Suppression of Nodule Development of One Side of a Split-Root System of Soybeans Caused by Prior Inoculation of the Other Side[1]

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ABSTRACT

In a split-root system of soybeans (*Glycine max* L. Merr), inoculation of one half-side suppressed subsequent development of nodules on the opposite side. At zero time, the first side of the split-root system of soybeans received *Rhizobium japonicum* strain USDA 138 as the primary inoculum. At selected time intervals, the second side was inoculated with the secondary inoculum, a mixture of *R. japonicum* strain USDA 138 and strain USDA 110. In a short-day season, nodulation by the secondary inoculum was inhibited 100% when inoculation was delayed 10 days. Nodulation on the second side was significantly suppressed when the secondary inoculum was delayed for only 96 hours. In a long-day season, nodule suppression on the second side was highly significant, but not always 100%. Nodule suppression on the second side was not related to the appearance of nodules or nitrogenase activity on the side of split-roots which were inoculated at zero time. When the experiments were done under different light intensities, nodule suppression was significantly more pronounced in the shaded treatments.

The nitrogen-fixing symbiotic association formed between leguminous plants and soil bacteria of the genus *Rhizobium* has recently become an area of intense scientific research because of the economic and agricultural benefits derived from cropping systems using nodulated legumes. One of the specific goals of recent investigations has been to understand the mechanisms involved in the formation of the legume-*Rhizobium* association. Although both the host plant and the bacteria contribute to the specificity of the association (6), the mechanisms by which each partner exerts its influence remain poorly understood (1, 4, 13). To date, relatively few studies have addressed the role which the host plays in regulating the symbiotic state. In this study, we used a split-root system (15) to separate strain/strain interactions from those involving the host. This system allowed us to partition these interactions both in space and time.

MATERIALS AND METHODS

Rhizobium Culture. Cultures of *Rhizobium japonicum* strains, USDA 110 and USDA 138, were obtained from E. L. Schmidt, University of Minnesota, St. Paul, MN. Stock cultures were maintained on YEM[2] (3) slants at 28°C. Inoculation consisted of injecting 1.0 ml (1 × 10⁹ cells) of 4-d YEM cultures into the seedling vessel at the designated times.

Split-Root System. A modification of the split-root system described by Singleton (15) was used in this study. Glass tubes (Bellco, 38 × 300 mm) were filled with 127 mm of coarse No. 3 gravel and overlaid with 170 mm of moist vermiculite. Sixty-five ml of 1:4 strength Hoagland nitrogen-free solution (5) was added to the gravel reservoir. A rope wick facilitated the movement of the solution into the vermiculite (Fig. 1). Water was added with a 19-gauge needle inserted into a glass tube (8 mm o.d., 185 mm length) fitted with a serum stopper. The entire apparatus was sterilized for 20 min at 121°C. PVC elbows (21.0 mm o.d., 6.0 cm sides) packed with moist vermiculite were sterilized by gamma-irradiation (1.5 × 10⁶ rads). After sterilization, the PVC elbows were placed into the tops of the growth vessels, covered with a 2-cm layer of paraffin-coated sand and sterile nonabsorbent cotton. A planting hole (10 mm diameter) was drilled into the joint of each elbow to receive seedling radicles.

Soybean seeds of cv Lee were obtained from H. H. Keyser, United States Department of Agriculture, Beltsville, MD. Seeds were surface-sterilized for 20 min in 4% calcium hypochlorite, washed five times with sterile H₂O, and soaked in H₂O for 2 h prior to germination in sterile moist vermiculite. Twenty-four-h-old seedlings with radicles 1 to 2 cm long were selected for all experiments. Before inserting the seedlings into the planting holes, the tips of the radicles (2–5 mm) were removed to induce branching. The seedlings were watered with sterile H₂O to ensure capillarity between the cut radicles and vermiculite. Twenty-four h after planting, the seedlings were transferred to the greenhouse where they were grown without artificial lighting. Seedlings were inoculated 1 week after transfer to the greenhouse. At zero time, the first side of the split-root systems received 1.0 ml (1 × 10⁹ cells/ml) of the primary inoculum, USDA 138. At different times afterwards, the second side was inoculated with 1.0 ml (1 × 10⁹ cells/ml) of the secondary inoculum, an equal mixture of strain USDA 110 and strain USDA 138. Plants were harvested 3 weeks after the inoculation of the second side.

Determination of Nitrogenase Activity. Nitrogenase activity was determined by the acetylene reduction assay (17). Acetylene reduction was determined at 25°C by incubating roots in jars with 10% (v/v) acetylene for 15 and 30 min. Gas samples were analyzed with a Bendix 2500 gas chromatograph equipped with a Porapak-T column (80–100 mesh).

Identification of Strains in Nodules. Immunofluorescence was used to type the strains within the nodules. Twenty-five % of the nodules from each replicate per treatment were identified. In cases where the number of nodules was less than ten, all of the nodules were examined. Nodule smears were stained with strain-specific FA according to the method of Schmidt *et al.* (12). Gelatin-rhodamine isothiocyanate (Rh-ITC) was used to sup-

[2] Abbreviations: YEM, yeast-extract mannitol; PVC, polyvinylchloride; FA, fluorescent antibodies.
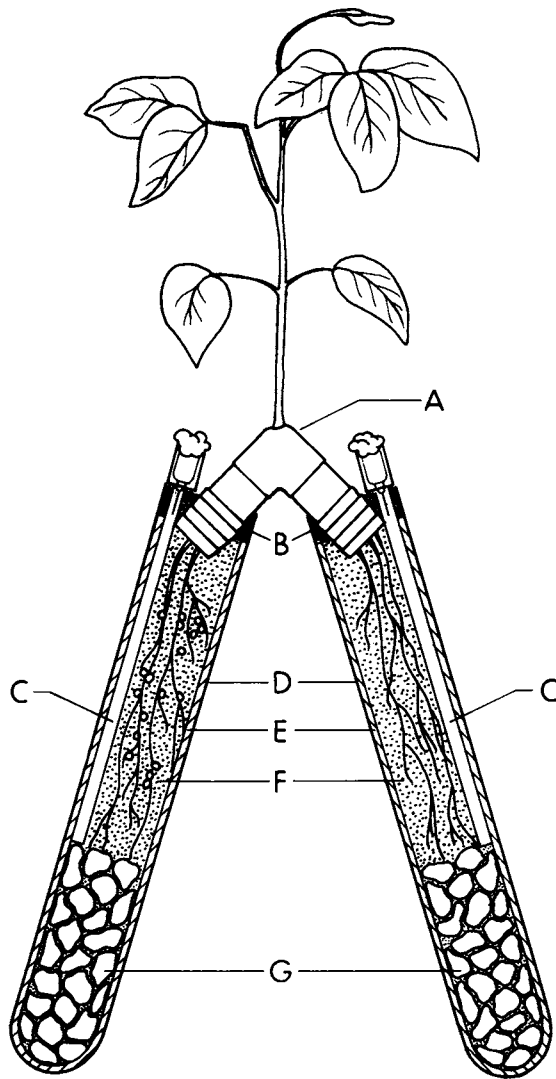
FIG. 1. Diagram of split-root assembly. (A), PVC elbow with planting hole; (B), layer of paraffin-coated sand and cotton; (C), glass tubing for watering with serum stopper and 19-gauge needle covered with cotton-plugged tygon tubing; (D), Bellco glass tubes; (E), rope wick; (F), vermiculite; (G), rock reservoir with Hoagland solution.

press nonspecific adsorption (2). Microscopy was done as described previously (7).

RESULTS

When soybeans were grown during a SD season, delayed inoculation of one side of the split-root system resulted in suppression of nodulation on that side (Table I). When both the first and the second sides were inoculated at zero time, nodule numbers and mass were approximately equal. A delay in inoculation of the second side for 4 d caused a significant suppression in nodule numbers and mass compared to the zero time treatment. When inoculation was delayed for 10 d, no nodules were formed on the second side. However, total nodule number and mass did not differ significantly between treatments. R. japonicum strain USDA 110 was a slightly better competitor than USDA 138 for both the zero time and 4-d delayed treatments (Table I).

In the second experiment, done during a LD season (July 1982), nodule suppression on the second side was significant, but not always 100% (Table II). Nodule mass did not differ significantly when only one side was inoculated at zero time, or when both sides were inoculated at zero time (Table II). However, when inoculation of the second side was delayed for 4, 6, 8, 10, or 14 d, nodule mass decreased significantly from the treatment in which both sides were inoculated at zero time. Suppression of nodule mass was also reflected in nitrogenase activity for the delayed treatments (Table II). A similar reduction in nodule numbers was also observed when inoculation of the second side was delayed for 4 d or longer (Table II). As in the previous experiment, USDA 110 was more competitive than USDA 138 and occupied 90% to 96% of the nodules on the second side (data not shown). Total nodule numbers and mass (Table II) did not differ significantly between treatments.

Shoot and root dry weights are summarized in Table III. Root dry weights for the first and second sides were approximately the same for the different treatments. An increase in total shoot and root dry weight was observed for the later harvest times.

In addition to the experimental treatments, 15 split-root systems were used to determine whether suppression of nodulation on the sides where inoculation was delayed was related to the appearance of nodules or the onset of nitrogenase activity. From this group, three split-root systems were harvested 6, 8, 9, 10, and 12 d after the inoculation of USDA 138 (zero time) to the first side of the split-root systems. Split-root systems harvested after 6 d were devoid of nodules, and no nitrogenase activity was

Table I. Suppression of Nodulation of Half-Root Systems Caused by Delayed Inoculation (SD Season, February 1981)

Four to five replicates used per treatment; all treatments harvested 3 weeks after inoculation of second side. Results are expressed as mean ± SE. Numbers ($^{b-i}$) in the same column flanked by the same letter are not significantly different (P = 0.05) as determined by the Mann-Whitney U test (16). Day length, 10 to 11 h; mean temperature, 25°C; light intensity for this time of the year, 1260 $\mu E/m^2 \cdot s$.

Treatment	First Side: Zero Time Inoculation with USDA 138 Nodule		Second Side: Delayed Inoculation						Total (1st + 2nd Sides) Nodule/Plant	
					Nodule					
	Mass	Number	Inoculum	Delay	Mass	Number	Occupancy[a]		Mass	Number
							110	138		
	mg			d	mg				mg	
1	58 ± 18	61 ± 15	None	0	0^b	0^c	0	0	58 ± 18^d	61 ± 15^e
2	39 ± 14	48 ± 14	110/138	0	32 ± 5^f	32 ± 8^g	57 NS	54 NS	71 ± 11^d	80 ± 18^e
3	49 ± 15	52 ± 21	110/138	4	15 ± 6^h	24 ± 7^i	70 NS	54 NS	61 ± 16^d	77 ± 21^e
4	71 ± 4	77 ± 18	110/138	10	0^b	0^c	0	0	71 ± 4^d	77 ± 18^e

[a] Not significant (P = 0.05) as determined by the t test for paired comparisons (16).

Table II. *Suppression of Nodulation of Half-Root Systems as a Result of Delayed Inoculation (LD Season, July 1982): Nodule Parameters*

Three to four replicates used per treatment; all treatments harvested 3 weeks after inoculation of second side. Results are expressed as mean ± SE. Numbers ([b-h]) in the same column flanked by the same letter are not significantly different (P = 0.10) as determined by the Mann-Whitney U test (16). Day length, 13 to 14 h; mean temperature, 33.5°C.

First Side: Zero Time Inoculation				Second Side: Delayed Inoculation					Total (1st + 2nd Sides) Nodule/Plant		
	Nodule					Nodule					
Inoculum	Mass	Number	N_2-ase activity[a]	Inoculum	Delay	Mass	Number	N_2-ase activity[a]	Mass	Number	N_2-ase activity[a]
	mg		*μmol/side·h*		*d*	*mg*		*μmol/side·h*	*mg*		*μmol/side·h*
138	80 ± 25	86 ± 32	5.0 ± 4.0	None	0	0[b]	0[d]	0	80 ± 25[a]	86 ± 32[h]	5.0 ± 4.0
110	70 ± 10	51 ± 16	ND	None	0	0[b]	0[d]	0	70 ± 10[a]	51 ± 16[h]	ND
110/138	84 ± 8	75 ± 13	5.0 ± 5.0	None	0	0[b]	0[d]	0	84 ± 8[a]	75 ± 13[h]	5.0 ± 5.0
None	0	0	0	110/138	14	93 ± 36[c]	94 ± 36[c]	2.0 ± 2.0	93 ± 36[a]	94 ± 36[h]	2.0 ± 2.0
138	33 ± 11	36 ± 12	0.3 ± 0.1	110/138	0	54 ± 16[c]	40 ± 7[f]	1.0 ± 0.1	87 ± 4[a]	76 ± 14[h]	1.3 ± 0.0
138	90 ± 3	59 ± 6	1.0 ± 0.3	110/138	4	<1[b]	3 ± 2[d]	0.1 ± 0.1	90 ± 3[a]	62 ± 8[h]	1.1 ± 0.2
138	87 ± 15	59 ± 14	4.0 ± 3.0	110/138	6	0[b]	0[d]	0	87 ± 15[a]	59 ± 14[h]	4.0 ± 3.0
138	99 ± 20	71 ± 7	1.0 ± 0.1	110/138	8	<1[b]	2 ± 1[d]	0.1 ± 0.1	99 ± 20[a]	73 ± 7[h]	1.1 ± 0.1
138	123 ± 12	75 ± 10	2.0 ± 0.7	110/138	10	3 ± 2[b]	8 ± 7[d]	0.1 ± 0.1	126 ± 11[a]	83 ± 4[h]	2.1 ± 0.8
138	100 ± 4	73 ± 20	0.7 ± 0.4	110/138	14	2 ± 2[b]	6 ± 5[d]	<0.1	102 ± 6[a]	79 ± 5[h]	0.7 ± 0.4
110	120 ± 3	79 ± 24	2.0 ± 1.0	110/138	14	<1[b]	2 ± 1[d]	<0.1	120 ± 3[a]	81 ± 24[h]	2.0 ± 1.0

[a] Two replicates used per treatment; ND, not determined.

Table III. *Suppression of Nodulation of Half-Root Systems as a Result of Delayed Inoculation (LD Season, July 1982): Plant Parameters*[a]

Three to four replicates used per treatment; all treatments harvested 3 weeks after inoculation of second side. Results are expressed as mean ± SE. ND, not determined.

First Side: Zero Time Inoculation		Second Side: Delayed Inoculation			Total (1st + 2nd Sides)	
Inoculum	Root dry wt	Inoculum	Delay	Root dry wt	Root dry wt	Shoot dry wt
	g/side		*d*	*g/side*	*g/plant*	*g/plant*
138	ND	None	0	ND	ND	0.46 ± 0.18
110	ND	None	0	ND	ND	0.39 ± 0.05
110/138	ND	None	0	ND	ND	0.45 ± 0.03
None	0.03 ± 0.00	110/138	14	0.08 ± 0.05	0.11 ± 0.05	0.77 ± 0.33
138	ND	110/138	0	ND	ND	0.49 ± 0.07
138	0.22 ± 0.17	110/138	4	0.09 ± 0.06	0.31 ± 0.11	0.87 ± 0.16
138	0.28 ± 0.20	110/138	6	0.28 ± 0.20	0.56 ± 0.30	1.20 ± 0.16
138	0.38 ± 0.10	110/138	8	0.19 ± 0.05	0.57 ± 0.13	1.40 ± 0.30
138	0.60 ± 0.06	110/138	10	0.31 ± 0.05	0.91 ± 0.10	1.80 ± 0.30
138	0.35 ± 0.21	110/138	14	0.16 ± 0.08	0.51 ± 0.13	1.80 ± 0.20
110	0.41 ± 0.15	110/138	14	0.22 ± 0.12	0.63 ± 0.20	1.80 ± 0.49

detected. Nodules were visible on split-root systems harvested 8 d after inoculation, but nitrogenase activity was negligible. Significant nitrogenase activity was not detected until 12 d after inoculation.

Another experiment was done to determine whether suppression of nodulation on one side of the split-root system was related to the quantity of light available to the soybean host. Split-root systems were grown during a LD season (August 1982) at three different light intensities. One group of plants were grown without shading (full sun), another group under 47% shade cloth, and a final group under 73% shade cloth. Results in Table IV indicate that there was incomplete suppression of nodule development on the delayed sides in plants grown without shading. However, both nodule numbers and mass on the sides where inoculation had been delayed for 10 d differed significantly from the treatments inoculated at zero time. In the shaded plants, no nodules developed on the delayed side of the split-roots. Microscopic examination of roots from the delayed sides showed that the process of nodulation had been initiated. Nodule primordia were visible on the delayed side of split-root systems grown under 47% shade, whereas only infection threads were observed on the split-roots of plants grown under 73% shade.

Total nodule mass, nitrogenase activity, and numbers (Table IV) did not differ significantly within each light intensity group. However, there was significant differences in nodule numbers and mass between the three light treatments. These differences were also reflected in shoot and root dry weights (Table V). As was observed in the previous two experiments, USDA 110 was the more competitive strain and formed the majority of the nodules regardless of the light intensity under which the soybeans were grown (data not shown).

DISCUSSION

Delayed inoculation of one side of a split-root system for 4 or more days resulted in partial or complete suppression of nodulation on that side. The degree to which nodulation of the second side was suppressed depended on the conditions under which the soybean host was grown. There was a complete suppression of nodule development on the second side when soybeans were

Table IV. *Effect of Light Intensity on Suppression of Nodulation of Half-Root Systems as a Result of Delayed Inoculation (LD Season, August 1982): Nodule Parameters*

Three to four replicates used per treatment; all treatments harvested 3 weeks after inoculation of second side. Results are expressed as mean ± SE. Numbers ($^{c-p}$) in the same column flanked by the same letter are not significantly different (P = 0.10) as determined by the Mann-Whitney U test (16). Day length, 13 to 14 h; mean temperature, 33.5°C.

Shading[a]	First Side: Zero Time Inoculation				Second Side: Delayed Inoculation					Total (1st + 2nd Side Nodule/Plant		
	Inoculum	Nodule			Inoculum	Delay	Nodule			Mass	Number	N$_2$-ase activity[b]
		Mass	Number	N$_2$-ase activity[b]			Mass	Number	N$_2$-ase activity[b]			
		mg		*μmol/side·h*		*d*	*mg*		*μmol/side·hr*	*mg*		*μmol/side·h*
None (6600 μE/m²·s)	None	0	0	0	110/138	0	45.0 ± 3^c	32 ± 9^g	0.51 ± 0.38	45.0 ± 3^j	32 ± 9^n	0.51 ± 0.38
	None	0	0	0	110/138	10	44.3 ± 13^c	43 ± 31^g	0.61 ± 0.20	44.3 ± 12^j	43 ± 31^n	0.61 ± 0.20
	138	58.7 ± 27	37 ± 14	0.51 ± 0.10	None	0	0^f	0^j	0	58.7 ± 27^j	37 ± 14^n	0.51 ± 0.10
	138	21.7 ± 19	19 ± 18	0.30 ± 0.15	110/138	0	20.7 ± 10^d	22 ± 15^g	0.19 ± 0.13	42.4 ± 11^j	41 ± 7^n	0.49 ± 0.02
	138	78.5 ± 12	58 ± 6	0.39 ± 0.05	110/138	10	1.5 ± 1^f	9 ± 4^h	0.02 ± 0.03	80.0 ± 13^k	67 ± 9^n	0.41 ± 0.08
47% (300 μE/m²·s)	None	0	0	0	110/138	0	21.3 ± 9^d	25 ± 9^g	0.35 ± 0.20	21.3 ± 9^l	25 ± 9°	0.35 ± 0.20
	None	0	0	0	110/138	10	23.5 ± 7^d	46 ± 20^g	0.30 ± 0.20	23.5 ± 7^l	46 ± 20^n	0.30 ± 0.20
	138	11.8 ± 7	10 ± 8	0.13 ± 0.01	None	0	0^f	0^j	0	11.8 ± 7^l	10 ± 8°	0.13 ± 0.01
	138	5.7 ± 5	6 ± 6	0.07 ± 0.02	110/138	0	10.0 ± 6^d	7 ± 6^h	0.12 ± 0.10	15.7 ± 6^l	13 ± 10°	0.19 ± 0.09
	138	36.8 ± 8	26 ± 2	0.15 ± 0.08	110/138	10	0^f	0^j	0	36.8 ± 8^l	26 ± 2°	0.15 ± 0.08
73% (140 μE/m²·s)	None	0	0	0	110/138	0	6.3 ± 4^e	7 ± 4^h	0.03 ± 0.00	6.3 ± 4^m	7 ± 4^p	0.03 ± 0.00
	None	0	0	0	110/138	10	10.8 ± 8^d	12 ± 12^h	0.15 ± 0.10	10.8 ± 8^l	12 ± 12°	0.15 ± 0.10
	138	5.0 ± 3	5 ± 4	0.02 ± 0.02	None	0	0^f	0^j	0	5.0 ± 3^m	5 ± 4^p	0.02 ± 0.02
	138	1.0 ± 1	2 ± 1	0.01 ± 0.00	110/138	0	51. ± 1^e	6 ± 1^h	0.02 ± 0.00	6.1 ± 2^m	8 ± 1^p	0.03 ± 0.00
	138	9.1 ± 13	9 ± 8	0.06 ± 0.00	110/138	10	0^f	0^j	0	9.1 ± 13^l	9 ± 8°	0.06 ± 0.00

[a] Average maximum light intensity over the course of the experiment. Determined by Spectra illumination meter, model OP400.

[b] Two replicates used per treatment.

Table V. *Effect of Light Intensity on Suppression of Nodulation of Half-Root Systems as a Result of Delayed Inoculation (LD Season, August 1982): Plant Parameters*

Three to four replicates used per treatment; all treatments harvested 3 weeks after inoculation of second site. Results are expressed as mean ± SE.

Shade Level	First Side: Zero Time Inoculation		Second Side: Delayed Inoculation			Total (1st + 2nd Side)	
	Inoculum	Root dry wt	Inoculum	Delay	Root dry wt	Root dry wt	Shoot dry wt
		g/side		*d*	*g/side*	*g/plant*	
None	None	0.13 ± 0.03	110/138	0	0.11 ± 0.06	0.24 ± 0.04	0.56 ± 0.04
None	None	0.13 ± 0.03	110/138	10	0.23 ± 0.06	0.36 ± 0.08	0.56 ± 0.22
None	138	0.18 ± 0.09	None	0	0.18 ± 0.07	0.36 ± 0.11	0.51 ± 0.08
None	138	0.22 ± 0.22	110/138	0	0.12 ± 0.02	0.34 ± 0.21	0.49 ± 0.01
None	138	0.49 ± 0.27	110/138	10	0.29 ± 0.14	0.78 ± 0.41	1.04 ± 0.05
47%	None	0.10 ± 0.10	110/138	0	0.07 ± 0.03	0.17 ± 0.04	0.38 ± 0.03
47%	None	0.08 ± 0.02	110/138	10	0.12 ± 0.06	0.20 ± 0.07	0.52 ± 0.10
47%	138	0.09 ± 0.07	None	0	0.09 ± 0.04	0.18 ± 0.06	0.30 ± 0.04
47%	138	0.09 ± 0.03	110/138	0	0.09 ± 0.03	0.18 ± 0.05	0.36 ± 0.06
47%	138	0.13 ± 0.06	110/138	10	0.09 ± 0.02	0.22 ± 0.06	0.61 ± 0.19
73%	None	0.08 ± 0.02	110/138	0	0.09 ± 0.04	0.17 ± 0.06	0.28 ± 0.03
73%	None	0.07 ± 0.02	110/138	10	0.07 ± 0.04	0.14 ± 0.04	0.39 ± 0.03
73%	138	0.09 ± 0.02	None	0	0.07 ± 0.02	0.17 ± 0.02	0.26 ± 0.04
73%	138	0.07 ± 0.01	110/138	0	0.08 ± 0.01	0.15 ± 0.01	0.27 ± 0.00
73%	138	0.05 ± 0.02	110/138	10	0.03 ± 0.00	0.08 ± 0.02	0.36 ± 0.06

grown during a SD season or under shade (Tables I and IV). A significant reduction in nodule numbers and mass on the second side occurred when soybeans were grown during a LD season (Table II). Suppression of nodulation on the second side was not related to the appearance of nodules or nitrogenase activity on the first sides inoculated at zero time. Suppression of nodulation had already occurred in those treatments in which the introduction of the two strains was delayed for 4 d. This was 4 d prior to the appearance of nodules and 8 d prior to the detection of significant nitrogenase activity on the sides which received the primary inoculum at zero time. Furthermore, suppression of nodulation was not strain related, since a 14-d delay in inoculation of the second side resulted in suppression of nodulation on that side with either *R. japonicum* strain USDA 138 or USDA 110 as the primary inoculum. In clover (9, 11) and alfalfa (10), it has also been observed that the appearance of the first group of nodules has a suppressive effect on further infection and nodule development. This effect was also shown to be unrelated to the supply of nitrogen (10, 11).

The total number of nodules and nodule mass supported by the host may be linked to the amount of light available to the host for photosynthesis, since significant differences in total nodule numbers and mass were observed between the three light intensity treatments (Table IV). The degree to which the process of infection proceeded was also found to vary with light intensity. In plants grown under full sun, there was a significant suppression in nodule number and mass on the sides where inoculation was delayed. In contrast, when plants were grown under 47% shade, the process of infection was stopped at the nodule primordia stage, while only infection threads were observed in plants grown under 73% shade. Host control of nodule numbers and mass has also been observed when soybeans were inoculated with varying ratios of effective and ineffective strains of *R. japonicum* (14). Singleton and Stockinger (14) found that the average size of an effective nodule was twice that of an ineffective one, and that the average weight of an effective nodule increased as the proportion of effective nodule tissue decreased.

The results from this study indicate that the number of infection sites which develop into functional nodules are controlled by the host. Control by the soybean host over the number of sites leading to successful infections appears to be exerted during the early stages of the infection process and to the amount of light available to the host for photosynthesis. Kosslak *et al.* (8) have previously shown that the early events in the process of infection of soybeans play a critical role in determining the nodule occupancy of two strains of *R. japonicum* competing for nodulation of the host legume. We found that when either a less competitive, or poorly competitive, ineffective strain was introduced into the soybean rhizosphere 6 or more hours before the introduction of a highly competitive strain, the per cent of nodules occupied by either of the less competitive strains increased significantly (8).

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