Suppression of Binding between Rhizobia and Soybean Roots by *Heterodera glycines*

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ABSTRACT

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Soybean seedlings inoculated with juveniles of race 1 of *Heterodera glycines*, the soybean cyst nematode (SCN), were incubated with a suspension of *Rhizobium japonicum* prepared from cultures grown in a synthetic medium containing D-(1-^{3}H) glucose. After washing to remove unbound rhizobia, the roots were oxidized, and the radioactivity of the resulting tritiated water was measured. Roots from SCN-inoculated seedlings had lower radioactivity on a per root or per unit weight basis than those from controls. Binding of *R. japonicum* to control soybean roots also was inhibited by pretreatment of roots with *N*-acetyl-D-galactosamine or D-galactose, the haptens of the 120,000-dalton soybean lectin, but not glucose. These results suggest that the soybean lectin is involved in binding *R. japonicum* to soybean roots, and that SCN infection suppresses the binding between roots and rhizobia. Scanning electron microscopy revealed that abundant rhizobia were on the surfaces of control soybean roots. Very few rhizobia were observed on root surfaces of SCN-infected plants. Nematode infection caused an increase in numbers of root hairs and therefore the surface area of total root system. In contrast, SCN infection caused a reduction in hemagglutination activity in root homogenates. We conclude that reduction in binding of rhizobia to SCN-infected soybean roots apparently was not due to the reduction in surface areas of infected roots but resulted from interference of the nematode with soybean lectin metabolism.

Additional key words: glycine max, nitrogen fixation, lectins of *Rhizobium japonicum*.

The soybean cyst nematode (SCN), *Heterodera glycines* Ichinohe, is one of the most destructive pests in many soybean-producing countries, including the United States (8). Infected plants are usually stunted and chlorotic when nematode population densities are high and environmental conditions are favorable for disease development. These symptoms are due primarily to nitrogen deficiency as a result of suppression of nodulation (indicated by a limited number of nodules per plant) and reduction in nitrogen-fixing efficiency (indicated by reduction in μmoles of C_2H_4 produced per gram of nodule per hour) (12,13).

Formation of root nodules is a complex process. The first step in nodule formation is the attachment of infective rhizobia to soybean root hairs. After attachment, the rhizobia penetrate root hairs and stimulate the host cells to form infection threads. The rhizobia multiply inside the infection thread as it ramifies toward the root cortex. Eventually the rhizobia are released into host cells where they form bacteroids. Invaded and uninvaded cells divide and expand, forming nodules (2). In our attempt to investigate when, where, and how nodulation and nitrogen fixation in soybeans are affected by *H. glycines*, we have found that leghemoglobin content in the nodules of infected soybeans is significantly reduced compared to those of control plants (10). Since the physiological function of leghemoglobins is to protect nitrogenase from inactivation by molecular oxygen, and there is a positive correlation between the amount of nitrogen fixed and the amount of leghemoglobins present in nodules (20), we attributed the reduction in leghemoglobin content as a major factor to the reduction in the nitrogen-fixing efficiency observed in nematode-infected soybeans.

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The objective of this study was to determine the factors that suppress nodule formation on nematode-infected plants with special emphasis on the binding of rhizobia to soybean roots.

MATERIALS AND METHODS

Plant materials. Soybean (*Glycine max* 'Ransom') seeds were surface sterilized by treatment with 0.525% sodium hypochlorite for 3 min. After repeated rinsing with tap water, the seeds were germinated in vermiculite for 4 days in a greenhouse. Each seedling was transplanted to a 180-ml Styrofoam cup containing 150 ml of 212-μm white quartz sand mixed with 0, 500, or 2,500 second-stage juveniles of race 1 of *H. glycines*. The seedlings were grown in a greenhouse for 3 days after inoculation and were used as plant materials throughout this study.

Preparation of rhizobia. *Rhizobium japonicum* strain 61A76 was obtained from J. Burton (The Nitragin Company, Milwaukee, WI 53209) and maintained on yeast-mannitol agar (19). Liquid cultures were grown in a defined medium (4) at 28 C for 4 days on a rotary shaker. The rhizobia were harvested by centrifugation at 10,000 *g* for 10 min, washed three times in a phosphate-buffered saline (PBS—7.2 g NaCl, 1.48 g Na_2HPO_4, 0.43 g KH_2PO_4, pH 7.2), and finally suspended in PBS and adjusted to an absorbance of 0.4 at 500 nm with a spectrophotometer. This value corresponded to 10^9 colony-forming units per milliliter as determined by dilution plating. When radioactive rhizobia were needed, the bacteria were prepared in a similar manner except that 200 μl of aqueous solution containing 200 μCi of D-(1-^{3}H) glucose (specific activity = 500 mCi/mmol, Amersham Corp., Arlington Heights, IL 60005) were added to 25 ml of rhizobial culture 1 day before harvesting.

Scanning electron microscopy of attachment of rhizobia to soybean root hairs. Soybean plants were removed from sand 3 days after inoculation with SCN and washed under running tap water. Sand was washed from soybean roots with PBS. Roots of the washed plants were dipped in rhizobial suspension for 1 hr. After

repeated rinsing with PBS, the roots were fixed in 4% glutaraldehyde solution and prepared for scanning electron microscopy (11).

Quantitative assay of binding of rhizobia to soybean roots. Soybean plants were harvested and washed as described previously. Roots of the washed plants were dipped in suspension of ^{3}H-labeled rhizobia for 1 hr. After repeated rinsing with PBS to remove unbound rhizobia, the roots were combusted at 900 C in the presence of oxygen in a biological oxidizer (R. J. Harvey Instrument Corp., Hillsdale, NJ 07642). The tritiated water was collected in a tritium trap submerged in liquid nitrogen. The total count of radioactivity associated with each root was determined in a liquid scintillation counter. In some experiments, a unit weight (usually 100 mg) of secondary and tertiary roots obtained from control and nematode-infected plants was used as root material in the binding assay. The effects of sugars on the binding of rhizobia to soybean roots were also determined in a similar manner except that secondary and tertiary roots of the washed plants were treated with 60 mM D-glucose (Glu), 60 mM D-galactose (Gal), or 30 mM *N*-acetyl-D-galactosamine (NAGalm) for 30 min prior to being dipped in rhizobial suspension.

Estimation of surface area of soybean root system. Soybean roots washed as previously described were immersed in a 0.1% aqueous methylene blue solution for 2 min to stain epidermal cells and root hairs. Measurements of root diameter, epidermal cells, and root hairs were made on secondary and tertiary roots with an ocular micrometer and a compound microscope. Surface areas of epidermal cells and root hairs were estimated according to the procedure of Carlson (7).

Estimation of lectin content in soybean roots. Weighed root tissue was cooled in liquid nitrogen and ground into fine powder in a mortar. The powder was extracted with 10 volumes of PBS for 10 min. After filtration through Miracloth, the filtrate was treated with $(NH_4)_2SO_4$ to 80% saturation. The precipitate was collected by centrifugation at 10,000 *g* for 10 min, resuspended in PBS, dialyzed against PBS, and stored frozen until use.

The hemagglutination assay procedure (9) was used to estimate lectin contents in root extracts. One hundred microliters of 4% human, type A, red blood cells in PBS (Sigma Chemical Co., St. Louis, MO 63178) was mixed with an equal volume of serially diluted root extract in a microtiter plate. Lectin purified from cultivar Ransom soybean seeds by using an Affi-Gel galactosamine column (Bio-Rad Laboratories, Richmond, CA 94804) according to the procedure of Allen and Neuberger (1) was also included in the assay. The hemagglutination titer of the root extract was the reciprocal of the greatest dilution that agglutinated red blood cells. The hemagglutination titer was converted to lectin concentration by using a standard curve developed from similar agglutinations performed with purified lectin.

RESULTS

Penetration of soybean roots by *H. glycines*. Soybean seedlings were harvested 3 days after inoculation with SCN, washed briefly under tap water, and stained with acid fuchsin (6). Nematodes that had penetrated soybean roots were counted under a dissecting microscope. The average numbers of nematodes per root on soybean plants inoculated with 500 and 2,500 juveniles were 63 and 192, respectively. These counts represented penetration rates of 12.5% for the low- and 7.7% for the high-density nematode inocula.

Effects of *H. glycines* on surface areas of secondary and tertiary roots. Surface areas of epidermal cells and root hairs were calculated from the measurements of total root length, average root diameter, and average area of the epidermal cells on 1-mg of tertiary roots. The data were analyzed by the SAS Institute's general linear models procedure (17). The results indicated that infection by SCN caused an increase in average diameter of secondary and tertiary roots but a decrease in root length. The total surface area of epidermal cells, however, was not significantly affected by nematode infection. There were more root hairs on secondary and tertiary roots and consequently more surface area of root hairs on SCN-infected soybean roots than on those of control plants (Table 1).

Effects of *H. glycines* on binding between rhizobia and soybean root as revealed by scanning electron microscopy. Abundant rhizobia were found on the surface of epidermal cells of secondary and tertiary roots, and on the root hairs of control soybean plants (Figs. 1 and 2). Although rhizobia attached to the entire root hair, tips of root hairs had more bacteria than other regions. The root hairs of soybean plants inoculated with either 500 or 2,500 juveniles bound significantly fewer rhizobia (Figs. 3 and 4).

Effects of *H. glycines* on binding between rhizobia and soybean roots as assayed by radioisotope technique. The specific activity of the rhizobia prepared according to the procedure described in this report varied from 102 to 253 cells per 1 cpm. The binding of ^{3}H-labeled rhizobia to soybean roots was determined for an entire root as well as for a unit weight of secondary and tertiary roots. The results indicated that in both assay systems, roots of control soybeans, whether pretreated with glucose or not, had higher radioactivity. NAGalm- or Gal-treated roots and those from nematode-inoculated plants had lower radioactivity (Table 2).

Effects of *H. glycines* on lectin contents in soybean roots. Based on six determinations on two replicates, control plants contained hemagglutination activity equivalent to 2.93 ± 0.65 μg of soybean lectin per gram fresh weight of root tissue. The agglutination activity decreased as the nematode inoculum densities increased. Hemagglutination activities equivalent to 2.80 ± 0.58, 1.34 ± 0.36, and 0.97 ± 0.25 μg of lectin were detected in a gram of root tissue from soybean plants inoculated with 500, 2,500, and 12,500 juveniles, respectively, of *H. glycines*.

DISCUSSION

The growth conditions reported herein allow the nematode to penetrate the root system which can be prepared for radioisotope assay and scanning electron microscopy essentially free of foreign matter. Results of preliminary experiments indicated that second-stage juveniles had a higher penetration rate in a sand-soil (1:1, v/v) mixture than in quartz sand, but it was difficult to wash adherent foreign materials off the root without severe damage to root hairs.

TABLE 1. Effects of *Heterodera glycines* on surface area of soybean roots[a,b]

Treatment	Av. root diam. (μm)	Root length (μm)	Surface area epid. cell (mm^2)	Av. epid. cell (μm^2)	Root hairs (no.)	Av. root hair (μm^2)	Surface area of root hairs (mm^2)	Total surface area (mm^2)
Control	517	85,657	137	96 × 17.2	82,980	292 × 12.3	938	1,075
Nematodes								
500	627*	71,056 NS	140 NS	68 × 19.5*	105,064*	330 × 12.3 NS	1,334*	1,474*
2,500	607*	68,989 NS	131 NS	72 × 17.1*	106,852*	288 × 12.3 NS	1,175*	1,306*

[a] Secondary and tertiary roots were removed from three plants of each treatment and mixed, and a 1-mg portion was measured with an ocular micrometer for root diameter, root length, and size of epidermal cells and root hairs.

[b] Number followed by NS is not significantly different than the control. Number followed by an asterisk (*) is significantly ($P = 0.05$) different than the control according to the SAS general linear models procedure (17).

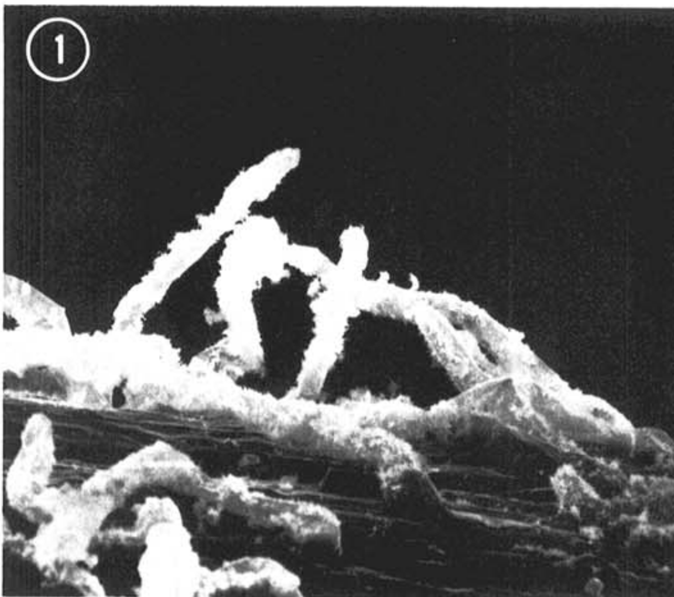
Soybean seedlings grew well in vermiculite, but the nematodes did not penetrate soybean roots in that medium.

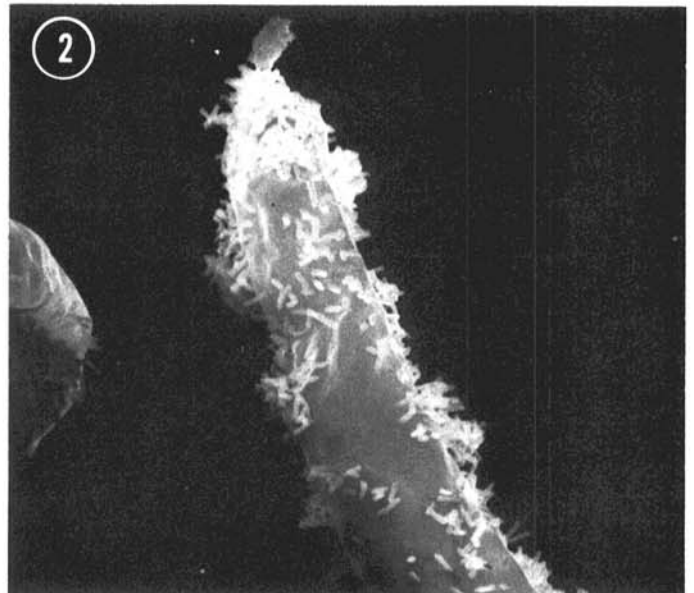
The binding between rhizobia and soybean root hairs is generally believed to be mediated by the rhizobial lipopolysaccharide and the 120,000-dalton soybean lectin (5,15). However, questions have been raised concerning the role of the soybean lectin (3,16). The inclusion of various sugar solutions in this study was intended to verify the role of soybean lectin in the rhizobia-soybean interaction under the experimental conditions reported herein. Soybean lectin has a high specificity to bind NAGalm and Gal but not Glu (14,18). If the lectin plays a role in rhizobia-soybean root interaction, soybean roots treated with either NAGalm or Gal should bind fewer rhizobia because some of the binding sites on root hairs will be occupied by the sugar. Glucose should not affect rhizobial attachment, because there is no affinity between soybean lectin and glucose. Our data (Table 2) show that NAGalm- or Gal-treated, but not Glu-treated, roots bound fewer rhizobia than the control roots on the basis of either whole root system or per unit weight of secondary and tertiary roots. This finding supports a role for soybean lectin in the binding between rhizobia and soybean roots.

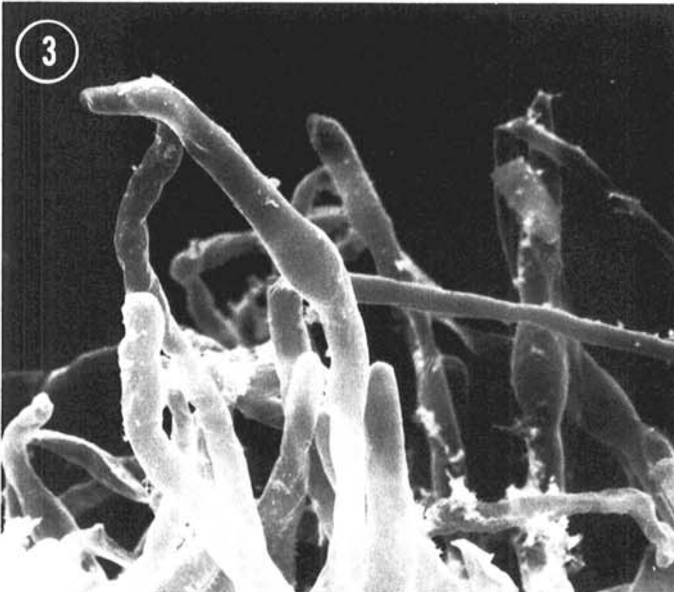
TABLE 2. Binding of ^{3}H-labeled cells of *Rhizobium japonicum* to soybean roots

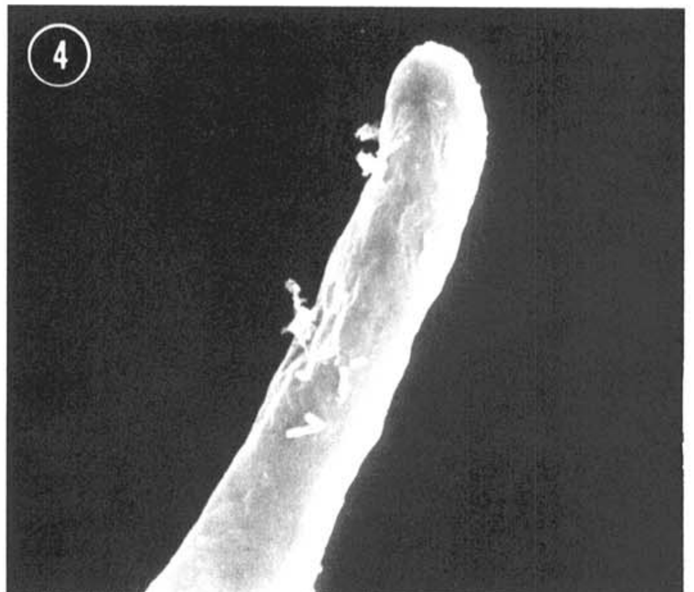
Treatment	Radioactivity (cpm)[v]	
	Whole root	2nd and 3rd root (100 mg)
Control roots		
Untreated	18,239 a	6,782 a
Glu-treated[w]	18,620 a	6,540 a
Gal-treated[x]	15,130 b	3,913 b
NAGalm-treated[y]	12,902 c	2,071 c
Roots inoculated with:		
500 nematodes[z]	11,391 d	3,243 c
2,500 nematodes[z]	10,250 d	3,257 c

[v] Values within columns not followed by the same letter are significantly different ($P = 0.05$) according to the Duncan's multiple range test.
[w] Treated 30 min with 60 mM D-glucose.
[x] Treated 30 min with 60 mM D-galactose.
[y] Treated 30 min with 30 mM *N*-acetyl-D-galactosamine.
[z] Juveniles of the soybean cyst nematode, *Heterodera glycines*.









Figs. 1–4. Scanning electron micrographs showing the effects of the soybean cyst nematode, *Heterodera glycines*, on binding between *Rhizobium japonicum* and soybean root hairs. **1 and 2,** Abundant bacteria on the surface of root hairs of control soybeans (×500 and ×2,000, respectively). **3 and 4,** Limited number of bacteria attached to root hairs of soybean plants inoculated with 2,500 nematode juveniles per plant (×500 and ×2,000, respectively).

Similar results have been reported for the binding between *G. soja* and *R. japonicum* (18).

The low radioactivity associated with nematode-infected soybean roots suggests that the number of binding sites is reduced as a consequence of nematode infection. There are two possible ways that nematode infection may limit the number of binding sites on soybean roots: by suppressing root growth, thereby reducing the surface area of the root system; and by reducing the lectin concentration in infected roots. Since there were more root hairs and surface area per unit weight of roots on nematode-infected soybeans than on uninoculated controls (Table 1), the suppression of binding cannot be attributed to a reduction in contact area on infected soybean roots. On the other hand, hemagglutination experiments suggest that nematode infection does affect lectin concentration in soybean roots and a negative correlation between hemagglutination activity and inoculum densities exists. We conclude that the differences in binding, as revealed in scanning electron microscopy (Figs. 1 to 4) and radioisotope assays (Table 2), apparently came from the differences in soybean lectins as a result of nematode infection.

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