Influence of *Heterodera glycines* on Leghemoglobins of Soybean Nodules

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ABSTRACT

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Four- to five-day-old soybean seedlings were dipped in a suspension containing 10^9 colony-forming units of *Rhizobium japonicum* and transplanted, seven per 20-cm-diameter clay pot containing a soil:sand (1:1, v/v) mixture. Three days after transplanting, some pots were inoculated with 2,500 juveniles of race 1 of *Heterodera glycines* per plant; other uninoculated pots served as controls. Plants were harvested 5 wk after nematode inoculation. Nodules harvested from nematode-infected soybeans had lower fresh weights per plant and lower specific nitrogenase activity (micromoles of C_2H_4 formed per gram of nodules per hour) as assayed by the acetylene reduction procedure. Leghemoglobins (Lb) were extracted from soybean nodules, purified in a Sephadex G-15 column, and separated into four components, Lb*a*, Lb*b*, Lb*c*, and Lb*d* by DEAE-cellulose column chromatography. Leghemoglobin content per gram of nodule was lower in nematode-infected soybeans than in the nodules of control plants. Lb*a* from nematode-infected and control plants had similar ultraviolet and visible light spectra and gel electrophoresis profiles, as did the Lb*b*, Lb*c*, and Lb*d*. The ratio of Lb*c*/Lb*a*, however, was higher from nematode-infected soybeans than from control plants. The significance of this difference is discussed.

Additional key words: *Glycine max*, nitrogen fixation, soybean cyst nematode.

Leghemoglobins (Lb) are the red pigments commonly found in the root nodules that develop on leguminous plants. They are hemoproteins consisting of an iron porphyrin and a peptide. The Lb from soybean nodules may be chromatographically fractionated into four components, Lb*a*, Lb*b*, Lb*c*, and Lb*d*, on a DEAE-cellulose column (1).

The physiological function of Lb is to facilitate O_2 diffusion within the nodule and into the bacteroids rapidly enough to support oxidative phosphorylation without damaging the functioning of nitrogenase (2).

Leghemoglobins extracted from the nodules of two dissimilar yellow lupins inoculated with the same strain of *Rhizobium lupini* were different as judged by DEAE-cellulose chromatography and polyacrylamide gel electrophoresis (8). When plants of a given line of yellow lupin were treated with two different rhizobial strains, the nodules had Lb of similar chromatographic and electrophoretic profiles (8). These results indicate that the type of Lb produced in a given symbiotic Rhizobium-legume interaction is plant specific. This finding has been extended to soybean (*Glycine max*), red kidney bean (*Phaseolus vulgaris*), broad bean (*Vicia faba*), and other legumes (6).

The efficiency of nitrogen fixation by nodules is influenced by various factors, including Lb concentration. There is a positive correlation between the intensity of nitrogen fixation and the amount of Lb present in root nodules (18). Acetylene reduction by bacteroid suspensions, a nitrogenase-mediated reaction, is dependent upon Lb concentration (3).

Infection of soybean by race 1 of the soybean cyst nematode (*Heterodera glycines* Ichinohe) causes a significant suppression in nitrogen-fixing efficiency by nodules as measured by the acetylene reduction procedure (14). The objective of this study was to determine the effects of nematode infection on Lb in soybean nodules.

MATERIALS AND METHODS

Preparation of nematodes, rhizobia, and nodules. Race 1 of *H. glycines* (14) was maintained on soybean (*G. max* Merr. 'Ransom') in the greenhouse. Nematode inoculum was prepared from cysts on soybean roots 40–50 days after nematode inoculation. Cysts were washed from the roots with a jet of water, filtered through a 710-μm sieve, collected on a 250-μm screen, and ruptured with a tissue grinder. Eggs thus released were hatched on a 75-μm screen partially submerged in water. Juveniles that penetrated through the screen were collected and counted with the aid of a microscope.

Rhizobium japonicum (Kirch.) Buch. strain 61A76, obtained from J. Burton (Nitragin Co., Milwaukee, WI 53209), was used throughout this study. Rhizobia were grown at 27 C for 5 days in a liquid medium (4) with constant shaking. The bacteria were harvested by centrifugation, and the concentration was determined with a spectrophotometer and adjusted to 10^9 colony-forming units per milliliter.

Soybean seeds were germinated in vermiculite for 4–5 days in a greenhouse. The seedlings were dipped in a rhizobial suspension for 30 min and transplanted, seven plants per 20-cm-diameter clay pot containing a steam-sterilized soil:sand (1:1, v/v) mixture.

Three days after transplanting, each pot received either 0 or 2,500 juveniles of *H. glycines* per plant. Plants were grown in a greenhouse with supplemental lights under a 16-hr photoperiod with day/night temperatures of 28 and 24 C, respectively. Plants were irrigated twice a week with Hoagland's solution (13) devoid of nitrogen and were harvested 42–45 days after seeding. Nodules were removed from roots, and their fresh weight was determined.

Determination of specific nitrogenase activity. Specific nitrogenase activity was determined by the acetylene reduction assay (12) and expressed as micromoles of C_2H_4 formed per gram fresh weight of nodules per hour. Nodules were incubated in 50-ml syringes containing 10% acetylene in air. After incubation at room temperature for 1 hr, 10-cm^3 samples were collected and stored in vacutainers (Vacutainer Systems, Rutherford, NJ 07070). Samples of 0.1–0.5 cm^3 were injected into a F & M model 700 gas chromatograph equipped with a flame ionization detector as described previously (14).

Leghemoglobin isolation. The isolation procedure described by Dilworth (9) was used with some modifications. Nodules were homogenized in four volumes of cold 0.1 M potassium phosphate buffer, pH 6.8, in the presence of polyvinylpyrrolidone (0.3 g/g of nodules). The homogenate was centrifuged at 10,000 *g* for 30 min and fractionated with solid ammonium sulfate between 55–80% saturation. The precipitate was dissolved in 0.1 M tris-HCl buffer,

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pH 7.7, containing 0.1 mM EDTA and dialyzed against the same buffer for 16 hr. After dialysis, the solution was centrifuged at 40,000 *g* for 30 min, and the supernatant mixed with 0.1 M tris-HCl buffer, pH 9.2, at a ratio of 3:1 (v/v). A small amount of solid $K_3Fe(CN)_6$ was added, and the solution stirred until O_2 evolution ceased. The mixture was then loaded onto a Sephadex G-15 column (30 × 1.5 cm) equilibrated with 0.1 M tris-HCl buffer, pH 9.2, at 4 C and eluted with the same buffer. Fractions containing red ferric Lb were pooled and dialyzed for 36 hr against 10 mM sodium acetate buffer, pH 5.2.

Leghemoglobin components, Lb*a*, Lb*b*, Lb*c*, and Lb*d*, were separated in a DEAE-cellulose column (30 × 1.5 cm) equilibrated with 10 mM sodium acetate buffer, pH 5.2, and eluted with a linear 10–100 mM sodium acetate buffer gradient, pH 5.2, in a total volume of 300 ml.

Determination of leghemoglobin concentration. Leghemoglobins were assayed by the pyridine haemochromogen method (15). Equal volumes of 4.2 M NaOH and leghemoglobin solution were mixed in the presence of a small amount of sodium hydrosulfite. Absorbance at 556 nm was read against a reagent blank in a Pye Unicam SP8-100 spectrophotometer. Leghemoglobin concentration was calculated using 16,000 as the average molecular weight of two major components, Lb*a*, and Lb*c* (10), and $E^{556\ nm}_{mM} = 34.6$ (9).

Spectrophotometry and gel electrophoresis. The ultraviolet and visible light absorption spectra of Lb components were measured in 0.1 M MES buffer, pH 5.2, with a Pye Unicam SP8-100 spectrophotometer. Disc electrophoresis of Lb components was performed at pH 8.3 in a 7% acrylamide gel according to the procedure of Davis (7). Each gel was loaded with 50 μg of Lb, run at 3 mA per tube, stained with 1% Amido Schwartz in 7% acetic acid for 1 hr, and destained with 7% acetic acid in a gel destainer.

RESULTS

Infection of soybean roots by *H. glycines*. One week after nematode inoculation, sample plants were removed from soil, washed, stained with acid fuchsin, and destained in glycerin. The number of nematodes in the roots was counted using a dissecting microscope (5). Approximately 20% of the 2,500 inoculated juveniles penetrated the roots of a given soybean plant.

Effects of *H. glycines* on nodule development and nitrogen fixation. Inoculation with *H. glycines* significantly suppressed nodular development and inhibited specific nitrogenase activity (Table 1). There was a 26% reduction in nodular fresh weight in nematode-infected soybean as compared to the control. Similarly, nodules from plants infected by *H. glycines* produced 61% less ethylene per gram nodule per hour than those from control plants.

Effects of *H. glycines* on leghemoglobin content and components. Infection of soybean by *H. glycines* also caused a significant reduction in leghemoglobin concentration. There was a 42% reduction in leghemoglobin content in plants parasitized by *H. glycines* as compared to that in the control soybeans (Table 1). Each Lb preparation was separated into five components by DEAE-cellulose chromatography. They were, in the order of elution, cytochrome c, Lb*a*, Lb*b*, Lb*c*, and Lb*d* (Fig. 1). Fractions for each Lb component were pooled, and the Lb concentration was determined (Table 1). About 90% of the Lb was in the forms of Lb*a* and Lb*c*.

TABLE 1. Effects of *Heterodera glycines* on nodule weight, specific nitrogenase activity, and leghemoglobin content of cultivar Ransom soybeans 5 wk after nematode inoculation

Treatment	Nodule wt.[a] (g)	Specific activity[b]	Leghemoglobin content[c]				
			Lb*a*	Lb*b*	Lb*c*	Lb*d*	Total
Control	0.413 ± 0.038	2.17 ± 0.45	34.2	2.7	39.0	5.4	81.3
H. glycines	0.307 ± 0.067	0.84 ± 0.46	16.0	1.3	27.4	2.5	47.2

[a] Grams per plant.
[b] Micromoles C_2H_4 per gram of nodules per hour.
[c] Micromoles per gram of nodules.

Comparison of leghemoglobins from soybean plants infected by *H. glycines* and uninfected control plants. All Lb components regardless of treatment had similar UV and visible light absorption spectra. Lb*a* and Lb*b* had maximal absorption wavelength at 403.0 nm, whereas Lb*c* and Lb*d* absorbed maximally at 403.5 nm. In polyacrylamide gel electrophoresis, Lb*d* was the most rapidly moving component, followed by Lb*c*, Lb*b*, and Lb*a* (Fig. 2). Lb*a* and Lb*c* from control and inoculated soybeans moved at the same rate and each moved as a single band. Lb*b* and Lb*d* gave multiple bands, but there was no difference in electrophoretic profiles between the components prepared from control and diseased plants.

DISCUSSION

The objective of this study was to determine the quantitative and qualitative effects of *H. glycines* on Lb in root nodules. Preliminary experiments were conducted to determine the conditions that allow this pathogen to exert a significant effect on soybean without completely inhibiting nodule development. When plants were

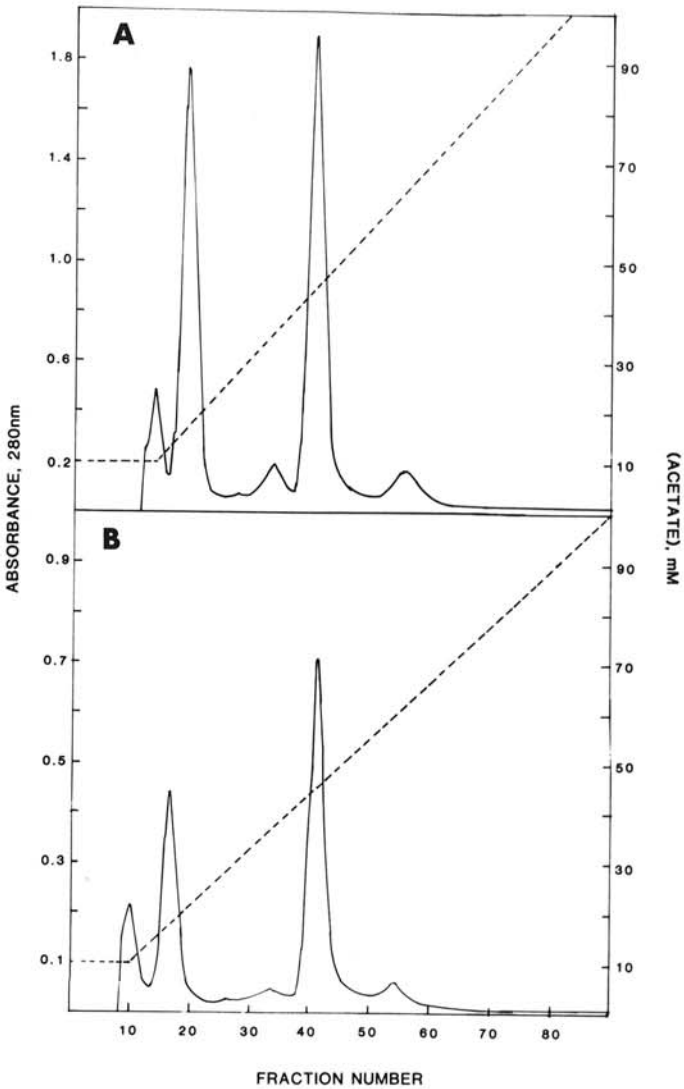

Fig. 1. Elution profiles for leghemoglobin (Lb) components on DEAE-cellulose columns. Leghemoglobins from nodules of **A**, control and **B**, from soybean plants infected by *Heterodera glycines* were loaded on DEAE-cellulose columns (30 × 1.5-cm) previously equilibrated with 10 mM sodium acetate, pH 5.2. The columns were eluted with a linear 10–100 mM sodium acetate buffer gradient, pH 5.2 (broken lines), and the effluents were monitored at 280 nm (solid lines). Each fraction was 3 ml. The five peaks from left to right are cytochrome c, and Lb*a*, Lb*b*, Lb*c*, and Lb*d*, respectively.

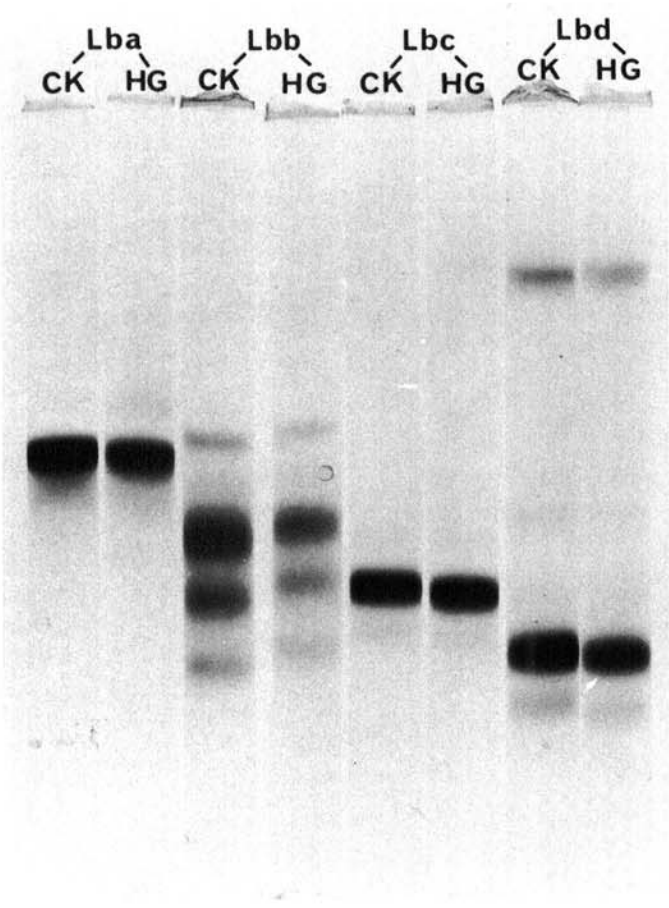

Fig. 2. Electrophoretic profiles of leghemoglobin (Lb) components. Lb*a*, Lb*b*, Lb*c*, and Lb*d* prepared from uninfected control soybean plants (CK) and from soybean plants infected by *Heterodera glycines* (HG) were applied to 7% polyacrylamide gels. Each gel was loaded with 50 μg of Lb, run at 3 mA per tube, and stained with 1% Amido Schwartz in 7% acetic acid.

inoculated simultaneously with a high density of nematodes (12,500 juveniles per plant) and rhizobia, complete inhibition of nodule development occurred. A 7-day delay in the introduction of low nematode inoculum density (500 juveniles per plant) resulted in no inhibition of nodulation. The conditions used in this study, 2,500 juveniles per plant inoculated 3 days after rhizobial treatment, inhibited nodulation approximately 30%.

The mean fresh weight of nodules collected from soybeans infected by *H. glycines* was lower than those of the control plants. This effect is in agreement with the earlier results reported by Lehman et al (14). Since there was no noticeable difference between the components prepared from control and nematode-infected soybeans, infection by *H. glycines* did not cause qualitative changes in leghemoglobins.

The Lb*c*/Lb*a* ratios for leghemoglobins obtained from nodules of control plants and plants parasitized by *H. glycines* were 1.14 and 1.71, respectively (Table 1). The former is in agreement with the ratio of 1.14 calculated from the data published by Appleby et al (1). The factor(s) which contributed to the observed higher Lb*c*/Lb*a* ratio in nodules of nematode-infected plants has not been determined. Different Lb components are coded for by different plant mRNA, and the relative levels of these mRNA change during root nodule development (17). Analysis of the in vitro translation products of mRNA from nodules of different ages has shown that Lb*c* is synthesized at a higher rate than Lb*a* in young nodules, and the reverse is a rule in mature nodules (17). The ratio of Lb*c*/Lb*a* in soybean root nodules, therefore, remains high in the early stages of soybean growth and decreases during flowering and fruiting (11). Since nodules from nematode-infected soybeans had a higher Lb*c*/Lb*a* ratio, this suggests that nodule development is impaired with nematode infection. The significant reduction in overall Lb content, however, indicates that nodules from nematode-infected plants are senescent. Although infection of soybean by the cyst nematode is known to limit nodule size (14), the cause, whether due to impairment of nodule development or acceleration of nodule senescence, remains to be determined. Uheda and Syono (16) have demonstrated that Lb*a* is more effective for oxygen binding and nitrogen fixation than Lb*c*. Therefore, the reduced nitrogen fixing efficiency of nodules from nematode-infected plants may be attributed to its higher Lb*c*/Lb*a* ratio.

LITERATURE CITED

1. Appleby, C. A., Nicola, N. A., Hurrell, J. G. R., and Leach, S. J. 1975. Characterization and improved separation of soybean leghemoglobins. Biochemistry 14:4444-4450.
2. Bergersen, F. J., and Turner, G. L. 1975. Leghemoglobin and the supply of O_2 to nitrogen-fixing root nodule bacteroids: Presence of two oxidase systems and ATP production at low free O_2 concentration. J. Gen. Microbiol. 91:345-354.
3. Bergersen, F. J., Turner, G. L., and Appleby, C. A. 1973. Studies of the physiological role of leghemoglobin in soybean root nodules. Biochim. Biophys. Acta 292:271-282.
4. Bishop, P. E., Guevara, J. G., Engelke, J. A., and Evans, H. J. 1976. Relation between glutamine synthetase and nitrogenase activities in the symbiotic association between *Rhizobium japonicum* and *Glycine max*. Plant Physiol. 57:542-546.
5. Byrd, D. W., Jr., Kirkpatrick, T., and Barker, K. R. 1982. An improved technique for clearing and staining plant tissue for detection of nematodes. J. Nematol. 15:142-143.
6. Cutting, J. A., and Schulman, H. M. 1971. The biogenesis of leghemoglobin. The determinant in the *Rhizobium*-legume symbiosis for leghemoglobin specificity. Biochim. Biophys. Acta 229:58-62.
7. Davis, B. J. 1964. Disc electrophoresis—II. Methods and application to human serum proteins. Ann. N.Y. Acad. Sci. 121:404-427.
8. Dilworth, M. J. 1969. The plant as the genetic determinant of leghemoglobin production in the legume root nodule. Biochim. Biophys. Acta 184:432-441.
9. Dilworth, M. J. 1980. Leghemoglobins. Meth. Enzymol. 69:812-823.
10. Ellfolk, N. 1972. Leghaemoglobin, a plant haemoglobin. Endeavour 31:139-142.
11. Fuchsman, W. H., Barton, C. R., Stein, M. M., Thompson, J. T., and Willett, R. M. 1976. Leghemoglobin: Different roles for different components? Biochem. Biophys. Res. Commun. 68:387-392.
12. Hardy, R. W. F., Holsten, R. D., Jackson, E. K., and Burns, R. C. 1968. The acetylene-ethylene assay for N_2 fixation: Laboratory and field evaluation. Plant Physiol. 43:1185-1207.
13. Hoagland, D. R., and Arnon, D. I. 1939. The water-culture method for growing plants without soil. Univ. Calif. Div. Agric. Sci. 347.
14. Lehman, P. S., Huisingh, D., and Barker, K. R. 1971. The influence of races of *Heterodera glycines* on nodulation and nitrogen-fixing capacity of soybean. Phytopathology 61:1239-1244.
15. Paul, K. G., Theorell, H., and Akeson, A. 1953. The molar light absorption of pyridine ferroprotoporhyrin (pyridine haemochromogen). Acta Chem. Scand. 7:1284-1287.
16. Uheda, V., and Syono, K. 1982. Effects of leghemoglobin components on nitrogen fixation and oxygen consumption. Plant Cell Physiol. 23:85-90.
17. Verma, D. P. S., Ball, S., Guerin, C., and Wanamaker, L. 1979. Leghemoglobin biosynthesis in soybean root nodules. Characterization of the nascent and released peptides and the relative rate of synthesis of the major leghemoglobins. Biochemistry 18:476-483.
18. Virtanen, A. I., Erkama, J., and Linkola, H. 1947. On the relation between nitrogen fixation and leghemoglobin content of leguminous root nodules. II. Acta Chem. Scand. 1:861-870.