearly an exception at 30°C.

Using wild-type strains, we have presented evidence relating competitiveness to nodule-forming efficiency as defined by numbers of nodules formed above the RTM. While the conditions used here were artificial, they avoided the confounding effects of biotic and abiotic soil factors and provide a base line of data with which the results of future studies under more realistic conditions can be compared.

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1120.
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