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Early Infection and Competition for Nodulation of Soybean by *Bradyrhizobium japonicum* 123 and 138†

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Interactions of soybean with *Bradyrhizobium japonicum* 123 (serogroup 123) and 138 (serogroup c1) were used to examine the relationship between early infection rates, competition for nodulation, and patterns of nodule occupancy. Both strains formed more infections in autoclaved soil (sterile soil) than in untreated soil (unsterile soil). Inoculation did not increase numbers of infection threads in unsterile soil-grown plants, where infection of proximal portions of primary roots was complete by 5 days after planting. Both strains infected and nodulated at similar rates in sterile soil. Nodules were always clustered on the upper root system, regardless of inoculation and soil treatment. Sixty-seven percent of the nodules of uninoculated plants grown in unsterile soil were occupied by rhizobia belonging to serogroups other than 123 or c1. Inoculation with strain 123 or 138 increased occupancy by that strain at the expense of residency by other rhizobia. Eighty-three percent of all nodules on plants dually inoculated with both strains in sterile soil contained strain 138. The corresponding value for plants inoculated in unsterile soil was 31%. Neither inoculum strain dominated occupancy of first-formed nodules in unsterile soil. It appears that north central Missouri soil may not have populations of highly competitive serogroup 123 and that early infection and nodulation rates do not contribute to the competitive success of strain 138.

The inoculation of soybean seed with highly effective *Bradyrhizobium japonicum* strains does not always result in higher yields. This failure often is due to the predominance of competitive, yet ineffective, indigenous soil rhizobia in nodules. The superior competitive ability of *B. japonicum* serogroup 123 has been demonstrated in soils from Ohio (36), Iowa (6), Minnesota (23, 25), Wisconsin (16), and Illinois (17). Most of these soils are silt loams with pH values ranging from 5.8 to 7.3. Serogroup 123 has been shown not to dominate nodulation in a Louisiana soil (Olivier silt loam, pH 5.8) (9) and in alkaline soils (pH 8.1 to 8.5) in Iowa (6), presumably because it is present in low numbers. The mechanism of the success of serogroup 123 is unknown but appears unrelated to rhizosphere populations (25, 31), tolerance to high soil temperatures (26), bacteroid viability (24), growth in sugar-amended soils (38), lectin-binding activity (30), nitrate sensitivity (20), or survival in soil (36).

Serogroup 123 is poorly competitive in occupying nodules under certain conditions. Kosslak and Bohlool (20) established serogroup 123 to be less competitive than serogroup 110 in vermiculite or *Rhizobium*-free soil from Hawaii. Hicks and Loynachan (15) found that phosphorus fertilization in the field reduced nodule occupancy by serogroup 123. These data indicate that the environment plays a critical role in competitiveness.

The work of Kosslak and her co-workers (20, 21) has shown that preexposure of one strain of *B. japonicum* to young seedlings can alter its pattern of competition with other strains. These results suggest that the early events in infection and nodulation are important in competition among *B. japonicum* strains. In this study we have examined the events of infection and nodulation as they pertain to the competition between an isolate of serogroup 123 (hereafter designated strain 123) and strain USDA 138 (serogroup c1) in soil. We specifically examined the role of early infections, nodulation, and nodule distribution as they relate to the competitiveness of strain 123 for nodulation. Our isolate of serogroup 123 was not competitive against strain 138, although both strains had similar rates of infection and nodulation. Inoculation strongly influenced the kinetics of nodulation and the spacing of nodules occupied by noninoculant indigenous strains.

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MATERIALS AND METHODS

Bacterial cultures. An isolate of *B. japonicum* serogroup 123 (not USDA 123) was obtained from E. L. Schmidt, University of Minnesota, St. Paul; *B. japonicum* USDA 138 (serogroup c1) was from D. F. Weber, U.S. Department of Agriculture-Agricultural Research Station, Beltsville, Md. Long-term storage of the bacteria was in 7.5% (vol/vol) glycerol at −70°C with short-term storage on yeast extract-mannitol (YEM) agar at 10°C. Cells for plant inoculations were grown to the midlog phase in YEM broth at 30°C and 125 rpm and adjusted turbidimetrically with sterile Jensen solution (37) to 2.5×10^7 cells per ml.

Soil treatments. Mexico silt loam (Udollic ochraqualf, pH 6.6) was collected at the University of Missouri Bradford Experimental Farm near Columbia. The field had a previous history of soybean cultivation. Fresh soil obtained from the upper 30 cm of the profile was sieved to uniform consistency with a no. 6 size sieve (335-mm mesh) and adjusted to 15% (wt/wt) moisture with deionized water. Soil was either used directly (unsterile soil) or after autoclaving for 2 h and cooling (sterilized soil). Autoclaving did not alter soil pH.

Plant inoculation and growth conditions. Soybean seeds (*Glycine max* L. Merr. cv. Williams 82; supplied by the Missouri Seed Improvement Association) were surface sterilized and sown in the appropriate soil treatment in plastic Cone-tainers (4-cm top diameter, 21 cm long; Cone-tainer Nursery, Canby, Ore.) with one seed per Cone-tainer. Seeds were inoculated immediately with strain 123 or 138 (2.5×10^7 cells per seed) plus 1 ml of sterile Jensen solution. Seeds

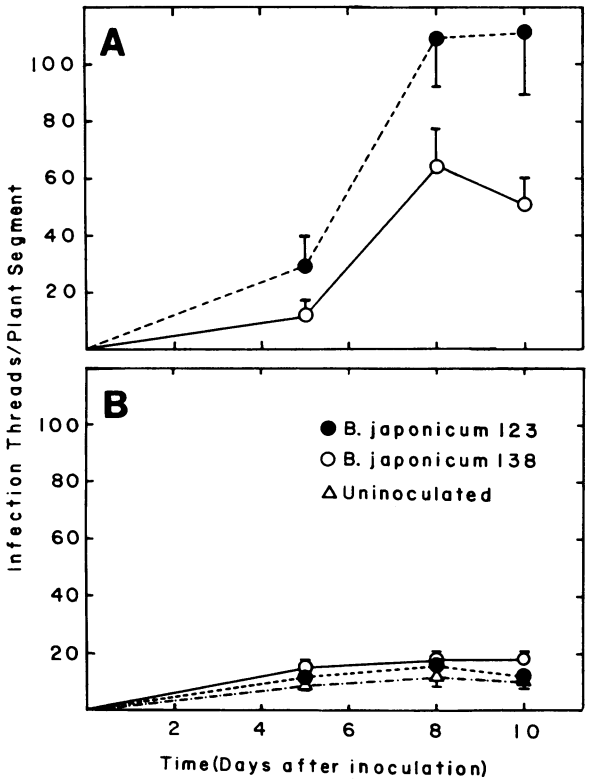

FIG. 1. Time course of infection thread formation on Williams 82 soybean roots. Seeds were inoculated at the time of planting with *B. japonicum* 123 or 138 in sterile (A) or unsterile (B) field soil. The upper 2 cm of the primary root was examined for infection threads. Each point represents the mean ± standard error of three independent experiments (seven to nine plants were examined per time period).

for competition experiments were inoculated with a mixture (1 ml of each) of both strains. Plants were irrigated daily with 5 to 10 ml of sterile deionized water and maintained in growth chambers at 25°C with a 12-h photoperiod at a light intensity of 500 $\mu E/m^2$ per s.

Infection thread enumeration. At 5, 8, or 10 days after inoculation root systems were harvested and rinsed free of adhering soil. Root systems then were dipped in 0.05% (wt/vol) toluidine blue, and infection threads were visualized by interference-contrast microscopy as previously described (27). Infection threads were enumerated on the upper 2 cm of the primary root.

Nodulation rates and nodule distribution. Plants were uprooted at each sampling time and carefully rinsed free of adhering soil. Nodules were counted and classified as primary (nodules on the primary root) or lateral (nodules not on the primary root). The location of each primary nodule to the nearest millimeter was mapped in relation to the junction of the primary and the first lateral root. The location of each lateral nodule was defined by the distance along the primary root (in millimeter) between the lateral root bearing the nodule and the first lateral root.

Nodule occupant analysis. Fluorescence microscopy was used to identify rhizobia in nodule smears by the method of Heron and Pueppke (13). At 16 days after inoculation, root systems were surface sterilized with 95% ethanol followed by 0.5% sodium hypochlorite and thoroughly rinsed with sterile distilled water. Primary and lateral nodules were selected at random, and nodule smears on glass microscope slides were incubated with serogroup-specific anti-123 and anti-138 rabbit antisera (kindly provided by H. P. Friedman, University of Missouri-St. Louis) followed by fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin (Sigma Chemical Co.), St. Louis, Mo.). Bacteria reacting with anti-123 antisera were classified as serogroup 123, and those reacting anti-138 antisera were classified as serogroup c1 (7). Nodule occupants were classified as 123 in nodule, 138 in nodule, both 123 and 138 in nodule, or neither 123 nor 138 in nodule.

RESULTS

Rates of infection and nodulation. Infection threads were readily detected in plants grown in unsterile or sterile soil inoculated with strain 123 or 138 (Fig. 1). In unsterile soil, the mean number of infection threads per root segment was between 10 and 20 on day 5 after inoculation. Infection thread numbers did not increase after this time. Inoculation of seeds with strain 123 or 138 failed to increase infection thread numbers relative to those in uninoculated unsterile soil (Fig. 1).

Infection thread numbers in inoculated plants grown in sterile soil were similar to those in unsterile soil on day 5, but they increased dramatically thereafter. The numbers of infection threads on 8- and 10-day-old plants inoculated with strain 123 were significantly greater than those on plants inoculated with strain 138. Under these conditions maximal infection thread numbers for strains 123 and 138 were nine times greater and four times greater, respectively, than those in unsterile soil.

Nodule numbers initially increased at similar rates in plants inoculated with strain 123 or 138 in sterile soil (Fig. 2). After an 8-day lag period, nodule numbers increased by about two per day in each treatment. Plants inoculated with strain 123 contained slightly more nodules than plants inoculated with strain 138 beginning on day 16. There was no obvious differences in the growth rates of the plants in any of the treatments.

Nodule distribution. Sixteen-day-old plants were used for determination of nodule occupancy, because the numbers of

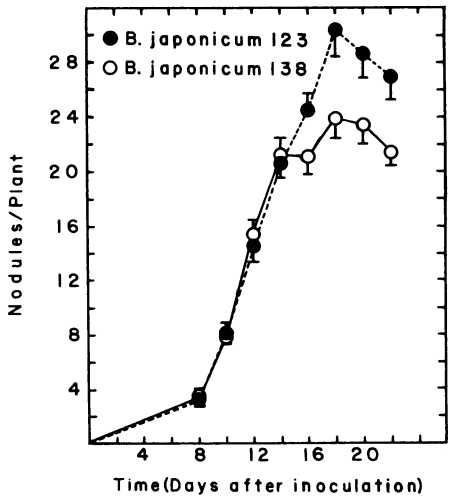

FIG. 2. Time course of nodule formation on Williams 82 soybean roots. Seeds were inoculated with *B. japonicum* 123 or 138 at planting in sterile soil. Each point represents the mean ± standard error of four independent experiments (39 to 41 plants were examined per time period).

TABLE 1. Nodule numbers of 16-day-old Williams 82 soybeans grown in unsterile or sterile soil

Treatment[a]	Mean no. of nodules ± SE on:					
	Primary roots		Lateral roots		All roots	
	Unsterile	Sterile	Unsterile	Sterile	Unsterile	Sterile
Uninoculated	17.9 ± 1.5	0	13.7 ± 1.4	0	31.6 ± 1.8	0
123	17.8 ± 1.3	9.4 ± 1.4	18.3 ± 2.1	11.9 ± 1.6	36.1 ± 2.1	21.4 ± 2.0
138	20.1 ± 1.6	9.5 ± 1.0	13.6 ± 1.1	11.1 ± 1.2	33.7 ± 1.7	20.6 ± 1.4
123 and 138	18.1 ± 1.4	9.7 ± 1.0	16.3 ± 1.3	8.2 ± 0.8	34.3 ± 1.7	17.9 ± 1.1

[a] Seeds were inoculated at time of planting with *B. japonicum* 123, 138, 123 and 138 (1:1), or left uninoculated; 25 to 40 plants were examined for each soil treatment in three independent experiments.

nodules were essentially constant after this time period. Numbers of primary, lateral, or total root nodules were not greatly influenced by inoculation treatment (Table 1). Plants grown in sterile soil, however, had significantly fewer nodules than those grown in unsterile soil, irrespective of inoculation treatment. The only exception was lateral roots of plants inoculated with strain 138, which contained equivalent numbers of nodules in unsterile and sterile soil. As expected, no nodules were found on uninoculated plants in sterile soil. In unsterile soil uninoculated plants formed just as many nodules per plant as did inoculated plants. Similar numbers of primary and lateral nodules were found within a treatment.

The nodules on the primary root were clustered asymmetrically near the crown (Fig. 3). Fifty percent or more of all primary nodules were within 30 mm of the junction of the primary and first lateral root of plants grown in unsterile soil. In sterile soil 65% or more of all primary nodules were found in this same region. This clustered distribution was similar for plants inoculated with strain 123 or 138 or both strains and for uninoculated plants in unsterile soil.

At least 86% of all lateral root nodules were present on the uppermost lateral root and other lateral roots within 20 mm below it (Fig. 4). Generally, plants in sterile or unsterile soil had similar distributions of lateral nodules, regardless of inoculation treatment. However, the first lateral root of plants grown in sterile soil inoculated with strain 138 had three times more nodules than did plants grown in unsterile soil. This difference was not apparent in plants inoculated with strain 123. Dually inoculated plants grown in unsterile soil had three times more nodules 5 to 10 mm below the first lateral root than did similarly treated plants growing in sterile soil.

Nodule occupancy. About two-thirds of the nodules on uninoculated plants grown in unsterile soil were occupied by rhizobia of serogroups other than 123 or c1 (Table 2). Although most of the remaining nodules contained serogroup c1, a few contained serogroup 123 and about 1% contained both serogroups. Inoculation of plants with strain 123 increased nodule occupancy by serogroup 123 almost eightfold. Increased residency by serogroup 123 came at the expense of residency by serogroup c1. Conversely, inoculation with strain 138 increased the percentage of nodules containing this serogroup, mainly due to displacement of rhizobia of serogroups other than 123 or c1. Inoculation had only minimal effects on the number of nodules simultaneously occupied by serogroups 123 and c1. We did not detect any selectivity in the occupancy of primary versus lateral nodules for either inoculant strain (data not shown).

Greater than 70% of all nodules from dually inoculated plants grown in sterile soil contained strain 138 (Table 3). This dominance was most readily apparent in lateral nodules, where strain 138 was found either alone or in combination with strain 123 in 98% of the nodules. Serogroup 123 occupied slightly more total nodules in unsterile soil (36%) than in sterile soil (30%). Indigenous *Bradyrhizobium* spp. not belonging to serogroup 123 or c1 occupied a slightly higher percentage of total nodules than did either strain 123 or 138 in unsterile soil. Dually occupied nodules were more frequent in sterile soil than in unsterile soil, where most of the dually occupied nodules were found on the primary root.

Figure 5 shows the distribution patterns of nodules occupied by different serogroups in unsterile soil. Nodules occupied by only serogroup 123, serogroup c1 or neither serogroup were distributed similarly on uninoculated plants, i.e.,

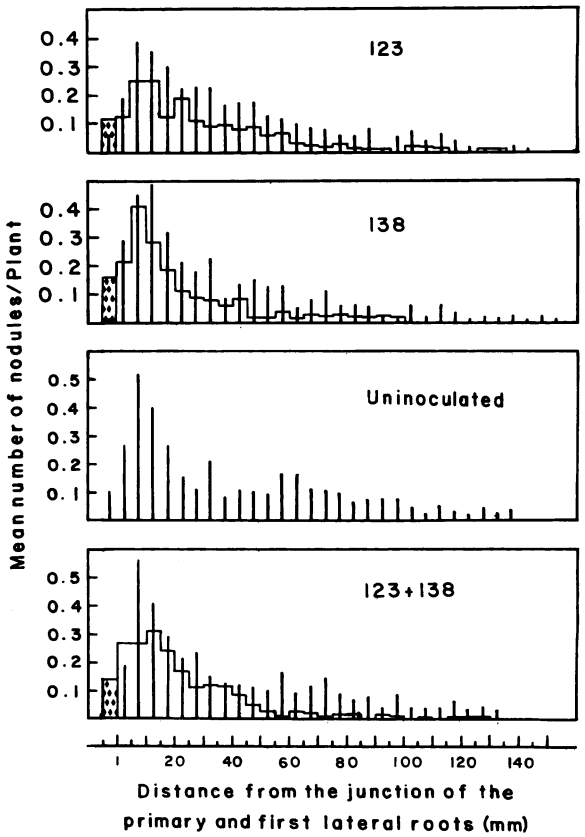

FIG. 3. Distribution of nodules on primary roots of 16-day-old Williams 82 soybeans. Seeds were inoculated at time of planting with *B. japonicum* 123 or 138 or with both strains (1:1) or left uninoculated. Symbols: |, plants grown in unsterile field soil (*n*, 34 to 40); □, plants grown in sterile field soil (*n*, 25 to 43); ⁝, position of the first lateral root. The direction of root growth is from left to right.

TABLE 2. Nodule occupancy for 16-day-old Williams 82 soybeans grown in unsterile soil

Treatment[a]	Mean % of nodules ± SE occupied by:			
	123	138	123 and 138	Neither
Uninoculated	5.9 ± 1.6	25.1 ± 4.1	1.1 ± 0.5	67.9 ± 5.0
123	46.1 ± 6.6	9.6 ± 1.8	3.2 ± 0.3	41.1 ± 5.1
138	2.4 ± 1.0	46.8 ± 6.8	0.5 ± 0.4	50.5 ± 6.5

[a] Seeds were inoculated at time of planting with *B. japonicum* 123 or 138 or left uninoculated; 31 to 38 plants and 361 to 390 total nodules per treatment in three independent experiments were used.

clustered near the root crown. Only a few nodules contained both serogroup 123 and c1, and they all were near the crown.

Inoculation had a distinct influence on nodule distribution patterns. Inoculation with strain 123, for example, produced a mixture of nodules at the crown. Some were occupied by serogroup c1, and some were occupied by serogroup 123. On the contrary, inoculation with strain 138 almost totally excluded serogroup 123 from nodules on the upper primary root. Inoculation with both strains resulted in serogroup 123 occupying more primary nodules than serogroup c1 (32% versus 20%), although nodules not occupied by serogroup 123 were found in the same region as serogroup 123-occupied nodules. Inoculation also influenced the number and distribution of dually occupied nodules. Although the majority of such nodules were on the upper 30 mm of the primary root in treatments containing strain 123, no dually occupied nodules were found on the upper 100 mm of the primary root in plants inoculated with strain 138 alone.

DISCUSSION

The upper 2 cm of the primary root was chosen for infection thread enumeration because this is the first-formed plant tissue and the region of the highest infection density in pouch-grown plants (3). The numbers and distribution of nodules in this tissue thus can give important clues about the earliest infection events. To our knowledge we are the first to quantify nodule clustering on the roots of soil-grown plants inoculated as seed. We believe that results from such plants are more relevant to the field situation than those from pregerminated, axenically grown seedlings. In the present study, we analyzed such tissue to determine whether competitiveness of *Bradyrhizobium* strains can be related to the extent of early infection and to initial nodulation rates. The influence of indigenous rhizobia and other soil factors was assessed by doing experiments in both sterilized and unsterile soil.

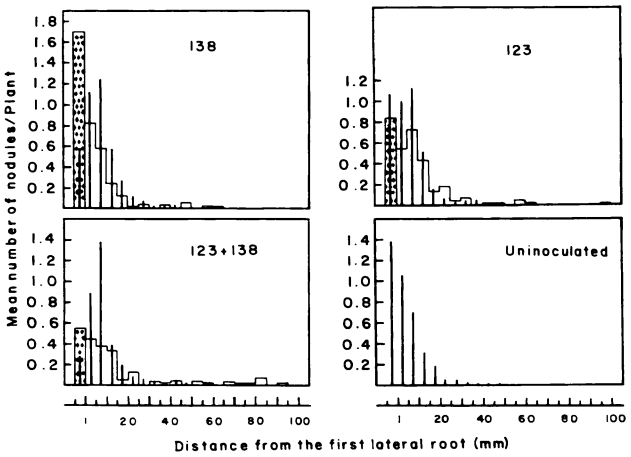

FIG. 4. Distribution of nodules on lateral roots of 16-day-old Williams 82 soybeans. Seeds were inoculated at time of planting with *B. japonicum* 123 or 138 or both strains (1:1) or left uninoculated. The plants are the same as those examined in Fig. 3, and the symbols are as in Fig. 3.

Sterile soil. Strain 138 was more highly competitive than strain 123 in sterile soil. This was surprising, given the reported highly competitive nature of strain 123 (see below). Strain 123 formed more early infections than did strain 138. Moreover, nodule distributions for strains 123 and 138 were similar, clearly indicating that early nodulation kinetics of a *Bradyrhizobium* strain cannot be used to predict its success in occupying nodules.

Both strains produced unusually high numbers of infections in sterile soil. This is probably due to the lack of antagonists in the rhizosphere. Data from sterile soil thus may be misleading compared with the behavior of rhizobia in nature. The survival of *B. japonicum* cells introduced into unsterile soil decreases over time (5), and soil microorganisms antagonistic to *B. japonicum* reduce both rhizobial soil populations and nodulation (1, 36). This decrease in *Bradyrhizobium* numbers in the rhizosphere undoubtedly influences infection thread and nodule development and may explain why infection thread formation was complete by 5 days in unsterile soil but continued up to 8 days in sterile soil. Alternatively, sterile soil may directly influence how the plant responds to rhizobia.

Both strains nodulated entire root systems at the same rate, which is similar to initial rates observed in the field by Zobel (39). Strain 123, however, produced slightly more nodules after 16 days than did strain 138. Strain 123 is an

TABLE 3. Nodule occupancy for dually inoculated 16-day-old Williams 82 soybeans grown in unsterile and sterile soil[a]

Roots	Soil	Mean % of nodules ± SE occupied by:			
		123	138	123 and 138	Neither
Primary	Unsterile	32.0 ± 7.1	20.4 ± 3.8	10.3 ± 2.9	37.3 ± 5.8
	Sterile	26.0 ± 11.2	60.9 ± 13.3	13.1 ± 2.2	0
Lateral	Unsterile	20.6 ± 4.3	31.9 ± 3.5	2.4 ± 0.6	45.1 ± 3.7
	Sterile	2.3 ± 1.2	84.7 ± 3.4	13.0 ± 2.7	0
All roots	Unsterile	28.3 ± 6.3	24.1 ± 3.2	7.7 ± 2.7	39.9 ± 5.6
	Sterile	16.8 ± 7.4	70.0 ± 9.1	13.2 ± 2.4	0

[a] Seeds were dually inoculated at time of planting with *B. japonicum* 123 and 138 (1:1); 34 plants were examined for each soil treatment in three independent experiments totaling 482 nodules in unsterile soil and 358 nodules in sterile soil.

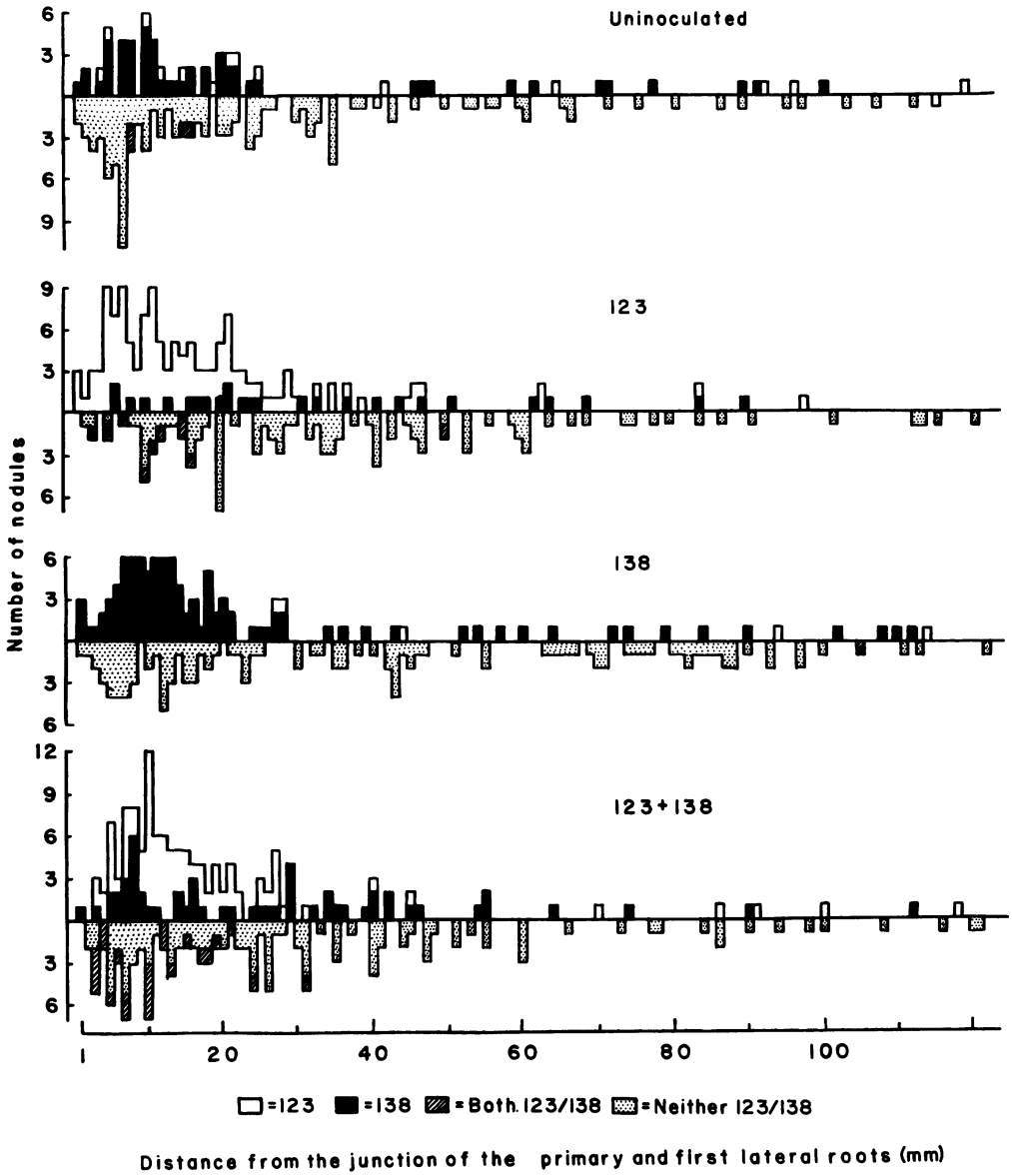

FIG. 5. Spatial distribution of strains in nodules on 16-day-old primary roots of Williams 82 soybeans. Seeds were inoculated at time of planting with *B. japonicum* 123 or 138 or both strains (1:1) or left uninoculated. Each distribution is the sum of three independent experiments; 31 to 34 plants and 226 to 272 nodules were used per treatment.

inefficient nitrogen fixer (35), and soybeans inoculated with this strain yield less than plants inoculated with strain 138 (12). The increase in nodule numbers thus may reflect compensation by the plant for unproductive nodules.

Unsterile soil. Serogroup c1 was more prevalent than serogroup 123 in nodules of uninoculated plants growing in untreated Mexico silt loam soil. By definition, this means that the former serogroup is more competitive than the latter (34). Under certain conditions in dually inoculated pouch-grown plants, strain 138 also occupies more nodules than serogroup 123 (25). This contrasts to work in Illinois and Minnesota, where strain 138 occupies few nodules in field-grown soybeans (17, 23). Serogroup c1 may be successful in Mexico silt loam soils because of the presence of unusually high numbers or, alternatively, highly competitive variants of the serogroup.

Like strain 138, strain 123 has been reported to vary in its ability to outcompete other rhizobia (4, 20). Although serogroup 123 usually occupies the majority of nodules in field-grown soybeans (23, 25), serogroup 31 has been recovered more frequently than serogroup 123 from nodules in 12 states. This indicates that rhizobia other than serogroup 123 can be competitive in field-grown soybeans (19). Our results suggest that although factors associated with steam sterilization strongly reduce the competitiveness of inoculant strain 123, plant growth conditions ultimately govern the competitiveness of serogroup 123 in unsterile soil.

The number of infections in inoculated plants grown in unsterile soil differed greatly from those in inoculated plants in sterile soil. Numbers at day 10 in unsterile soil were similar to values obtained by Ranga Rao and Keister (29) for field-grown soybeans treated with commercial inoculum. This similarity validates use of the convenient Cone-tainer system in examining infection in soil. It is significant that

inoculation had no effect on infection thread numbers in unsterile soil. Apparently, seed inoculation in unsterile soil may influence nodule occupancy but not numbers of infections.

The clustering of nodules on the upper lateral roots and top regions of the primary root confirms the observations of Grubinger et al. (11), who worked with mature field-grown soybeans. Similar nodule distributions have been reported for pouch-grown plants, where primary nodules most frequently appear near the region occupied by the root tip at time of inoculation (2, 3). It must be noted that soybean nodule distributions in pouch-grown plants can differ from those in sterile soil-grown plants (28). We found that nodules occupied by serogroup c1 or 123 in unsterile soil appeared in similar regions of the primary root, regardless of the total percentage of nodules occupied by each strain. This suggests that when a strain is competitive (i.e., occupies a high percentage of nodules), it does not totally exclude other strains from the region of the root where primary nodules initially form. It follows that first-formed nodules are not dominated by any given strain.

Conclusions. Our results suggest that unlike soils in other midwestern states, a soil representative of north central Missouri may not have populations of highly competitive serogroup 123. Variability in serogroup 123 has been described in Iowa (10, 14), Minnesota (33), and Wisconsin (16). Keyser and co-workers (18, 32) have subdivided isolates of serogroup 123 into three groups according to their nodulation characteristics on two soybean lines. Although the isolates were serologically distinguishable and genetically diverse, they all were equally competitive against strain 110 for nodulation of greenhouse-grown Williams soybeans. In contrast, strain 123 as an inoculant clearly fares poorly against strain 138 in sterile Mexico silt loam soil but does better in unsterilized soil. It may be possible that competition is being influenced by strain-cultivar interactions. Early infection and nodulation rates do not appear to contribute to the success of strain 138. Success also cannot be attributed to the ability of strain 138 to exclude strain 123 from the first formed nodules. Recently Kosslak et al. (22) demonstrated that genes involved in early events in nodulation are induced in strain USDA 123 by isoflavones from soybean roots. Perhaps work with the response of strain 138 to these and other compounds, as suggested by Djordjevic and coworkers (8), may reveal a possible mechanism.

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