Effect of *Rhizobium japonicum* Nodulation on Severity of Phytophthora Root Rot of Soybean

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ABSTRACT

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Phytophthora root rot of soybeans (*Glycine max*), caused by *Phytophthora megasperma* f. sp. *glycinea* (*Pmg*), is more severe on susceptible plants nodulated by *Rhizobium japonicum* than on plants not nodulated. Shoot growth and shoot and root dry weight means of inoculated Harosoy plants were lower and root rot scores were higher on nodulated plants than on those not nodulated. Disease severity of inoculated Harosoy 63 plants did not differ with or without nodulation. The *Pmg*-inoculated Harosoy plants had fewer nodules than did uninoculated Harosoy and Harosoy 63 plants, and *Pmg* colonized nodules of Harosoy but not Harosoy 63 soybeans.

The interactions of root-rotting fungi, nitrogen-fixing rhizobia, and leguminous plants have been the subject of many investigations (5,8,13,14,17,18). The effect of rhizobia and/or nodulation on the development of root rot in legumes is not clear. Higher levels of free-living rhizobia in soil are associated with decreased severity of Phytophthora root rot of soybeans (*Glycine max* (L.) Merr.) (17) caused by *Phytophthora megasperma* Dreschs. f. sp. *glycinea* (Kuan & Erwin) (*Pmg*) (11) and with the decreased incidence of Phytophthora root rot of alfalfa (18) (*Medicago sativa* L.) caused by *P. megasperma* Dreschs. f. sp. *medicaginis* (Kuan & Erwin). Rhizoctonia root rot of soybeans (caused by *Rhizoctonia solani* Kühn) is less severe on nodulated than on unnodulated soybeans (13). On the other hand, Gray and Hine (8) reported an increase in Phytophthora root rot of alfalfa in seedlings from rhizobia-treated seed planted in artificially infested soil and an association of root nodules with early infections.

These contrasting results and Tu's (17) report that increased levels of free-living rhizobia are associated with reduced severity of Phytophthora root rot of soybeans led us to compare the severity of Phytophthora root rot on soybeans with and without nodulation and to determine whether *Pmg* colonized root nodules. A preliminary report of this work has been published (2).

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MATERIALS AND METHODS

Plant material. Seeds of soybean cultivars Harosoy and Harosoy 63, which are susceptible and resistant, respectively, to *Pmg* race 1, were surface disinfested (0.05% sodium hypochlorite for 10 min) and incubated for 3 days (27 C) on sterile, moist filter paper. Harosoy 63, used as a control in these experiments, is a near-isogenic cultivar to Harosoy differing in resistance to *Pmg* race 1 (1,12). Clay pots 10 cm in diameter were filled with vermiculite, pot surfaces were covered with filter paper and aluminum foil, and pots were autoclaved for 1 hr. Foil was removed from pots, and 3-day-old seedlings were planted 3 cm deep in the sterile vermiculite. Seedlings emerged through a small slit cut in the filter paper and were thinned to one plant per pot. Experiments were conducted in a growth chamber at 25 C (14 hr of light) and 20 C (10 hr of dark) (7) under light intensities of 129 $\mu E/m^2$ per second at bench level. Seedlings were watered twice daily with tap water and fertilized once after *Pmg* inoculation with Crone's (16) nitrogen-free nutrient solution.

Rhizobial inoculum. Flasks of yeast-extract mannitol broth (19) were seeded with cells of *Rhizobium japonicum* Kirchner (Buchanan) (strain 110 from USDA Rhizobium Culture Collection) and incubated 4 days at 25 C on a rotary shaker (3 rpm). Cell concentration was determined from a standard curve of absorbance at 580 nm vs. number of cells per milliliter from counts on a Petroff-Hauser-Helber bacterial counting chamber. Inoculum diluted with tap water to provide concentrations of 10^7 cells per cubic centimeter of vermiculite was poured onto one-half of the 3-day-old seedlings in pots at the time of planting. Unseeded broth diluted with tap water was poured onto the remaining seedlings in pots as a control.

Fungal inoculum. Race 1 of *Pmg* isolated from diseased soybeans in Maryland (3) was maintained on lima bean agar medium and transferred monthly. Fungal inoculum was prepared by filtering 5-day-old V-8 juice broth (V-8 juice at 200 ml/L, $CaCO_2$ at 3 g/L) cultures of the fungus, determining wet weight of mycelium, resuspending the mycelium in V-8 juice broth, and chopping mycelium for 15 sec in a Waring Blendor (Waring Products Corp., Winsted, CT). Three-week-old, nodulated and unnodulated plants were removed from pots. Shoot height and number of nodules per plant were determined. The mean number of nodules per plant was equal at this time. Plants were repotted, and the root medium of nodulated and unnodulated plants was infested with chopped mycelium of *Pmg* in V-8 juice broth that had been diluted with tap water to provide a concentration of 800 mg of mycelium (wet wt) per pot. Other nodulated and unnodulated plants served as uninoculated controls.

Data collection and analysis. Two weeks after *Pmg* inoculation (5-wk-old plants), shoot height (centimeters), shoot and root dry weight (milligrams), degree of root nodulation, and degree of Phytophthora root rot were determined. Shoot growth was the difference between shoot height measurements of 5-wk-old and 3-wk-old plants. Root rot per plant was rated (15) as follows: 0 = no apparent root rot, 1 = up to 35% root reduction compared with control, 2 = 35–75% reduction, and 3 = >75% reduction. Root nodulation per plant was rated as follows: 0 = no nodules, 1 = 1–10 nodules, 2 = 11–20 nodules, and 3 = >20 nodules per plant.

The experiment was repeated four times, and the unbalanced data sets were analyzed for variance using the Statistical Analysis System General Linear Models procedure. The mean number of plants per treatment was 32. Means were separated by Duncan's multiple range test.

Nodule histopathology. Root nodules excised from *Pmg*-inoculated and uninoculated 5-wk-old Harosoy and Harosoy 63 plants were fixed in 3% glutaraldehyde in 0.025 M sodium phosphate buffer (pH 7.4) for 24 hr, dehydrated in a graded ethanol series, and embedded in paraffin. Sections 10 μm thick were mounted on glass slides, dewaxed, stained overnight (about 18 hr in 1% safranin in 95% ethanol diluted 1:1 with distilled water), counterstained (0.5% fast green in 95% ethanol) for 20 sec, destained with clove oil:absolute ethanol:xylene (2:1:1) (10), and observed using bright-field optics on a Leitz Dialux 20 Research Microscope.

RESULTS AND DISCUSSION

Results are presented as means for shoot growth, shoot dry weight, root dry weight, root rot ratings, and root nodule ratings from nodulated and unnodulated Harosoy and Harosoy 63 soybeans (Table 1). We conclude from these data that disease was more severe on nodulated, *Pmg*-susceptible plants in the greenhouse than on those plants not nodulated. Mean shoot growth and mean shoot and root dry weights of *Pmg*-inoculated Harosoy plants were significantly ($P = 0.05$) lower on nodulated than on unnodulated Harosoy plants. Growth parameters of uninoculated Harosoy plants and of plants in all the Harosoy 63 treatments were significantly greater than of nodulated, *Pmg*-inoculated Harosoy plants.

That mean root rot severity ratings of *Pmg*-inoculated Harosoy plants were significantly ($P = 0.05$) greater on nodulated than on unnodulated plants supports growth parameter data indicating less growth on *Pmg*-susceptible, rhizobial-nodulated Harosoy plants. The low root rot ratings of *Pmg*-inoculated Harosoy 63 plants are consistent with the resistance of this cultivar to *Pmg* race 1.

The lower root nodule scores of *Pmg*-inoculated Harosoy plants may be the result of destruction of both roots and root nodules by the fungus. Nodules on *Pmg*-inoculated plants were dark brown and often collapsed. The degree of root nodulation on Harosoy 63 plants was not affected by *Pmg*, and no nodule discoloration was noted.

P. megasperma f. sp. *glycinea* colonized nodules from Harosoy roots (Fig. 1A and B). Nodular outer cortex cells and portions of the sclerenchyma cell layer had disintegrated and were stained darkly in *Pmg*-colonized Harosoy nodules (Fig. 1A). Oospores were present in cortical parenchyma cells in the inner layer of the nodule and in bacteroidal cells in the nodular central tissue (Fig. 1B). Bacteroidal cell degeneration, characterized by the absence of both discernible nuclei and intact cell walls, was evident in the central tissue of nodules from *Pmg*-inoculated Harosoy plants. Central tissue from nodules of uninoculated Harosoy and Harosoy 63 plants and of *Pmg*-inoculated Harosoy 63 plants (Fig. 1C) contained apparently healthy bacteroidal cells. No mycelium or oospores were observed in the nodules of Harosoy 63 plants.

Although a previous report (17) suggests that increased levels of free-living rhizobia in soil are associated with a decrease of Phytophthora root rot on soybeans, our work indicates that Phytophthora root rot is more severe on plants nodulated by *R. japonicum* than on those not nodulated. Gray and Hine (8) reported similar results showing an increase in the incidence of Phytophthora root rot of alfalfa on nodulated plants. The interaction of free-living rhizobia, *Pmg*, and legume roots is apparently different from the interaction between *Pmg* and nodulated plants.

In contrast to our work with *Phytophthora*, Orellana et al and Orellana and Worley (13,14) reported a decrease in Rhizoctonia root rot on nodulated soybeans concomitant with the restriction of fungal colonization to the outer cortex of nodules on 26-day-old soybeans. They postulated that ramification of the fungus to nodular central tissue may have been impeded by the sclerenchyma layer in the nodular tissue. Our results show that *Pmg* colonized nodules from 35-day-old Harosoy soybeans beyond the outer cortex into the nodular central tissue. The sclerenchyma cell layer apparently did not prevent ramification by *Pmg*. Fungal penetration of Harosoy nodular tissue may have occurred from the outer nodular cortex or via the region of the nodule connected to the root. Nodules from Harosoy 63 plant roots were not colonized by the fungus. Evidently, Phytophthora root rot resistance was expressed in Harosoy 63 nodule tissue. Differences between this work and that of Orellana et al and Orellana and Worley (13,14) may be a reflection of the mode of infection by the two pathogens, the different plant-fungus interaction, and experimental procedures.

We speculate that several factors explain increased disease severity on nodulated plants. Nodule exudates may differ qualitatively or quantitatively from other root exudates such that motile zoospores are attracted to the nodule surface, fungal growth in the rhizosphere is stimulated, or dormant oospores of *Pmg* are stimulated to germinate. Nodulation may lead to morphological changes that render nodules more easily penetrable by *Pmg*. Gray and Hine (8), for example, associated initial infections

Table 1. Changes in plant growth and severity of Phytophthora root rot caused by *Phytophthora megasperma* f. sp. *glycinea* (*Pmg*) on soybeans nodulated with *Rhizobium japonicum* or on unnodulated soybeans

Cultivars and treatment	Shoot growth[w] (cm)	Shoot dry weight (mg)	Root dry weight (mg)	Root rot rating[x]	Root nodule rating[y]
Harosoy					
Pmg					
Nodulated	0.7 c[z]	160 c	60 d	3 a	1 b
Not nodulated	2.6 b	300 b	130 c	2 b	0 c
No *Pmg*					
Nodulated	4.6 a	400 a	260 ab	0 c	2 a
Not nodulated	3.8 ab	400 a	250 ab	0 c	0 c
Harosoy 63					
Pmg					
Nodulated	4.5 a	480 a	240 b	0 c	2 a
Not nodulated	3.3 ab	460 a	270 ab	0 c	0 c
No *Pmg*					
Nodulated	4.6 a	450 a	280 a	0 c	2 a
Not nodulated	3.8 ab	410 a	270 ab	0 c	0 c

[w] Difference between shoot height at 3 and 5 wk.

[x] Least square means; 0 = all roots apparently healthy, 1 = up to 35% root reduction compared with control, 2 = 35–75% root reduction compared with control, 3 = greater than 75% root reduction compared with control.

[y] Least square means; 0 = no nodules, 1 = 1–10 nodules, 2 = 11–20 nodules, 3 = more than 20 nodules.

[z] Means in a column followed by the same letter are not significantly different ($P = 0.05$) according to Duncan's multiple range test (mean number of plants per treatment is 32).

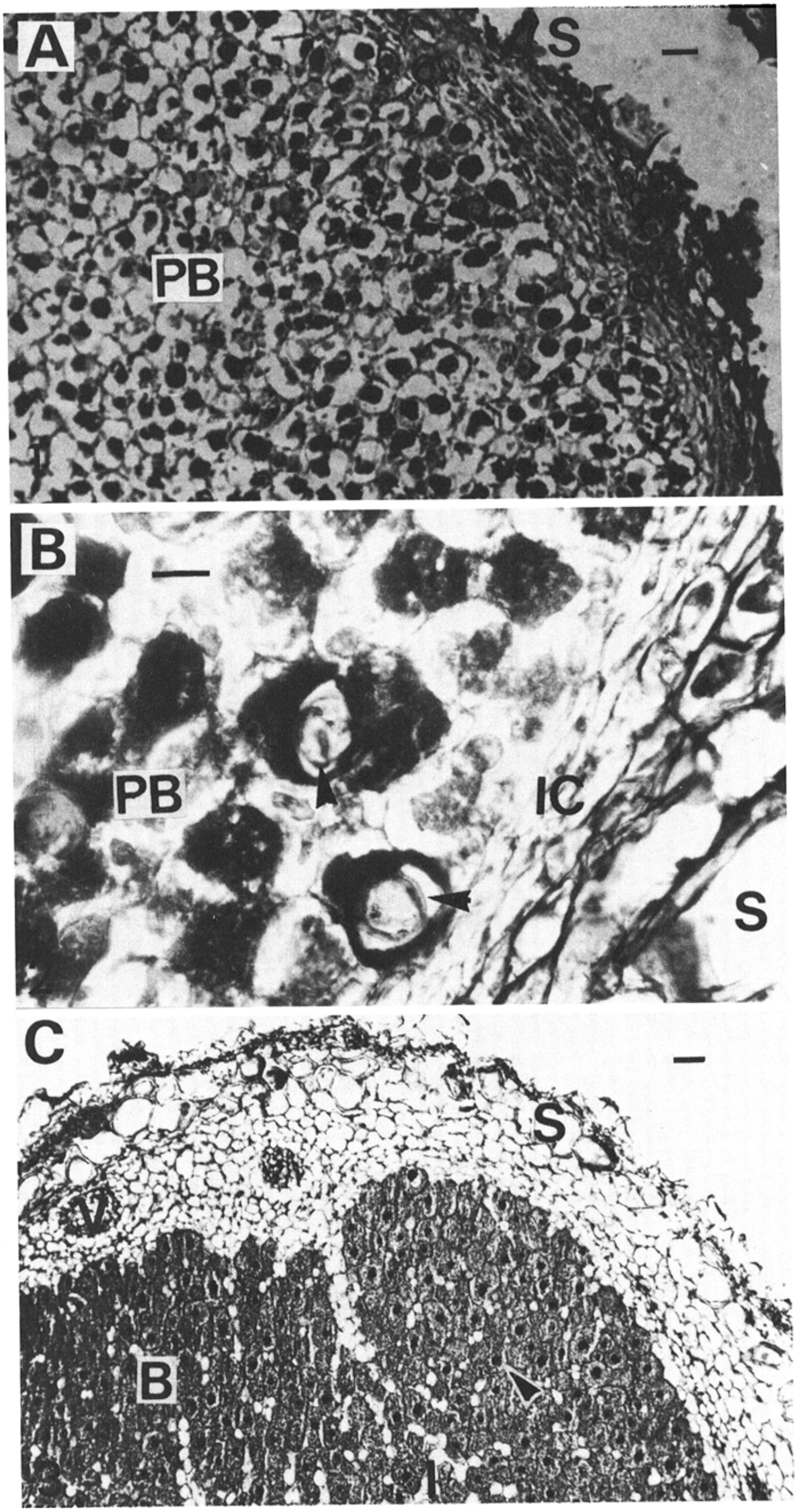

Fig. 1. Light micrographs of nodules incited by *Rhizobium japonicum* on soybean inoculated with *Phytophthora megasperma* f. sp. *glycinea*: **(A)** Cross section of Harosoy nodule showing plasmolyzed bacteroidal cells (PB) in the central tissue and degeneration of the sclerenchyma layer (S). Bar = 80 μm. **(B)** Cross section of Harosoy nodule showing oospores (arrows) in plasmolyzed bacteroidal cells (PB) in the central tissue; nuclei are not distinguishable. S = sclerenchyma cell, IC = inner cortical layer. Bar = 30 μm. **(C)** Cross section of Harosoy 63 nodule showing lack of bacteroidal cell (B) degeneration in the central tissue, presence of nuclei (arrow), and intact sclerenchyma layer (S). V = vascular bundle, I = interstitial cell. Bar = 80 μm.

of alfalfa roots by *P. megasperma* with root nodules. Nodulation may affect the host defense mechanisms such that normal mechanisms for limiting fungal activities are altered.

Increased susceptibility of nodulated Harosoy plants to *Pmg* may be associated with the nitrogen level in the host tissue. Although no direct measurements of nitrogen content in nodulated vs. unnodulated host tissue were made, we consistently observed that 5-wk-old nodulated plants had dark green foliage, whereas unnodulated plants were pale green (indicative of a nitrogen deficiency). Higher levels of nitrogenous fertilizers have been reported to increase the severity of many diseases caused by soilborne pathogens (9), including *Pmg* (4,6). The mechanisms involved in increased disease severity resulting from the addition of nitrogenous fertilizers to soil may be similar to those involving the effect of nodulation on disease severity.

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