

Competition of *Rhizobium japonicum* Strains in Early Stages of Soybean Nodulation

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The effects of preexposure of soybean (*Glycine max* L. Merrill) roots to *Rhizobium japonicum* strains and subsequent establishment of other strains in the nodules were investigated by using combinations of effective strains (USDA 110 and USDA 138) and effective-ineffective strains (USDA 110 and SM-5). Strain USDA 110 was a better competitor than either USDA 138 or SM-5 on cultivars Lee and Peking. However, when either of the two less-competitive strains was inoculated into 2-day-old seedlings before USDA 110 was, their nodule occupancy increased significantly on both cultivars. With USDA 138 as the primary inoculum and USDA 110 delayed for 6, 48, and 168 h, the incidence of USDA 138 nodules increased on cultivar Peking from 6% (at zero time) to 28, 70, and 82% and on cultivar Lee from 17% (at zero time) to 32, 88, and 95% for the three time delays, respectively. Preexposure of 2-week-old roots of cultivar Lee to USDA 138 had essentially the same effect: the incidence of USDA 138 nodules increased from 23% at zero time to 89 and 97% when USDA 110 was delayed for 24 and 72 h, respectively. When the ineffective strain SM-5 was used as the primary inoculum, followed by USDA 110 72 h later, the percentage of nodules containing SM-5 increased from 7 to 76%. These results indicate that the early events in the nodulation process of soybeans are perhaps the most critical for competition among *R. japonicum* strains.

Competition among *Rhizobium* strains for nodulation of their legume host is an important aspect of the ecology of the root nodule bacteria. This can be of agronomic concern when the desired inoculum strain fails to establish in nodules because of competition from indigenous, and often less efficient, rhizobia. This problem is highlighted in the soybean-growing regions of the United States, where inoculation with highly effective strains does not always result in yield increases. This is partly because of the failure of the inoculum strains to compete with resident rhizobia (6, 10).

The mechanisms that confer competitive advantage to a strain are poorly understood. It is clear that many factors of both the microsymbiote and the host play important roles in determining which strain succeeds in occupying the majority of the nodules (for a review, see reference 5). Some of the characteristics of *Rhizobium* species that have been studied are effectiveness (4, 8, 14), numbers (12, 15, 20), and bacteriocinogenicity (17). Other studies (3, 4, 11, 13, 19) have indicated that the host plant can play an important role in selecting the successful strain. None of these studies, however, have identified the essential factors or the critical

stages in the infection process responsible for success or failure of a strain.

In this study we used delayed inoculation with specific strains to pinpoint the period of time in the nodulation process most critical to competition among strains. We report that preexposure of soybean roots to a poorly competitive strain of *Rhizobium japonicum* results in a significant increase in nodule occupancy by that strain.

MATERIALS AND METHODS

In one study, two effective strains of *R. japonicum*, USDA 110 and USDA 138 (from E. L. Schmidt's collection, University of Minnesota, St. Paul), were used with soybean (*Glycine max* L. Merrill) cultivars Lee and Peking (from D. Weber, U.S. Department of Agriculture, Beltsville, Md.). Lee is a commercial cultivar, and Peking is an unimproved cultivar reported to exclude USDA 110 under field conditions if other *R. japonicum* strains are present (3, 9). In another study, an ineffective strain, SM-5 (from W. Brill, University of Wisconsin, Madison), was used in competition against USDA 110 on soybean cultivar Lee.

Rhizobium cultures were maintained on yeast extract-mannitol (2) slants, and for inoculation, 1 ml of 4-day-old broth cultures was injected into the plant chambers at the designated times.

Seeds were surface sterilized for 20 min in 4%

calcium hypochlorite, washed exhaustively in sterile water, and pregerminated in sterile vermiculite. Two-day-old seedlings (radicles of 2 to 3 cm) were selected for the experiments. They were planted in sterile vermiculite moistened with 1:4 strength Hoagland nitrogen-free solution in 250-ml Erlenmeyer flasks (7). After the primary inoculation with the desired strain(s), the top of the vermiculite was covered with a 2-cm layer of sterile perlite and a 2-cm layer of paraffin-coated sand. A 19-gauge needle, pushed through a rubber stopper into the flask, facilitated inoculation and watering.

In the time course experiment with USDA 110 and USDA 138, three different treatments were used for 2-day-old seedlings. At zero time, one group (six plants) received both strains, another group received only USDA 110, and the last group received only USDA 138. At selected times (6, 48, and 168 h) thereafter, three flasks each from groups 2 and 3 received the other strain as the secondary inoculum. Controls consisted of uninoculated seedlings and seedlings inoculated with only one strain at each time period. Plants were grown in a Sherer model CEL4-7 controlled-environment growth chamber at 27°C with a light intensity of 170 micro einsteins/cm² per s and a photoperiod of 16 h. Four weeks after the secondary inoculation, the plants were harvested, and the nodules were separated into early (taproot) and late (lateral root) groups based on their location on the root and into large (>1 mm) and small (<1 mm) groups based on their size. For each treatment, 40 to 65 nodules were analyzed. Of these, approximately 32 to 72% were classified as early nodules. The strains in the nodules were identified by immunofluorescence with strain-specific fluorescent antibodies (16). Gelatin-rhodamine isothiocyanate (1) was used to suppress non-specific adsorption. The microscopy techniques used have been described elsewhere (11).

A similar experiment was done with 2-week-old plants instead of seedlings. The design of the experiment with the ineffective strain SM-5 was essentially the same, except that the secondary inoculum was introduced 72 h after the primary one.

RESULTS

Nodule occupancy of strains USDA 110 and USDA 138 did not differ for early (small or large) or late (small or large) nodules. In groups receiving both strains simultaneously, USDA 110 occupied the majority of the nodules, regardless of size and location. Likewise, in the delayed-inoculation experiments, the pattern of nodule occupancy of the strains was found to be the same. Therefore, the percentages of nodules occupied by the two strains were pooled (Fig. 1).

When seedlings were inoculated with both strains at the same time, USDA 110 was more competitive and occupied the majority of the nodules on both cultivar Lee (52 ± 16.5% [standard error]) and cultivar Peking (87 ± 16.5%). Approximately 30% of the nodules on cultivar Lee and 10% of the nodules on cultivar Peking contained both strains. When USDA 138 was allowed a head start in the root zone (Fig. 1B

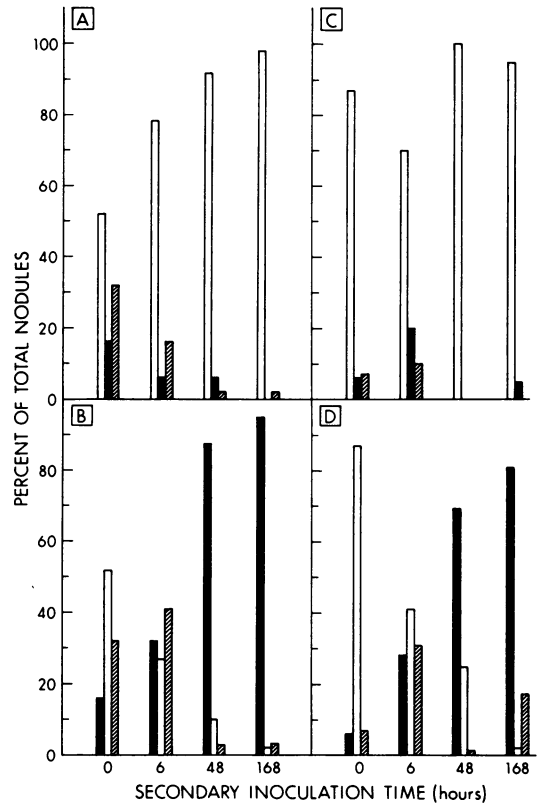


FIG. 1. Effect of preexposure of roots to an effective strain of *R. japonicum* on nodule occupancy by another effective strain. (A and C) *G. max* cv. Lee and Peking, respectively, with USDA 110 as the primary inoculum and USDA 138 as the secondary inoculum; open bars, USDA 110; solid bars, USDA 138; hatched bars, USDA 110 and USDA 138. (B and D) *G. max* cv. Lee and Peking, respectively, with USDA 138 as the primary inoculum and USDA 110 as the secondary inoculum; solid bars, USDA 138; open bars, USDA 110; hatched bars, USDA 110 and USDA 138.

and D), its ability to compete against USDA 110 was improved. The 48-h delay in the introduction of USDA 110 enabled USDA 138 to become dominant and occupy 69% of the nodules on cultivar Peking and 87% of the nodules on cultivar Lee. Even with a 6-h delay in the introduction of USDA 110, the nodule occupancy of USDA 138 increased substantially. When USDA 110 was used as the primary inoculum (Fig. 1A and C), it completely out-competed USDA 138 at every time point.

In the experiment with 2-week-old plants (cultivar Lee), the nodule occupancy of USDA 138 increased significantly when USDA 138 was introduced 24 h before USDA 110 (Table 1). A 72-h preexposure to USDA 138 resulted in virtual elimination of USDA 110 from the nodules.

TABLE 1. Effect of preexposure of 2-week-old roots to a poorly competitive strain (USDA 138) on nodule occupancy by a highly competitive strain (USDA 110) of *R. japonicum*

Inoculum ^a		No. of nodules per plant ^b	Nodule occupancy (% of total) ^c		
Primary (zero time)	Secondary (h)		USDA 110	USDA 138	USDA 110 and USDA 138
USDA 110	None	ND	100 ^d	0 ^e	0 ^f
USDA 138	None	ND	0 ^g	100 ^h	0 ^f
USDA 110 and USDA 138	None	104 ± 24	47 ⁱ	23 ^j	30 ^k
USDA 138	USDA 110 (24)	87 ± 14	8 ^g	89 ^h	3 ^f
USDA 138	USDA 110 (72)	121 ± 7	0 ^g	97 ^h	3 ^f

^a All uninoculated controls were devoid of nodules.

^b Values are the means of three replicates ± standard error. ND, Not determined.

^c Values are the means of three replicates.

^{d-k} Numbers followed by the same letter within a given column do not differ significantly ($P = 0.05$) as determined by the Duncan multiple-range test.

Another experiment was designed to determine if the establishment of an ineffective strain, SM-5, would affect the subsequent establishment of USDA 110 on cultivar Lee. When the two strains were introduced together (Table 2), USDA 110 occupied the majority of the nodules. However, when SM-5 was introduced 72 h before USDA 110, its nodule occupancy increased to 76%. The total number of nodules formed by SM-5 was greater than that formed by USDA 110, whereas the total nodule mass per plant was much less for SM-5-formed nodules than for USDA 110-formed nodules. Plants which were inoculated with SM-5 at zero time and then with USDA 110 72 h later remained chlorotic (yellow) for approximately 1 week or more longer than those which were inoculated with both strains at zero time or which received USDA 110 as the primary inoculum.

DISCUSSION

The competitive superiority of USDA 110 over USDA 138 and SM-5 was demonstrated in

all of the experiments in which the strains were introduced simultaneously. USDA 110 out-competed USDA 138 on both cultivars. This is particularly surprising with cultivar Peking, as this cultivar had been previously shown to exclude USDA 110 in soils containing other *R. japonicum* strains (3, 9).

Delaying the introduction of USDA 110 into the root zone resulted in an enhancement of nodule occupancy by either USDA 138 or SM-5. When either of the two less-competitive strains was introduced for 48 h or longer before USDA 110, it formed the majority of the nodules. The effect of delayed inoculation on nodule occupancy of *Rhizobium* strains has also been shown by others (18, 21). Skrdleta (18) found that the delayed introduction of one strain of *R. japonicum* favored the strain applied at sowing. However, the shortest period for delayed inoculation chosen by Skrdleta (18) was 3 days. In our studies, we show that even a short preexposure (6 h or less) is adequate to significantly alter the competition pattern. Skrdleta (18) also found that all of the taproot nodules were formed by

TABLE 2. Effect of preexposure of roots to an ineffective strain (SM-5) on nodule occupancy by an effective strain (USDA 110) of *R. japonicum*

Inoculum ^a		Total nodule mass (mg) ^b	No. of nodules per plant ^b	Nodule occupancy (% of total) ^b		
Primary (zero time)	Secondary (72 h)			USDA 110	SM-5	USDA 110 and SM-5
USDA 110	None	53 ^{c,d}	25 ^e	100 ^f	0 ^g	0 ^h
SM-5	None	32 ^d	57 ^{i,j}	0 ^k	100 ^l	0 ^h
None	USDA 110	64 ^c	40 ^{e,j}	100 ^f	0 ^g	0 ^h
None	SM-5	39 ^d	73 ⁱ	0 ^k	100 ^l	0 ^h
USDA 110 and SM-5	None	49 ^{c,d}	28 ^e	89 ^m	7 ⁿ	3 ^h
None	USDA 110 and SM-5	53 ^{c,d}	31 ^e	92 ^{m,o}	9 ⁿ	0 ^h
USDA 110	SM-5	44 ^{c,d}	18 ^e	96 ^{f,o}	4 ^{g,n}	0 ^h
SM-5	USDA 110	52 ^{c,d}	41 ^{e,j}	18 ^p	76 ^q	5 ^h

^a All uninoculated controls were devoid of nodules.

^b Values are the means of three replicates.

^{c-q} Numbers followed by the same letter within a given column do not differ significantly ($P = 0.05$) as determined by the Duncan multiple-range test.

the primary strain, whereas in our studies, the majority of both taproot and lateral root nodules were formed by the primary strain. Winarno and Lie (21) showed that nodulation of pea cultivar Afghanistan by a nodulating strain of *R. leguminosarum*, TOM, was suppressed by a non-nodulating strain, PF2. They found that when TOM was introduced 24 h before PF2, normal nodulation occurred.

In midwestern soils of the United States the use of highly effective strains as inocula has not resulted in increases in soybean yields when other indigenous strains of *R. japonicum* have been present. In particular, strains belonging to the USDA 123 serogroup form the majority of the nodules on soybeans grown in this area (5, 15, 20). Although USDA 123 out-competes USDA 110 in most midwestern soils, we have found that in vermiculite and in soils devoid of indigenous *R. japonicum* strains, USDA 110 out-competes USDA 123 (data not shown). In a time course design similar to that described in this paper, we preexposed young seedlings to USDA 110 and then transplanted them into pots containing a midwestern soil. Our results (data not shown) showed that even a 2-h preexposure to USDA 110 increased the percentage of nodules formed by this strain. Longer periods of preexposure resulted in the majority of nodules containing USDA 110.

The results from these studies indicate that interactions which occur during the early period of infection between the soybean host and its microsymbiote are perhaps the most critical for competition among *R. japonicum* strains. The role of the host in determining the outcome of competition among strains was highlighted by the observation that young seedlings with only a small (1- to 2-cm) radicle present at the time of primary exposure to one strain became predisposed to that strain for further nodulation. The majority of the nodules formed on the mature plant, regardless of their location on the root, were initiated by the strain to which the host was preexposed. This occurred whether the primary inoculum was an effective or ineffective strain. The localization of the particular processes which are affected during the early period of infection would greatly aid our understanding of the complex events which occur in the establishment of an effective nitrogen-fixing symbiosis between the legume host and its microsymbiote.

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