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Nodulation of Soybeans as Affected by Half-root Infection with *Heterodera glycines*[1]

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Abstract: A split-root technique was applied to soybean, *Glycine max* (L.) Merr. cv. Lee 68, to characterize the nature of the nodulation suppression by race 1 of the soybean cyst nematode (SCN), *Heterodera glycines.* Root-halves of each split-root plant were inoculated with *Rhizobium japonicum,* and one root-half only was inoculated with various numbers of SCN eggs. Nodulation (indicated by nodule number, nodule weights, and ratio of nodule weight to root weight) and nitrogen-fixing capacity (indicated by rate of acetylene reduction) were systemically and variously suppressed on both root-halves of the split-root plant 5 weeks after half-root inoculation with 12,500 SCN eggs. Inoculation with 500 eggs caused this suppression only on the SCN-infected (+NE) root-half; nodulation on the companion uninfected (−NE) root-half was stimulated slightly. The +NE root-halves inoculated with 5,000 eggs were excised at 2-week intervals; nodulation on the remaining −NE root-halves was not different from that of the noninoculated control when measured 6 weeks after the SCN inoculation. Thus, the systemic suppression of nodulation was reversible upon the removal of the SCN. Similarly, application of various levels of KNO_3 to the −NE root-halves of the split-root plant did not alleviate the suppressed nodulation on the companion +NE root-halves, even though plant growth was much improved at certain levels of nitrogen (125 μg N/g soil). This indicated that the localized suppression of nodulation by SCN was caused by factors in addition to poor plant growth.

Key words: soybean cyst nematode, *Rhizobium japonicum, Glycine max,* nitrogen fixation, split-root technique.

Much of the damage inflicted on soybean, *Glycine max* (L.) Merr., by certain races of the soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, results from the disruption of the symbiotic nitrogen-fixation process. This interaction can occur by suppression of nodule formation (10,13,14,18) or by dysfunction of existing nodules such as the impediment of leg-hemoglobin biosynthesis (12). Dysfunction of the symbiotic process in soybeans and in other legumes also occurs with viral and fungal infections (7,23,24), but the physiological basis for such phenomenon has received little study. The formation and function of nodules can be disrupted by an alteration in the host, the rhizobia, and/or the environment (9,25). For instance, the low nodule weight per soybean plant infected with soybean mosaic virus and/or bean pod mottle virus is related to poor plant growth (28). Others (24) attribute similar effects on soybean infected with *Rhizoctonia solani* to the severe root necrosis and root-hair decay. Plant-parasitic nematodes alter the morphology, anatomy, and biochemistry of their hosts, including the hosts' compatibility to other pathogens (15,19,20). Prior exposure of one-half of a split-root to one species of nematodes has been shown to suppress the population build-up of other nematode species in the opposite half-roots (21). This response is regarded as evidence that a translocatable metabolite detrimental to the nematode is involved, although the origin of this metabolite is unknown (20,21).

The present investigation was initiated, using the split-root technique, to determine if the SCN suppression of nodulation on soybeans was systemic and reversible upon removal of the nematodes and if the suppression could be overcome with the application of nitrogen fertilizer. Information gained from such experiments should add insight to the nature of the soybean–nematode interaction and to the mechanism of nodulation. A portion of this work has been preliminarily reported (16).

Materials and Methods

Nematode inoculum: SCN (Race 1), originating from a population in Wilmington, North Carolina, was used throughout this study. Nematode egg inoculum was prepared by crushing the cysts gently in a

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Ten-Broeck homogenizer. The cysts were obtained from greenhouse-grown SCN-infected soybeans ('Lee 68') after 2 months. The crushed eggs were cleared of soil and plant debris by centrifugation at 500 *g* for 3 minutes into a cushion of 20% sucrose (w/w) in a swinging bucket rotor. The eggs, banded at the interface between the aqueous and sucrose layers, were collected in a 26-μm sieve, rinsed with water, and diluted to the desired densities.

Rhizobium inoculum: Rhizobium japonicum (Kirchner) Buchanan, strain 61A76, obtained from J. Burton of the Nitragin Company, Milwaukee, Wisconsin, was grown in yeast-extract manitol medium (27) at 25 C on a rotary shaker (150 rpm) for 4–5 days. The cells were harvested in the exponential phase of growth by centrifugation at 10,000 *g* for 15 minutes. After being washed twice in saline, the cells were diluted to the desired cell density, using a colorimeter.

Split-root soybean plants: Selected seeds of Lee 68 soybeans were surface sterilized in a mixture of ethanol, commercial bleach (containing 5.25% sodium hypochlorite), and water (1:2:7, v/v/v) for 3 minutes. After several rinses in sterile water, the seeds were germinated in vermiculite for about 3 days at 25 C. When the roots of the seedlings had attained a length of 4–5 cm, the terminal 4 cm of each root was halved longitudinally with a sterile razor blade. The divided roots were kept apart with small pieces of spacer (cork, size 00) and then placed in moist sterile sand for an additional 4 days before being transferred individually into paired 10-cm-d clay pots containing a 1:1 soil-sand mixture (sandy loam). The split-root plants (1-week-old) were inoculated with *R. japonicum* and nematode eggs at the time of transplanting.

Influence of nematodes on nodulation in split-root systems: Each root-half of the split-root plants was inoculated with 1×10^8 colony-forming units (CFU) of *R. japonicum.* In addition, one root-half of each split-root plant received 0, 500, 2,500, or 12,500 SCN eggs. Thus, in the 0-egg treatment, both root-halves were without nematodes (−NE/−NE), whereas in other treatments, there was one root-half with nematode (+NE root-half) and the companion root-half without nematode (−NE root-half). The treatments, each with five replicates, were arranged in a complete randomized block design in the greenhouse, and the experiment was performed three times.

All plants were grown in the greenhouse with supplemental lights under a 16-hour photoperiod at about 28 C and an 8-hour dark period at ~23 C. The pots received water twice daily and Evan's nitrogen-deficient nutrient solution (27) biweekly. The plants were harvested 5 weeks after inoculation. Fresh weights of shoot, root, and nodules, as well as the number of nodules for each half-root system were recorded. The nitrogen-fixing capacity of the nodules from each excised half-root system was determined, using the acetylene-ethylene assay (13). The development of SCN in roots was monitored on the 10th or 30th day by harvesting samples (five replicates) from all treatments. Roots harvested on day 10 were stained with acid-fuchsin lactophenol (8), and those harvested on day 30 were washed with a high pressure spray of water for recovery of cysts (1).

Effects of nematode removal on nodulation: Each split-root soybean plant was inoculated with *Rhizobium* (1×10^6 CFU) in each root-half and also with either 0 (noninoculated, with −NE/−NE root-halves) or 5,000 SCN eggs in one of the root-halves (SCN inoculated, with −NE/+NE root-halves). At 0, 2, 4, and 6 weeks after inoculation, the entire +NE root-half of the SCN-inoculated plant—or, in case of the noninoculated plant, one of the −NE root-halves—was removed by excision with a sharp razor blade. Growth and nodulation parameters previously mentioned were measured on the remaining root-halves (i.e., the −NE root-halves) 6 weeks after the nematode inoculation (or at 6, 4, 2, and 0 weeks, respectively, after the half-root excision). Plants with root-halves excised at week 6 were referred to as the unexcised controls, since in these cases, measurements were taken immediately after the excision; among these plants, those with half-root infection of SCN were referred to as the unexcised, inoculated controls (UIC), and those without half-root infection of SCN were referred to as the unexcised, noninoculated control (UNC). These treatments, each with eight replicates, were arranged in the greenhouse in a split-plot

design, with nematode levels being the whole plots and time of excision the subplots.

Influence of application of nitrate on nodulation: Each split-root soybean plant was inoculated similarly with *Rhizobium* and either 0 (noninoculated control, with −NE/−NE root halves) or 2,500 (SCN-inoculated, with −NE/+NE root-halves) SCN eggs. Potassium nitrate (KNO_3) solutions were applied to the −NE root-half of the nematode-inoculated split-root plant or to either −NE root-half of the control to establish four nitrogen treatments (+NIT): 0, 25, 125, and 625 μg N/g air-dried soil. The growth and nodulation parameters on the companion nonfertilized (−NIT) root halves were measured 5 weeks after inoculation. These treatments, each with five replicates, were also arranged in the greenhouse in a split-plot design, with nematode levels being the main plots and level of nitrate the subplots.

Results

Preliminary experiments were conducted to determine the possible effects of splitting roots on the growth and nodulation of the plant. Intact and split-root soybeans inoculated with 1×10^8 CFU of *R. japonicum* were grown in 10-cm-d clay pots in the greenhouse. At the end of 6 weeks, the fresh shoot weights for the normal and split-root plants were 15.5 and 22.4 g, respectively, whereas the corresponding fresh root weights were 13.0 and 19.6 g. Thus, the fresh weights of either the shoot or the root of the split-root plants were greater ($P = 0.05$) than those of the normal plants. Presumably, the better growth for the split-root plant was attributed to the additional soil available for root growth. Nonetheless, the growth stage (number of nodes on the stem), the nodule fresh weights, the number of nodules, the ratio of nodule weight to root weight, and the shoot/root ratios of the split-root and normal plants were not different ($P = 0.05$). The split-root soybean plants were, therefore, suitable for the purpose of the experiments.

Influence of nematodes on the growth and nodulation of split-root soybeans: At each inoculum level, 32–40% of SCN eggs hatched and penetrated the roots 10 days after inoculation, and 16–38% of these eggs finally developed to mature cysts 30 days after inoculation. The correlations between number of eggs inoculated and the number of juveniles or cysts found in the root tissues were 1.00 or 0.96, respectively.

At the time of harvest, even though only one of the half-root systems was infected with SCN, the growth and nodulation of the split-root plant as a whole were suppressed progressively as the level of SCN inoculum increased from 0 to 12,500 eggs (Table 1). The negative effects of SCN on nodule weight were greater than those on plant weight; for example, a 90% inhibition of nodule weight/plant corresponded to only 55% suppression of plant growth (indicated by total plant weight) when the nematode inoculum level shifted from 0 to 12,500 eggs. Nodule number, nitrogen-fixing capacity, and the average weights of the nodules were 86%, 83%, and 43% lower than the respective controls at the 12,500-egg inoculum level (Table 1). The

Table 1. Growth and nodulation of split-root soybean, *Glycine max* (L.) Merr. cv. Lee 68, as affected by half-root infection with *Heterodera glycines* race 1.*

Growth or nodulation parameters	Inoculum (eggs/root-half)			
	0	500	2,500	12,500
Shoot height (cm)	69.3 a	66.5 b	61.6 c	49.2 d
Shoot weight (g)	11.2 a	10.4 b	8.3 c	4.7 d
Total root weight (g)	9.8 a	9.2 ab	8.2 b	4.8 c
Total no. of nodules/plant	144.0 a	118.0 b	64.0 c	20.0 d
Total weight of nodules/plant (g)	1.2 a	0.9 b	0.5 c	0.1 d
Weight (mg) of nodules/g root	125.7 a	107.8 b	59.3 c	27.5 d
Nitrogen-fixing capacity (μmoles C_2H_2 reduced/plant/day)	69.8 a	70.2 a	49.8 b	12.0 c
Average weight of nodules (mg/nodule)	8.2 a	7.7 ab	6.7 b	4.7 c
Nodular efficiency (μmoles C_2H_2 reduced/g nodule/day)	68.8 b	89.1 ab	157.2 a	115.9 ab

* Means of five replicates. Means followed by the same letter in the same rows are not different ($P = 0.05$) according to the Waller-Duncan *K*-ratio *t*-test.

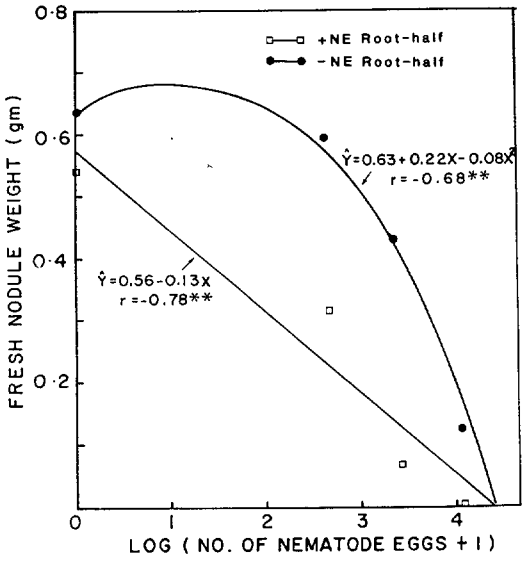

FIG. 1. Dose-response curve of fresh nodule weights of soybean (*Glycine max* cv. Lee 68) root-halves to half-root inoculation with various numbers of *Heterodera glycines* race 1 (SCN) eggs. Nematode (NE) eggs were inoculated onto the +NE root-halves only when the seedlings were 7 days old. Each point (mean of five replications) was taken at 5 weeks after inoculation.

leaves also became increasingly chlorotic as the level of SCN increased, an indication of increasing nitrogen stress resulting from suppression of nodulation.

There was a difference between nodulation response on +NE root-halves and that on companion −NE root-halves of the same plants (Figs. 1, 2). A linear decline in fresh nodule weight to zero was observed on +NE root-halves as the logarithm of SCN egg numbers (from 0 to 12,500 eggs) increased (Fig. 1). In the companion −NE root-halves, the nodule weight was not decreased at low SCN numbers (500 eggs), but decreases sharply thereafter with increasing SCN inocula (Fig. 1). Thus, the response of nodule weight to the level of nematode infection in −NE root-halves could be described by a quadratic model ($P = 0.01$) (Fig. 1). Similar patterns of nodulation response were observed for the ratio of nodule weight to root weight (mg nodules/g root) (Fig. 2). Rate of acetylene reduction, number of nodules per gram root, and average weight per nodule were less systemically affected at the 2,500-egg treatment than at the 12,500-egg treatment (Fig. 2). In general, these nodulation parameters were highest on the −NE root-halves of the 500-egg treatment, being always slightly above those for the 0-egg treatment; thereafter, the nodulation parameters of the 2,500- and 12,500-egg treatments were equal to, or much less ($P = 0.05$) than, those for the 0-egg treatment (Fig. 2). Also, the nodulation parameters on the +NE root-halves were always less than those on the companion −NE root-halves, regardless of nematode inoculum level. For example, at the 12,500-egg treatment, the rate of acetylene reduction was suppressed almost 100% in the +NE root-halves, whereas it was suppressed only 67% in the companion −NE root-halves (Fig. 2B).

Effects of excising nematode-infected half-root systems on the growth and nodulation of split-root soybean: For the split-root plants with −NE/−NE root-halves (noninoculated), excising one of the −NE root-halves at later stages of growth resulted in better shoot growth (Table 2). Fresh shoot weight was the highest (22.4 g) for the plants with one of the −NE root-halves excised on week 6 (UNC). The time of half-root excision made no difference among the fresh root weights, nodule weights, ratios of nodule weight to root weight, or number of nodules on the remaining −NE root-halves of these noninoculated plants (Table 2). For the split-root plant with −NE/+NE root-halves (SCN-inoculated), there was no difference ($P = 0.05$) in shoot growth between the plants with +NE root-halves excised on week 6 (UIC) and the plants with root-halves excised at earlier times. Also, the fresh root weight, nodule weight, ratio of nodule weight to root weight, and the number of nodules on the remaining −NE root-halves of these UIC plants were the lowest (Table 2).

Relative to those of the UNC, fresh nodule weight and milligrams of nodules per gram of root on the remaining −NE root-halves of UIC were inhibited by 51% and 30%, These inhibitions were not observed on the −NE root-halves of the SCN-inoculated plants with excised +NE root-halves (Table 2). The initial chlorotic appearance of the leaves of these SCN-inoculated plants also disappeared after excision of the +NE root-halves at the time of harvest, whereas those of the UIC remained chlorotic.

Influence of nitrate on the growth and nodulation of split-root soybean: Application of

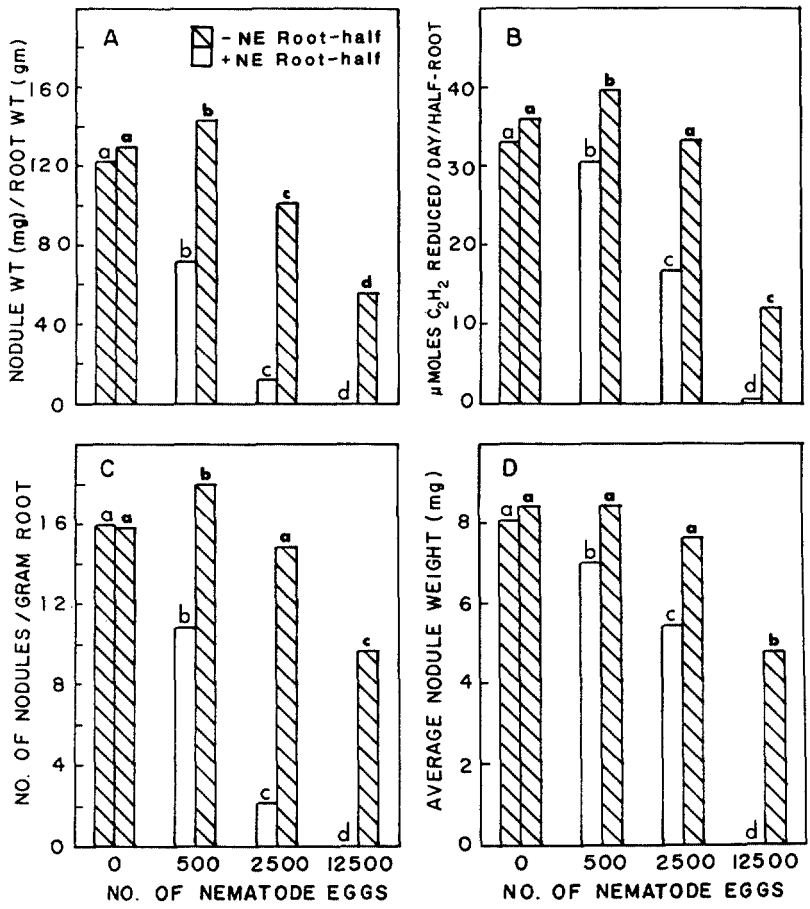

FIG. 2. Nodulation responses of soybean (*Glycine max* cv. Lee 68) root-halves to various levels of *Heterodera glycines* race 1 (SCN) infestation. A) Nodule weight (mg) per gram root. B) Rate of acetylene reduction (μmoles $C_2H_2 \cdot day^{-1} \cdot half\text{-}root^{-1}$). C) Number of nodules per gram root. D) Average weight (mg) per nodule.

SCN eggs (NE) were inoculated onto the +NE root-halves only when the seedlings were 7 days old. Bars marked with the same letters with the same root-half treatment are not significantly different ($P = 0.05$) according to Waller-Duncan *K*-ratio *t*-test. All data (means of five replicates) were taken at 5 weeks after inoculation.

increasing levels of nitrate to either −NE root-halves of −NE/−NE treatments or to the −NE root-half of −NE/+NE treatments generally improved plant growth (Fig. 3). There was one exception, however: at a high level of nitrate (625 μg N/g soil), the roots in physical contact with nitrate showed signs of morphological changes and plant growth was inhibited under the additional stress of SCN (Fig. 3). At a medium level of nitrate (125 μg N/g soil), the growth and green color of these SCN-inoculated plants were comparable to those of the noninoculated plants, with a total plant fresh weight 96% of the latter. Despite the general improvement of growth, nodulation on +NE root-halves of these plants was still suppressed by 82.4% (Table 3), suggesting that poor plant growth, resulting from nitrogen deficiency, was not the sole cause of nodulation suppression.

In order to check the effects of nitrate itself on nodulation, weights of nodules per gram root on +NIT root-halves (roots directly in contact with nitrate) of the SCN-noninoculated plants were also determined. The respective nodule weights for the treatments containing 0, 25, 125, and 625 μg N/g soil were 82.9, 91.6, 40.6, and 0.4 (mg/g), whereas those on the companion −NIT root-halves (roots remote from

TABLE 2. Growth and nodulation of soybean cv. Lee 68 root-halves as affected by time of removal of the companion root-halves under various treatments with *Heterodera glycines* race 1 (SCN).*

SCN treatment on soybean root-halves	Root-half being excised	Time of excision (wk)†	Growth or nodulation parameter on remaining (−NE) root-halves: Fresh shoot weight (g)	Fresh root weight (g)	Fresh nodule weight (g)	Weight of nodules per gram root (mg)	Number of nodules
Noninoculated (−NE/−NE)	−NE	0	15.3 b	11.9 a	1.43 a	120 a	153.6 a
		2	16.1 b	11.2 a	1.44 a	128 a	105.7 a
		4	19.6 ab	10.9 a	1.47 a	136 a	122.9 a
		6 (UNC)	22.4 a	10.1 a	1.10 a	105 a	98.4 a
Inoculated (+NE/−NE)	+NE	0	15.3 a	11.9 a	1.43 a	120 a	157.6 a
		2	14.5 a	10.8 b	1.49 a	137 a	188.2 b
		4	14.0 a	9.8 b	1.38 a	140 a	145.4 a
		6 (UIC)	14.1 a	7.1 c	0.49 b	69 b	66.0 c

* Readings taken 6 wk after inoculation. Means (of eight replicates) with same letters in same column within same nematode treatment are not different ($P = 0.05$) according to the Waller-Duncan K-ratio t-test. −NE, root-halves inoculated with 0 SCN eggs; +NE, root-halves inoculated with 5,000 SCN eggs; UNC, unexcised noninoculated control; UIC, unexcised inoculated control.

† Weeks after the SCN inoculation.

nitrate) were 86.4, 96.3, 91.7, and 63.3 (mg/g), respectively; therefore, at 625 μg N/g soil, nitrate seemed to suppress nodulation systemically. At 125 μg N/g soil, nodulation was suppressed 51% on the +NIT root-half but was stimulated 6% on the −NIT root-half over that at 0 μg N/g soil. Thus, nitrate at the 125-μg N/g soil level gave only localized suppression of nodulation on the +NIT root-halves and had no effect on the opposite −NIT root-halves, the root systems on which the nodulation measurements used in Table 3 were made.

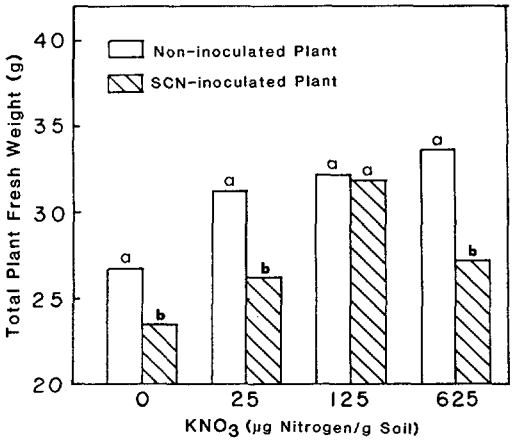

FIG. 3. Growth of split-root soybeans (*Glycine max* cv. Lee 68) as affected by application of KNO_3 and inoculation of *Heterodera glycines* race 1 (SCN) to different root-halves of the same plants. Various concentrations of KNO_3 were applied to one of the root-halves of the SCN-inoculated or noninoculated plants. The remaining root-halves of these plants were inoculated with 2,500 or 0 SCN eggs, respectively, when the seedlings were 7 days old. Plant fresh weights were taken at 5 weeks after inoculation. Bars marked with the same letter within the same KNO_3 treatment are not significantly different ($P = 0.05$) according to Waller-Duncan K-ratio t-test.

DISCUSSION

The method described in these experiments can be used to establish soybeans with split-root systems in less than a week and is suitable for early development stud-

TABLE 3. Effect of half-root application of nitrate on the nodulation of the companion half-root systems in the presence or absence of *Heterodera glycines* race 1 (SCN).*

Level of KNO_3 applied to the −NE† root-half (μg N/g soil)	Nodulation on companion root-half with treatment of: −NE† (mg nodules/g root)	+NE† (mg nodules/g root)	Inhibition of nodulation in presence of SCN (%)
0	86.4 a	9.7 a	88.8
25	96.3 a	14.4 b	85.0
125	91.7 a	16.1 b	82.4
625	63.3 b	0.0 c	100.0

* Means of five replicates. Means followed by same letters in same column are not different ($P = 0.05$) according to the Waller-Duncan K-ratio t-test.

† −NE, root-halves inoculated with 0 SCN eggs; +NE, root-halves inoculated with 2,500 SCN eggs.

ies of nodules. A similar split-root technique, set up by training lateral roots, has been used on soybeans to determine the effects of combined nitrogen on nodulation (11). However, this latter method has the disadvantage that 2–3 weeks are required to develop the split-root plant, during which time the physiology of the plant conducive to nodulation could have been altered (4).

SCN infection causes a suppression in growth and nodulation of the split-root soybeans, similar to the effects reported for soybean plants with intact roots (1,12,14). The greater suppression on nodule weights than on plant growth seems to agree with the idea that nitrogen (N_2) reduction may not be the limiting factor for the growth of legumes (25).

With the split-root technique it is possible to partition the responses of the infected and uninfected root tissues. The results of our experiments demonstrate that the influence of *H. glycines* on nodulation may be localized or systemic, depending on the level of nematode inoculum. Apparently, at low nematode numbers, the plant is able to compensate for nodules lost in the infected tissues by producing more nodules in the uninfected tissues. This response is consistent with the fact that growth of existing nodules, initiation of new nodules, and elimination of senescent nodules are so adjusted that nodule mass per root becomes a highly predictable quantity for a given *Rhizobium*–legume association at a particular stage of development (25).

Theoretically, if the effect of SCN on nodulation is purely systemic, one would expect the dose-response curves (Fig. 1) for each half-root system of the same plant to have about the same slope. In contrast, if the effect is purely localized, one would expect a flat horizontal curve for the −NE half-roots and a downward slope for the +NE half-roots. The dissimilar shapes and slopes of the dose-response curves for nodule weights (Fig. 1), deviating from the theoretical, are indicative of the mixed localized and systemic effects of the nematode. The systemic effect is probably operating on the −NE half-root system because of its remoteness from contact with the nematodes. In addition to the systemic effects, localized effects may also be operating on the roots in contact with the nematodes, giving rise to a more severe suppression of nodulation on these roots (Fig. 2).

The nematode causes localized disruption of root cortex and may interfere with the entry of the rhizobial infection thread into the suitable cortical cells, thereby preventing the establishment of symbiosis. Cyst nematodes induce cellular changes at the site of infection, such as the formation of syncytia that become nutrient sinks (15). Since nodulation is governed strongly by the availability of photosynthate (9), nodulation in the presence of SCN may, in part, be limited by the availability of photosynthate and minerals. Mobilization of minerals (3) and incorporation of photosynthate (5) to infection sites of root-knot nematode have been reported. Whether this is also true with cyst nematodes awaits further investigation. It is also possible that a translocatable inhibitor of nodulation has been produced as a result of the plant–nematode interaction. There have been cases where biologically active compounds are produced or initiated by the nematodes (6,22), although the effect of these compounds may be localized.

Excision of half of the root system itself does not cause a suppression in nodulation, although it causes a slight inhibition in plant growth. Suppression of nodulation is negated upon the removal of nematodes in the infected root-halves. This result also indicates that the nematode acts as a powerful nutrient sink or producer of inhibitors. Excisions of effective nodules from clover (9,25) or pods from soybeans (17), both powerful sinks for carbohydrates, has also been shown to stimulate further nodule production or nitrogen-fixing activity of the existing nodules.

It has been reported that the damage to soybeans by SCN can be lessened with the application of nitrogen fertilizer, NH_4NO_3 (26), indicating that combined nitrogen can improve the growth of the diseased plant. However, combined nitrogen, such as nitrate at a high level, is a powerful inhibitor of nodulation (9,25) and also has an adverse effect on the development of SCN (2). Therefore, it is necessary to use the split-root technique to determine whether the general improvement of growth of the plant would alleviate SCNs suppression of nodulation. One of the half-root systems serves as feeder roots for the nitrogen fer-

tilizer while nodulation is tested on the remaining half-root systems with or without the nematode infection. The results clearly indicate that the localized suppression of nodulation cannot be eliminated by the application of nitrate or by the general improvement of growth of the plant. Possibly, apart from the inadequate supply of nitrogen or photosynthate, nodulation is further impeded by the nematode's more direct effect on root morphology or physiology.

It would be interesting to determine whether the systemic suppression of nodulation could be relieved with the general improvement of growth of the plant. Results from such an experiment would give insight not only to the validity of the nutrient sink or chemical inhibitor hypothesis but also to the question of whether there are, indeed, separate localized and systemic effects operating to suppress the nodulation of soybeans.

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