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Infection by *Heterodera glycines* Elevates Isoflavonoid Production and Influences Soybean Nodulation[1]

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Abstract: High-performance liquid chromatography and *Sinorhizobium fredii* USDA191 *nodC-lacZ* gene fusion were used to monitor changes in the isoflavonoid content of soybean roots infected with *Heterodera glycines* isolate TN1. Isoflavonoid concentrations in infected roots of both *H. glycines*-resistant Hartwig and susceptible Essex soybean were two to four-fold higher than those of uninfected roots 2 and 3 days after inoculation. The isoflavonoids produced activated the transcription of *nodC-lacZ* fusion. The most abundant isoflavonoids were identified as daidzein and genistein by HPLC and GC/MS. *Heterodera glycines* increased the number of nodules formed on Essex roots inoculated with *B. japonicum* (USDA110) but reduced shoot weight and decreased the net nitrogenase activity of the nodules. *Heterodera glycines* infection of resistant Hartwig did not affect the total number of nodules or their nitrogen-fixing capacity.

Key words: β-galactosidase, *Heterodera glycines,* host-parasite relationship, HPLC, isoflavonoid, nematode, nodulation, soybean, soybean cyst nematode.

Soybean (*Glycine max* (L.) Merr.) is an important legume crop and serves as a protein and oil source. Two groups of bacteria, *Bradyrhizobium* and *Sinorhizobium* (rhizobia), enter into symbiotic association with soybean to form nodules that are able to convert atmospheric nitrogen into ammonia, which is used by the plant for its growth and development. This biologically important association results in more than 1×10^9 kg of fixed nitrogen per year in the United States. In contrast, the soybean cyst nematode, *Heterodera glycines* Ichinohe, can cause serious damage to soybean. For example, soybean disease loss estimates for the top 10 soybean-producing countries in 1994 attributed the greatest losses worldwide to *H. glycines* (Wrather et al., 1997). Cyst nematodes are known to influence the nodulation of legumes. For example, infection by *H. goettingiana* on peas and *H. trifolii* on white clover resulted in fewer nodules on their host roots (Jones and Moriarty, 1956; Taha and Raski, 1969), while fumigation experiments established an inverse relationship between the densities of *H. glycines* and the number of nodules on soybean (Endo and Sasser, 1958; Epps and Chambers, 1962). Different races of *H. glycines* have different effects on soybean nodulation. Race 1 reduced nodulation and nitrogen-fixing capacity and caused severe chlorosis on soybean. In contrast, races 2 and 4 did not affect the growth of soybean severely (Lehman et al., 1971). In a split-root study, nodulation suppression was related to the population density of race 1, and the suppression of nodulation could be reversed by removal of the nematodes (Ko et al., 1984). In general, the *H. glycines* interaction with soybean leads to reduced nodulation and reduced nitrogen fixation.

Studies conducted during the past 10 years have established the existence of an intricate signal-exchange mechanism between rhizobia and legumes (Denarie et al., 1996). Soybean plants secrete isoflavonoids into the rhizosphere, and these metabolites are perceived by the rhizobia (Phillips, 1992). The isoflavonoids interact with a regulatory protein, NodD, which then activates the transcription of several *nod* genes, including *nodABC* (Smit et al., 1992). The result is the production of lipochitooligosaccharide return signal, called the nod factor, which at very low concentrations induces the formation of nodules on soybeans (Stokkermans and Peters, 1994). These observations

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imply a crucial role for isoflavonoids in soybean-rhizobia interactions.

Flavonoids, which are phenylpropanoid-derived metabolites, are widely distributed in plants and have a protective function (McClure, 1975). Several biotic and abiotic factors can influence the production of flavonoids (Graham, 1989, 1991). Even though it is known that nematode infection adversely affects nodulation, it is not known if this interaction also affects isoflavonoid production. Our study was undertaken to determine the effect of *H. glycines* infection on isoflavonoid production and to examine the effects of infection on nodulation of soybean cultivars susceptible and resistant to *H. glycines*.

Materials and Methods

Preparation of roots for flavonoid analysis: Soybean cyst nematode isolate TN1 (race 5) was maintained on the susceptible cultivar Essex in pots in a 27 °C water bath in the greenhouse. Seeds of Essex and the resistant cultivar Hartwig were surface-sterilized by immersion for 5 minutes in 95% ethanol followed by 5% sodium hypochlorite. The seeds were rinsed with deionized water and germinated in petri plates containing 1% water agar at 30 °C for 2 days. Two-day-old soybean radicles of both cultivars were transplanted into 13-cm-diam. × 10-cm clay pots (10 seedlings per pot) containing 100 cm^3 sterilized field sand (loamy sand; 84% sand, 4% silt, 12% clay, pH 7.9, 0.3% organic matter) and inoculated the same day with 2,000 surface-sterilized second-stage juveniles (J2) of *H. glycines* isolate TN1 per plant. Pots were then maintained at a constant temperature of 27 °C within a water bath in a greenhouse (Yen et al., 1995). Two and 3 days after planting, seedlings were removed from the sand and roots were harvested and used for isoflavonoid extraction. In order to examine if wounding could also influence the isoflavonoid content of soybean roots, we made 20 pricks with fine-tipped forceps on 2-day-old Essex roots. The wounded plants were transferred to pots that were maintained at 27 °C as described above.

HPLC analysis of soybean root flavonoids: Freshly harvested soybean roots were ground with a pestle and mortar to a fine paste (1 g root/5 ml solvent) in 50% ethanol. The slurry was transferred to 125-ml flasks and placed on a mechanical shaker overnight. The ethanolic extracts were centrifuged at 12,000*g* for 15 minutes. The clear supernatant was sterilized by passage through 0.45-µm filters and stored at −20 °C until used. Further purification of flavonoids was carried out with HPLC. The crude flavonoids were dried *in vacuo,* re-suspended in 5 ml 10% ethanol, and loaded on C18 Sep-pak cartridges (Waters Associates, MA). The cartridges were washed with 5 ml 10% ethanol, and the bound flavonoids were eluted with 5 ml 50% ethanol. This flavonoid fraction was filtered through 0.2-µm membranes, and 100-µl aliquots were loaded onto a Gilson HPLC (Gilson, Middleton, WI) fitted with a C18 reversed-phase column. A 60-minute linear gradient from 25% to 100% methanol was used to elute the flavonoids at a flow rate of 2 ml/minute. The eluting compounds were monitored at 262 nm. Two peaks that were highly potent in inducing the *S. fredii USDA191 nodC-lacZ* fusion (described in the following paragraph) were further characterized by gas chromatography and mass spectrometry, essentially as described earlier (Krishnan, 1998). Root preparations examined by HPLC were Essex inoculated with *H. glycines* and Essex inoculated with water (control). Three extractions were prepared and analyzed for each treatment.

Influence of H. glycines on nod gene induction and nitrogenase activity: Sinorhizobium fredii USDA191mm17 carries a *lacZ* fusion in *nodC* (Krishnan and Pueppke, 1991). This construct was grown in yeast extract-mannitol (YEM) medium (Vincent, 1970) for 3 days at 30 °C. A 200-µl aliquot of 3-day-old culture was transferred to 5 ml of YEM media to which the soybean root flavonoids (100 to 200 µl) had been added. The cultures were grown overnight at 30 °C in an orbital shaker. β-galactosidase assays were carried out with *o*-nitrophenyl β-D-galactopyranoside (ONP) as the substrate (Miller, 1972).

The assays were conducted on triplicate samples, and each experiment was repeated twice.

Influence of H. glycines on nodulation: Nodulation tests were performed on 2-day-old soybean radicles dipped into a suspension of *B. japonicum* USDA110 containing approximately 10^8 cells/ml. The seedlings were immediately transferred to 13-cm-diam. × 10-cm clay pots containing autoclaved loamy sand. In order to study the effect of *H. glycines* on nodulation, soybean seedlings that had and that had not been inoculated with *B. japonicum* USDA110 were planted in pots infested with and in pots not infested with 2,000 J2 of *H. glycines* isolate TN1. Four replicate pots were included for each of the four combinations. Plants were grown at 28 °C in a growth chamber at 400 μmol photons/m^2/sec with a 12-hour photoperiod. The plants were irrigated with sterile water as needed. The number of *H. glycines* in soybean roots 33 days after transplanting was determined from examination of acid fuchsin-stained roots under a microscope (Byrd et al., 1982). Nitrogen-fixation capacity of soybean nodules was measured by their ability to reduce acetylene (Schwinghamer et al., 1970). The experiment was repeated once. Fresh weights of shoots also were measured.

Data analysis: Means separation was performed with the least square means (LSMEANS) option of general linear model (GLM) procedure of SAS (SAS Institute, Cary, NC). The level of probability used for identification of significant differences between means was 0.05.

Results

HPLC analysis of soybean root flavonoids: Flavonoids isolated from Essex 2 days after transplanting were resolved into several peaks (Fig. 1A). A similar profile was also obtained for infected roots 3 days after transplanting, except that there was a measurable increase in peaks that eluted between 30 and 40 minutes (Fig. 1C). These peaks were higher in *H. glycines*-infected roots at both 2 and 3 days after soil infestation (Fig. 1B,D). When HPLC peaks were collected individually and assayed for their ability to induce the *S. fredii* USDA191 *nodC* gene, the bulk of the inducing activity was present in two peaks that eluted at 32 and 34 minutes. Authentic daidzein and genistein (Sigma) eluted at 32 and 34 minutes, respectively. The identity of the active compounds as genistein and daidzein was verified by gas chromatography and mass spectrometry. Electrostatic spray mass spectrum revealed peaks at m/z 271 and 255, which corresponded to the pronated genistein and daidzein, respectively. Daidzein levels increased four- or three-fold in infected roots over uninfected roots at 2 and 3 days after soil infestation. Genistein levels increased about two-fold in infected roots over uninfected roots at both 2 and 3 days. There was also an increase in the peak heights of genistein and daidzein in 3-day-old uninfected roots compared with 2-day-old roots (Fig. 1). Similar HPLC profiles were obtained from two other tissue extractions.

Influence of H. glycines on nod gene induction and nitrogenase activity: The crude ethanolic root extract from Essex 2 days after inoculation induced the expression of *nodC-lacZ* fusion; however, extracts from soybean roots infected with *H. glycines* had a higher inducing activity (Fig. 2). Similar results were obtained from extracts of Hartwig roots. The *nodC*-inducing activity was also greater in roots 3 days after inoculation, except that inducing activity was much less evident on Hartwig than on Essex (Fig. 2). We also found that wounding soybean Essex roots also elevated the *nodC-lacZ* fusion. Ethanolic extract from Essex roots 2 days after wounding had *nodC*-inducing activity of 322 ± 34 Miller units in comparison to the control treatment, which exhibited 126 ± 16 Miller units.

Influence of H. glycines on nodulation: Bradyrhizobium japonicum USDA110 was able to nodulate both Essex and Hartwig efficiently. Essex had an average of 19 and Hartwig 13 nodules per plant. Inoculation with *H. glycines* increased the number of nodules in the susceptible cultivar Essex. The number of nodules on seedlings in-

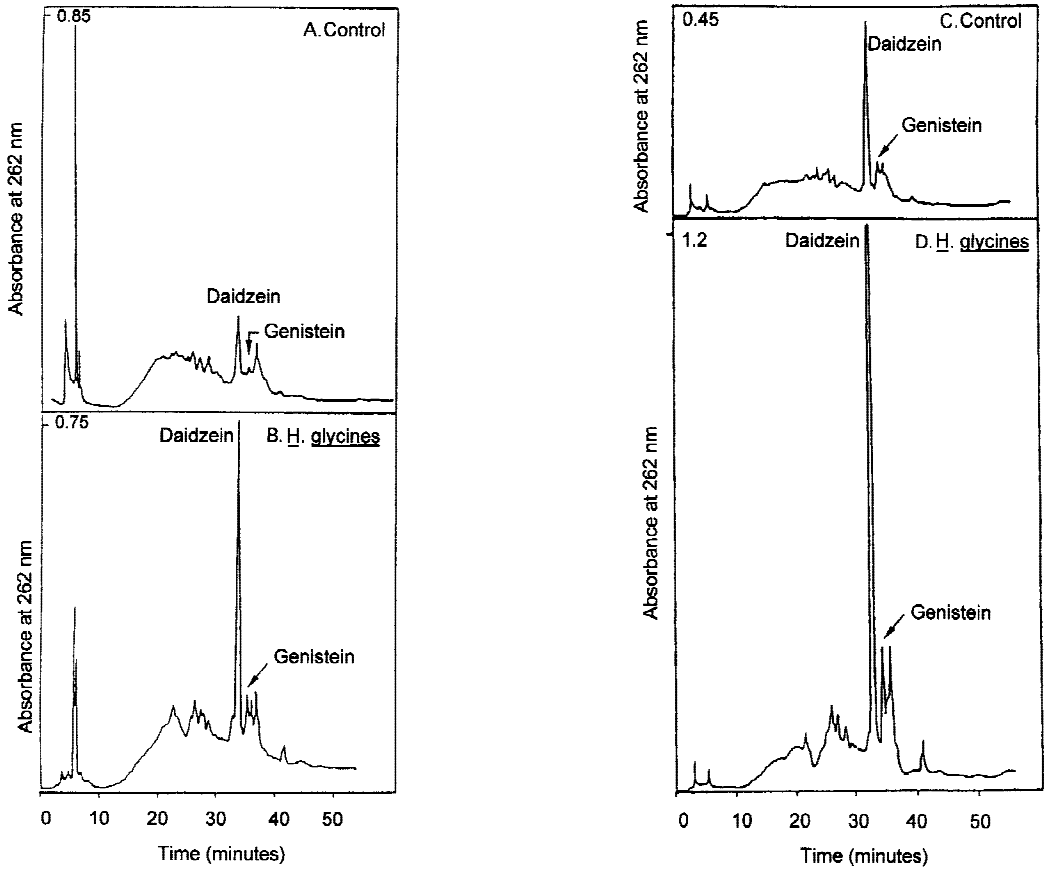

FIG. 1. Representative HPLC chromatogram from ethanolic root extracts showing elevation of daidzein and genistein levels in soybean roots infected with *Heterodera glycines*. Two-day-old radicals of soybean cultivar Essex were inoculated with 2,000 second-stage juveniles (J2) of *H. glycines* isolate TN1. A) Four days after transplant, no nematodes. B) Four days after transplant, 2 days after J2 inoculation. C) Five days after transplant, no nematodes. D) Five days after transplant, 3 days after J2 inoculation.

fected with both *H. glycines* and *B. japonicum* was 53% greater than on seedlings without *H. glycines;* however, the nodules were smaller and weighed 37% less than the nodules produced on nematode-free roots (Table 1). Acetylene reduction assays indicated that nodules from plants infected with both *H. glycines* and *B. japonicum* had 29% lower nitrogenase activity than nodules on plants inoculated with *B. japonicum* alone (Table 1). In addition, although inoculation with both *H. glycines* and *B. japonicum* led to an increase in the number of nodules on Essex, the increase was not reflected in shoot weights. Plants infected with *H. glycines* alone and *B. japonicum* plus *H. glycines* had fresh weights by 24% and 6% lower, respectively, than control plants. Inoculation of Essex with *B. japonicum* alone increased shoot fresh weight by 47% over that of controls (Table 1). In contrast, the resistant cultivar Hartwig showed no changes in shoot weights among the treatments, with an average shoot weight of 2.2 g. Only a small increase in the shoot fresh weight (from 2.1 to 2.2 g) was noted for plants inoculated with *B. japonicum.* Similarly, the nitrogen-fixing capacity of the nodules, measured in terms of nitrogenase activity, was not significantly affected by *H. glycines* infection (Table 1). Hartwig plants at 33 days after infection with *H. glycines* contained very few nematodes in their roots. In contrast, Essex plants inoculated with *H. glycines* had an average of 1,250 nematodes/plant and those with both *H. glycines* and *B. japonicum* had 950.

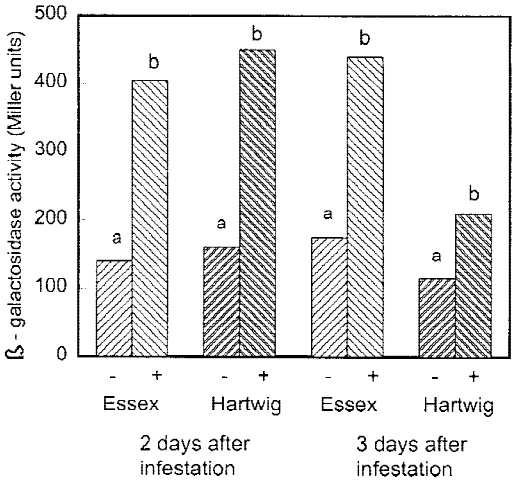

FIG. 2. Enhancement of isoflavonoid production by *Heterodera glycines* infection. Two-day-old soybean radicles of cultivar Essex and Hartwig were exposed to 2,000 second-stage juveniles of *H. glycines* isolate TN1 of water, and were grown in soil at 27 °C (+ = infested with *H. glycines* J2; − = uninfected). Isoflavonoid production was measured as β-galactosidase activity from ethanolic root extracts incubated with *S. fredii* USDA191 containing the reporter gene *lacZ* fused to the common nodulation gene *nodC*. Bars in each treatment followed by a different letter are significantly different at $P = 0.05$.

DISCUSSION

The *nod* genes of the soybean symbionts *B. japonicum* and *S. fredii* are activated by isoflavonoids (Krishnan and Pueppke, 1991; Smit et al., 1992). Thus, changes in the concentration of isoflavonoids in soybean roots can be indirectly measured by monitoring the activity of *nod* genes that have been fused to a reporter gene such as β-galactosidase. In this study, we used *S. fredii* USDA191 *nodC-lacZ* fusion to detect changes in isoflavonoids from soybean roots. Our results confirm that daidzein and genistein are abundant in soybean root extracts and show that their concentrations are elevated by *H. glycines* infection. The latter observation is consistent with those of an earlier study, which demonstrated an increase in the transcription of enzymes involved in the later stages of the phenylpropanoid pathway in soybean cultivars inoculated with *H. glycines* (Edens et al., 1995).

Daidzein and genistein were identified previously as the most abundant isoflavonoids in both seed and root exudates of soybean (Graham, 1991; Grady et al., 1995; Pueppke et al., 1998; Smit et al., 1992). In addition to their well-established role as inducers of *nod* genes, they can attract rhizobia (Caetano-Anolles et al., 1988), influence polysaccharide production (Dunn et al., 1992), and protect plants against pathogens (Graham, 1989). The elevated levels of flavonoids detected in our study in response to *H. glycines* infection raise the question of whether these compounds have a protective role against *H. glycines* infection. Our observation that the isoflavonoid content in-

TABLE 1. Effect of *Heterodera glycines* and *Bradyrhizobium japonicum* on nodulation, growth, and nitrogen-fixing capacity of 'Essex' (*H. glycines* susceptible) and 'Hartwig' (*H. glycines* resistant) soybean 33 days after inoculation with nematodes and rhizobium.

Treatment	Nodules per plant	Nodule fresh weight (mg)	Shoot fresh weight (g)	Nitrogenase activity[a]
Essex control	—	—	1.7a	—
Essex + *H. glycines*[b]	—	—	1.3a	—
Essex + *B. japonicum*[c]	19a	9.8a	2.5b	24.0a
Essex + *B. japonicum* + *H. glycines*	29b	6.2b	1.8a	17.1b
Hartwig control	—	—	2.1a	—
Hartwig + *H. glycines*[b]	—	—	2.2a	—
Hartwig + *B. japonicum*[c]	13a	11.0a	2.2a	19.8a
Hartwig + *B. japonicum* + *H. glycines*	15a	10.6a	2.2a	18.6a

Values are the average of two experiments with four replications each. Means separation was performed with the least square means option of general linear model procedure of SAS. Values in the same column followed by the same letter are not significantly different at $P = 0.05$.

[a] µmoles per gram fresh weight per hour.

[b] Two-day-old soybean seedlings were planted in 100 cm^3 sterilized soil infested with 2,000 second-stage juveniles.

[c] Two-day-old soybean radicles were dipped into a suspension of *B. japonicum* USDA110 containing 10^8 cells/ml.

creases in both resistant and susceptible cultivars in response to *H. glycines* infection suggests that they do not. Several other studies have clearly shown that flavonoid concentrations can be influenced by several biotic and abiotic stresses. Nitrogen levels, light intensity, and developmental stage of the plant have all had pronounced effects on the concentration of flavonoids (Graham, 1991). The interaction of rhizobia with legume roots can also lead to an increase in the flavonoid content of the roots (Pueppke et al., 1998). We have also found that wounding elevates the isoflavonoid content in soybean roots. Thus, increased isoflavonoid content results from a wide variety of factors and appears non-specific.

Several investigators have studied the effect of *H. glycines* infection on nodulation (Barker et al., 1972a, 1972b; Huang and Barker, 1983; Hussey and Barker, 1976; Ko et al., 1984; Lehman et al., 1971). In addition to nematodes, other pathogens, such as *Rhizoctonia* (Orellana et al., 1976), soybean mosaic virus, and bean pod mottle virus (Tu et al., 1970), have been shown to affect nodulation. In general, a deleterious effect occurs. However, it has been shown that several factors can influence the *H. glycines*-soybean interaction. The time of *H. glycines* inoculation had significant influence on nodule development. The greatest inhibition of nodule formation occurred when the soybean roots were simultaneously inoculated with rhizobia and *H. glycines* (Barker et al., 1972a). This inhibition was also dependent on the *H. glycines* host race present, with some races showing only marginal inhibition of nodulation (Lehman et al., 1971).

Different nematodes affect nodulation differently. Endoparasites, such as *Meloidogyne hapla* and *Pratylenchus penetrans,* greatly stimulated nodule formation, whereas an ectoparasite, *Belonolaimus longicaudatus,* had only a marginal effect (Hussey and Barker, 1976). In the present study, we observed that while *H. glycines* infection increased the number of nodules on soybean cultivar Essex, the accumulative nitrogen-fixing capacity of these nodules was reduced. Other workers (Orellana et al., 1978) have also reported deleterious effects of pathogens on nitrogen-fixing capacity in soybean. The lower nitrogen-fixing capacity of nodules from *H. glycines*-infected plants has been attributed to alteration in the content of leghemoglobin, an abundant protein that plays a crucial role in facilitating oxygen diffusion within the nodules (Huang and Barker, 1983). The varied effect of *H. glycines* on soybean nodulation reported in the literature may be due to several factors including soybean cultivar, nematode population density, soil type, growth conditions, temperature, soil moisture, light, *H. glycine* host race, time of inoculation, and the concentration of rhizobia. More research is needed to evaluate the significance of these factors to soybean yield losses caused by *H. glycines*.

Literature Cited

Barker, K. R., D. Huisingh, and S. A. Johnston. 1972a. Antagonistic interaction between *Heterodera glycines* and *Rhizobium japonicum* on soybean. Phytopathology 62:1201–1205.

Barker, K. R., P. S. Lehman, and D. Huisingh. 1972b. Influence of nitrogen *and Rhizobium japonicum* on the activity of *Heterodera glycines*. Nematologica 17:377–385.

Byrd, D. W., Jr., T. Kirkpatrick, and K. R. Barker. 1982. An improved technique for clearing and staining plant tissue for detection of nematodes. Journal of Nematology 15:142–143.

Caetano-Anolles, G., D. K. Crist-Estes, and W. D. Bauer. 1988. Chemotaxis of *Rhizobium meliloti* to the plant flavone luteolin requires functional nodulation genes. Journal of Bacteriology 170:3164–3169.

Denarie, J., F. Debelle, and J. C. Prome. 1996. *Rhizobium* lipo-chitooligosaccharide nodulation factors: Signaling molecules mediating recognition and morphogenesis. Annual Review of Biochemistry 61:503–535.

Dunn, M. F., S. G. Pueppke, and H. B. Krishnan. 1992. The *nod* gene inducer genistein alters the composition and molecular mass distribution of extracellular polysaccharides produced by *Rhizobium fredii* USDA193. FEMS Microbiology Letters 97:107–112.

Edens, R. M., S. C. Anand, and R. I. Bolla. 1995. Enzymes of the phenylpropanoid pathway in soybean infected with *Meloidogyne incognita* or *Heterodera glycines*. Journal of Nematology 27:292–303.

Endo, B. Y., and J. N. Sasser. 1958. Soil fumigation experiments for the control of the soybean-cyst nematode, *Heterodera glycines*. Phytopathology 48:571–574.

Epps, J. M., and A. Y. Chambers. 1962. Effects of seed inoculation, soil fumigation, and cropping sequences on soybean nodulation in soybean cyst nematode-infested soil. Plant Disease Reporter 46:48–51.

Grady, H., R. G. Palmer, and J. Imsande. 1995. Isoflavonoids in roots and hypocotyl of soybean seedlings

(*Glycine max,* Fabaceae). American Journal of Botany 82:964–968.

Graham, T. L. 1989. Constitutive conjugates of daidzein and genistein may play multiple roles in early race-specific antibiotic resistance in soybean. Phytopathology 79:1199.

Graham, T. L. 1991. Flavonoid and isoflavonoid distribution in developing soybean seedling tissues and in seed and root exudates. Plant Physiology 95:594–603.

Huang, J. S., and K. R. Barker. 1983. Influence of *Heterodera glycines* on leghemoglobin of soybean nodules. Phytopathology 73:1002–1004.

Hussey, R. S., and K. R. Barker. 1976. Influence of nematode and light sources on growth and nodulation of soybean. Journal of Nematology 8:48–52.

Jones, F. G. W., and F. Moriarty. 1956. Further observation on the effects of peas, beans, and vetch upon soil population levels of pea root eelworm, *Heterodera gottingiana* Liebscher. Nematologica 1:268–273.

Ko, M. P., K. R. Barker, and J.-S. Huang. 1984. Nodulation of soybeans as affected by half-root infection with *Heterodera glycines*. Journal of Nematology 16:97–105.

Krishnan, H. B. 1998. Identification of genistein, an anticarcinogenic compound, in the edible tubers of the American groundnut (*Apios americana* Medikus). Crop Science 38:1052–1056.

Krishnan, H. B., and S. G. Pueppke. 1991. Sequence and analysis of the *nodABC* region of *Rhizobium fredii* USDA257, a nitrogen-fixing symbiont of soybean and other legumes. Molecular Plant-Microbe Interactions 4:512–520.

Lehman, P. S., D. Huisingh, and K. R. Barker. 1971. The influence of races of *Heterodera glycines* on nodulation and nitrogen-fixing capacity of soybean. Phytopathology 61:1239–1244.

McClure, J. W. 1975. Physiology and functions of flavonoids. Pp. 970–1055 *in* J. B. Harborne, I. J. Marbry, and H. Mabry, eds. The flavonoids. New York: Academic Press.

Miller, J. H. 1972. Experiments in molecular genetics. New York, Cold Spring Harbor. Cold Spring Harbor Laboratory.

Orellana, R. G., F. Fan, and C. Sloger. 1978. Tobacco ringspot virus and *Rhizobium* interaction in soybean: Impairment of leghemoglobin accumulation and nitrogen fixation. Phytopathology 68:577–582.

Orellana, R. G., C. Sloger, and V. L. Miller. 1976. *Rhizoctonia-Rhizobium* interactions in relation to yield parameters of soybean. Phytopathology 66:464–467.

Phillips, D. A. 1992. Flavonoids: Plant signals to soil microbes. Recent Advances in Phytochemistry 26:201–231.

Pueppke, S. G., M. C. Bolanos-Vasquez, D. Werner, M-P. Bec-Ferte, J-C. Prome, and H. B. Krishnan. 1998. Release of flavonoids by the soybean cultivars McCall and Peking and their perception as signals by the nitrogen-fixing symbiont *Sinorhizobium fredii*. Plant Physiology 117:599–608.

Schwinghamer, E. A., H. J. Evans, and M. D. Dawson. 1970. Evaluation of effectiveness in mutant strains of *Rhizobium* by acetylene reduction relative to other criteria for N_2 fixation. Plant and Soil 33:192–212.

Smit, G. V., V. Puvanesarajah, R. W. Carlson, W. M. Barbour, and G. Stacey. 1992. *Bradyrhizobium japonicum nodD*1 can be specifically induced by soybean flavonoids that do not induce the *nodYABCUIJ* operon. Journal of Biological Chemistry 267:310–318.

Stokkermans, T. J. W., and N. K. Peters. 1994. *Bradyrhizobium elkanii* lipooligosaccharide signal induces complete nodule structures on *Glycine soja* Siebold et Zucc. Planta 193:413–420.

Taha, A. H. Y., and D. J. Raski. 1969. Interrelationships between root-nodule bacteria, plant-parasitic nematodes and their leguminous host. Journal of Nematology 1:201–211.

Tu, J. C., R. E. Ford, and S. Quiniones. 1970. Effect of soybean mosaic virus and/or bean pod mottle virus infection on soybean nodulation. Phytopathology 60: 518–523.

Vincent, J. M. 1970. A manual for the practical study of root-nodule bacteria. Oxford, UK: Blackwell Scientific Publications.

Wrather, J. A., T. R. Anderson, D. M. Arsyad, J. Gai, L. D. Ploper, A. Porta-Puglia, H. H. Ram, and J. T. Yorinori. 1997. Soybean disease loss estimates for the top 10 soybean-producing countries in 1994. Plant Disease 81: 107–110.

Yen, J. H., T. L. Niblack, and W. J. Weibold. 1995. Dormancy of *Heterodera glycines* in Missouri. Journal of Nematology 27:153–163.