Soybean Stem Colonization by Genotypes A and B of *Cadophora gregata* Increases with Increasing Population Densities of *Heterodera glycines*

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ABSTRACT

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Growth chamber experiments were conducted to investigate whether parasitism by increasing population densities of *Heterodera glycines*, the soybean cyst nematode, increases the incidence and severity of stem colonization by the aggressive genotype A and the mild genotype B of *Cadophora gregata* (*Phialophora gregata*), causal agents of brown stem rot of soybeans. Soybean genotypes with three combinations of resistance and susceptibility to *H. glycines* and genotype A of *C. gregata* were inoculated with each genotype of *C. gregata* alone or each genotype with two population densities of *H. glycines* eggs, 1,500 or 10,000 per 100 cm³ of soil. Stems of two *H. glycines*-susceptible soybeans were more colonized by both aggressive and mild genotypes of *C. gregata* in the presence of high than in the presence of low *H. glycines* population density.

Additional keywords: disease progress, pathogen interaction, soilborne

Brown stem rot (BSR) is a widespread (25) and economically important disease of soybeans (*Glycine max* (L.) Merr.) in the north-central United States. It is caused by the vascular pathogen *Cadophora gregata* Harrington & McNew (5) (*Phialophora gregata* (Allington & D.W. Chamberlain) W. Gams (AC)) (1). The soybean cyst nematode, *Heterodera glycines* Ichinohe, is also economically important and widely distributed throughout the mid-western United States (25).

Annual yield loss estimates for the United States from 1999 to 2002 ranged from 188,038 to 315,899 metric tons for BSR and from 3,605,962 to 4,299,382 metric tons for the soybean cyst nematode (SCN) (26). The current recommended management strategy for both pathogens is the use of resistant soybean cultivars combined with rotation to nonhost crops (17,23,24,27). In the Midwest, *Cadophora gregata* is composed of two genotypes (3,6) that differ in their aggressiveness and ability to cause BSR symptoms on certain soybean genotypes (6,8). Genotype A is generally recognized as the more aggressive based on symptom expression on soybeans without known BSR resistance genes (3,6,8). However, it is not known which genotype of the fungus causes the

most yield loss in soybeans. Both genotypes of the BSR fungus are widely found in the north-central United States (6).

The effect of *H. glycines* on increasing incidence and severity of BSR stem symptoms has been reported (12,18,19,22). In 2003, Tabor et al. (19) reported that *H. glycines* not only increases incidence and severity of BSR stem symptoms but also increases incidence and severity of stem colonization by genotype A of *C. gregata*. They also reported that infection by *H. glycines* breaks resistance to genotype A in many BSR-resistant soybeans (19). Whether *H. glycines* increases incidence and severity of stem colonization by genotype B of *C. gregata* is yet to be investigated.

The timing of infection by *C. gregata* may be important in determining yield loss. Difference in the timing of colonization by the fungus appears to be an important difference between genotype A-resistant and genotype A-susceptible soybeans. Tabor et al. (20) demonstrated in growth chamber experiments that stems of the genotype A-susceptible cultivar Sturdy were colonized earlier than stems of the genotype A-resistant cultivar BSR101. In the presence of *H. glycines*, *C. gregata*-resistant soybean cultivars may be colonized by the fungus as early as genotype A-susceptible cultivars are colonized by the fungus in the absence of *H. glycines*.

Infection by *H. glycines* is known to affect the progression of colonization of soybean stems by *C. gregata*. In growth chamber experiments using genotype A of *C. gregata* and one population density of *H. glycines*, Tabor et al. (19) have shown

that *H. glycines* can cause earlier colonization of soybean stems by genotype A of *C. gregata*. Thus, the potential of various population densities of *H. glycines* to affect the progression of colonization of soybeans by both genotypes of *C. gregata* should be determined.

The objective of this research was to determine if increasing population densities of *H. glycines* affect the colonization of soybean stems by genotypes A and B of *C. gregata* over time.

MATERIALS AND METHODS

Three growth chamber experiments (experiments 1A, 1B, and 1C) were conducted to determine the effect of *H. glycines* on colonization of stems by *C. gregata* over time in genotype A-resistant and genotype A-susceptible soybean cultivars.

Soybean cultivars. The soybean cultivars tested in the three experiments were the genotype A-resistant and SCN-susceptible BSR101 (21), the genotype A- and SCN-susceptible Sturdy (16), and the genotype A- and SCN-resistant Jack (14). Seeds of each genotype were planted in an autoclaved mix of sand and soil (1:1) in 4 × 21 cm plastic, cone-shaped containers (Stuewe and Sons, Corvallis, OR). There was one plant per container, and plants were grown in a growth chamber at a constant temperature of 22°C. In all experiments, plants were grown under 16 h light and were fertilized weekly.

***H. glycines* inoculum production.** *H. glycines* was increased on *H. glycines*-susceptible Kenwood 94 soybeans in a greenhouse. Eggs were obtained by dislodging female nematodes and cysts from roots of infected plants with a stream of water, and wet-sieving and decanting (4,13) of *H. glycines*-infested soil. Females and cysts were recovered on a 250-μm-pore sieve nested below an 850-μm-pore sieve. Eggs were released from females and cysts by crushing them in water with a motorized pestle (13), and eggs were collected on a 25-μm-pore sieve nested under a 75-μm-pore sieve. Eggs were separated from plant debris and soil particles by centrifugal flotation (9) and counted by direct microscopic observation. Inoculum population densities used in the experiments were 0, 1,500, and 10,000 *H. glycines* eggs per plant.

***C. gregata* inoculum production.** Genotype A of *C. gregata* strain (OH2-3) used in the study (19) was a single-spore isolate of strain OH2 provided by Cecil

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Nickell at the University of Illinois. Genotype B, 98A5-6, (8) was provided by Craig Grau at the University of Wisconsin-Madison. The genotypes of the isolates were confirmed by the molecular method used by Chen et al. (3) and by comparing the distinct colony morphology of each isolate on green bean extract (GBE) medium (35 g of ground frozen *Phaseolus vulgaris* L. green pods and 20 g of agar per liter). Cultures were started on GBE medium supplemented with 50 mg of ampicillin per liter and were incubated at room temperature (21 to 23°C) until abundant sporulation was evident. Conidia were suspended in sterile deionized water, mixed with twice-autoclaved, shredded soybean straw, and incubated for 25 to 30 days at room temperature in the dark. The number of *C. gregata* colony forming units (CFU) per gram of straw inoculum was determined by serial dilution and plating on GBE agar.

Inoculation and incubation. In all experiments, inoculum was incorporated into an autoclaved mix (100 cm³) of equal volumes of sand and soil. The soil and inoculum mix was placed in 4 × 21 cm plastic, cone-shaped containers before planting of soybean seeds. Pathogen treatments per 100 cm³ of soil were genotype A alone, genotype A + 1,500 *H. glycines* eggs, genotype A + 10,000 *H. glycines* eggs, genotype B alone, genotype B + 1,500 *H. glycines* eggs, genotype B + 10,000 *H. glycines* eggs, and 10,000 *H. glycines* eggs alone as a negative control (not included in experiment 1A). The *H. glycines*-only treatments received equal amounts of twice-autoclaved, *C. gregata*-free shredded straw. Plants were grown in a growth chamber at a constant temperature of 22°C and 16 h light. Colonization of stems by *C. gregata* was monitored over time and plants were sampled repeatedly from 4 to 12 weeks. The *C. gregata* inoculum population densities used in each of the experi-

ments is listed in the tables and figure legends.

Experimental design. In all experiments, containers were arranged in a randomized complete block design, and blocks were locations in the growth chamber. In all experiments, there were four blocks, and a treatment combination occurred once per block and comprised one plant in a container.

Incidence and severity assays. To assess percentage of stems colonized and severity of stem colonization, stems were severed at the soil line and were immersed for 3 min in 70% ethanol, followed by 5 min in 0.5% sodium hypochlorite and a final rinse in sterile, deionized water. Stem length was measured, and the stems were cut into 2-cm-long pieces. The pieces were plated on GBE agar supplemented with ampicillin (50 mg/liter) and incubated at 15°C in the dark for 15 days. The emerging fungal mycelia were examined microscopically for conidia and conidiophores with morphology characteristic of *C. gregata*. A stem (plant) was considered colonized if *C. gregata* was recovered from any portion of the stem piece. In all instances, stems were colonized starting from the base up to the maximum height colonized without gaps. Consequently, severity of colonization was calculated by dividing the maximum height colonized by the total stem height. The genotypes of the isolates were confirmed by the molecular method used by Chen et al. (3) and also by their distinct colony morphology on GBE medium. Colonization data were collected without knowledge of the treatments.

Data analyses. All statistical analyses were conducted using the SAS software package version 9.1 (SAS Institute, Cary, NC). Results from the three experiments were similar, and thus, data were pooled. For each experiment, data on incidence and severity of *C. gregata* colonization over time were converted to area under

disease progress curve (AUDPC) using (2) the formula:

$$\text{AUDPC} = \sum_{i=1}^{n-1} [(X_i + X_{i+1})/2][t_{i+1} - t_i]$$

in which X_i = disease incidence or severity expressed as a percentage of the i th observation, t_i = time (weeks after planting) at the i th observation, and n = total number of observations. AUDPC data were analyzed using the mixed model (10) analysis of fixed effects (Mixed Procedure) treating experiments and blocks as random effects and pathogen as a fixed effect. For each cultivar, orthogonal comparisons were made among pathogen treatment means. To determine whether it is the time (intercept) or the rate (slope) of the progression of colonization affected by *H. glycines*, data on incidence and severity of *C. gregata* colonization over time were analyzed by logistic regression (7) using the Logistic procedure. Chi-square analysis was used to test slope and intercept differences of the regression of *C. gregata* colonization over time between pathogen treatments within a soybean genotype.

RESULTS

Analyses of data using AUDPC and logistic regression suggest that, in some cultivars, the incidence and/or severity of colonization by both genotypes of *C. gregata* increased with increasing population densities of *H. glycines*.

AUDPC. Genotype A. As population densities of *H. glycines* increased, the incidence and severity of stem colonization by genotype A of *C. gregata* increased ($P < 0.001$) in Sturdy and BSR101 but not ($P > 0.10$) in Jack (Table 1).

Genotype B. As population densities of *H. glycines* increased, the incidence and severity of stem colonization by genotype B of *C. gregata* increased ($P < 0.001$) in Sturdy and BSR101. For Jack, there was a trend ($P \leq 0.10$) of increased colonization

Table 1. Colonization of soybean stems by genotypes A and B of *Cadophora gregata* (*C. g.*) in the presence three population densities of *H. glycines* (*H. g.*)

Soybean genotype	Disease ^z	AUDPC ^{w,x}					
		Genotype A ^y			Genotype B ^y		
		<i>C. g.</i> alone	<i>C. g.</i> + 1,500 <i>H. g.</i> eggs	<i>C. g.</i> + 10,000 <i>H. g.</i> eggs	<i>C. g.</i> alone	<i>C. g.</i> + 1,500 <i>H. g.</i> eggs	<i>C. g.</i> + 10,000 <i>H. g.</i> eggs
BSR101	Incidence	83 a	283 b	505 c	63 a	297 b	463 c
	Severity	50 a	188 b	343 c	48 a	157 b	316 c
Jack	Incidence	217 a	225 a	305 a	275 a	275 a	383 a
	Severity	116 a	116 a	155 a	201 ab	176 a	277 b*
Sturdy	Incidence	409 a	546 b	703 c	204 a	391 b	625 c
	Severity	338 a	463 b	634 c	156 a	294 b	495 c

^w Area under the disease progress curve. $\text{AUDPC} = \sum_{i=1}^{n-1} [(X_i + X_{i+1})/2][t_{i+1} - t_i]$ where X_i = disease incidence or severity expressed as a percentage of the i th observation, t_i = time (weeks after planting) at the i th observation, and n = total number of observations.

^x AUDPC data for each cultivar were analyzed using mixed model analysis of fixed effects (Mixed Procedure) considering experiment and blocks as random effects. Treatments were compared using orthogonal comparisons. Each number is a mean from a total of 84 plants, three experiments each with four replications and one plant per replication.

^y Numbers with different letters within a row are significantly different ($P < 0.001$, * = $P \cdot 0.1$).

^z *C. gregata* inocula were 1.5×10^9 , 1×10^8 , and 5×10^7 CFU of genotype A and 1.9×10^9 , 1.9×10^7 , and 3.7×10^7 CFU of genotype B in experiments 1, 2, and 3, respectively.

by genotype B with increased population density of *H. glycines* (Table 1).

To determine whether it is the time (intercept) or the rate (slope) of progression of colonization affected by *H. glycines*,

data on incidence and severity of *C. gregata* colonization over time were also analyzed by logistic regression. Probability values for comparing differences between logistic regression parameters of pathogen

treatments for each soybean cultivar are presented in Table 2.

Incidence and severity of colonization by genotype A. The higher the population density of *H. glycines*, the higher the inci-

Table 2. The $P > \chi^2$ for comparison of logistic regression parameters (intercepts and slopes) from analysis of incidence and severity of *Cadophora gregata* (*C. g.*) colonization in the presence of three population densities (0, 1,500, and 10,000) of *Heterodera glycines* (*H. g.*) in growth chamber experiments^y

Comparison ^z	Incidence of colonization						Severity of colonization					
	BSR101		Jack		Sturdy		BSR101		Jack		Sturdy	
	Intercept (time)	Slope (rate)	Intercept (time)	Slope (rate)	Intercept (time)	Slope (rate)	Intercept (time)	Slope (rate)	Intercept (time)	Slope (rate)	Intercept (time)	Slope (rate)
A vs. (A + 1,500)	0.1006*	0.3583	0.4984	0.7357	0.2683	0.7483	0.0582*	0.1840	0.2016	0.2682	0.0055*	0.1158
A vs. (A + 10,000)	0.0899*	0.4836	0.2459	0.4497	0.8300	0.7057	0.0368*	0.1793	0.1259	0.2084	0.0004*	0.0652*
(A + 1,500) vs. (A + 10,000)	0.1011	0.7777	0.1711	0.3266	0.0531*	0.5656	0.0165*	0.1561	0.1654	0.2943	0.0002*	0.0770*
B vs. (B + 1,500)	0.6839	0.3752	0.6277	0.3716	0.4749	0.3786	0.2806	0.9882	0.7516	0.5008	0.1154	0.8599
B vs. (B + 10,000)	0.3139	0.5654	0.8216	0.7149	0.5885	0.2037	0.1399	0.8172	0.7672	0.7548	0.0119*	0.4394
(B + 1,500) vs. (B + 10,000)	0.1726	0.9677	0.3853	0.7834	0.8002	0.1697	0.0933*	0.9589	0.4086	0.9161	0.0019*	0.1558

^y Numbers are $P > \chi^2$ from analysis of incidence and severity of *C. gregata* colonization over time by logistic regression using the Logistic Procedure. Chi-square analysis was used to test regression coefficients intercept (time) and slope (rate) differences between two pathogen treatments in a soybean cultivar. * = statistically significant.

^z A and B represent genotypes A and B of *C. gregata*, respectively; numbers 1,500 and 10,000 represent the number of *H. glycines* eggs in the treatment.

Fig. 1. **A,** Incidence of *Cadophora gregata* (*C. g.*) colonization of genotype A-resistant and soybean cyst nematode (SCN)-susceptible BSR101, genotype A- and SCN-resistant Jack, and genotype A- and SCN-susceptible Sturdy soybean cultivars in the absence or presence of two population densities of *Heterodera glycines* (*H. g.*) in three experiments. *C. gregata* inocula were 1.5×10^9 , 1×10^8 , and 5×10^7 CFU of genotype A in experiments 1A, 1B, and 1C, respectively, and 1.6×10^9 , 1.9×10^7 , and 3.7×10^7 CFU of genotype B in experiments 1A, 1B, and 1C, respectively. Data points are means of three experiments each with four replicates each with one plant. Error bars are standard errors. **B,** Severity of *C. gregata* (*C. g.*) colonization of genotype A-resistant and SCN-susceptible BSR101, genotype A- and SCN-resistant Jack, and genotype A- and SCN-susceptible Sturdy soybean cultivars in the absence or presence of two population densities of *H. glycines* (*H. g.*) in three experiments. *C. gregata* inocula were 1.5×10^9 , 1×10^8 , and 5×10^7 CFU of genotype A in experiments 1A, 1B, and 1C, respectively, and 1.6×10^9 , 1.9×10^7 , and 3.7×10^7 CFU of genotype B in experiments 1A, 1B, and 1C, respectively. Data points are means of three experiments each with four replicates each with one plant. Error bars are standard errors.

dence and/or severity of colonization by genotype A in Sturdy and BSR101 but not in Jack (Fig. 1A and B and Table 2). For Sturdy and BSR101, incidence of *C. gregata* colonization occurred earlier ($P \leq 0.10$) in the presence of high (10,000 eggs) than of low (1,500 eggs) *H. glycines* population density (Fig. 1A and Table 2). The low *H. glycines* population density did not significantly ($P > 0.10$) increase the incidence of colonization compared with the *C. gregata*-only treatment in Sturdy. In BSR101, colonization occurred earlier ($P \leq 0.10$) in the low *H. glycines* compared with the *C. gregata*-only treatment. For Jack, the incidence of *C. gregata* colonization did not increase ($P > 0.10$) in the presence of any level of *H. glycines* (Fig. 1A and Table 2).

For Sturdy, severity of *C. gregata* colonization progressed faster ($P = 0.0770$) and occurred earlier ($P = 0.0002$) in the presence of higher than of lower *H. glycines* population density (Fig. 1B and Table 2). For BSR101, severity of *C. gregata* colonization occurred earlier ($P = 0.0165$) in the presence of higher than of lower *H. glycines* population density (Fig. 1B and Table 2). In Sturdy and BSR101, the lower (1,500) population density of *H. glycines* increased ($P \leq 0.10$) severity of colonization compared with *C. gregata*-alone treatments; early severe colonization occurred. For Jack, the severity of *C. gregata* colonization did not increase ($P > 0.10$) in the presence of either low or high *H. glycines* population densities (Fig. 1B and Table 2).

Incidence and severity of colonization by genotype B. For all soybean cultivars tested, the incidence of *C. gregata* colonization did not progress faster ($P > 0.10$), nor did it occur earlier ($P > 0.10$) in the presence of higher than of lower *H. glycines* population density (Fig. 1A and Table 2). However, there was a trend of increased colonization with increased population density of *H. glycines* for all cultivars.

For Sturdy and BSR101, severity of *C. gregata* colonization occurred earlier ($P \leq$

0.10) in the presence of high than of low *H. glycines* population density (Fig. 1B and Table 2). For Jack, severity of colonization by genotype B did not increase ($P > 0.10$) in the presence of high or low *H. glycines* population density (Fig. 1B and Table 2). However, there was a trend of increased colonization by genotype B with increased population density of *H. glycines* for Jack.

DISCUSSION

We have demonstrated that with increasing population density of *H. glycines* in the soil, colonization of certain soybean cultivars by both genotypes of *C. gregata* increases (Table 1). This increase is manifested mainly as severe colonization occurring earlier in the presence of high population density than in the presence of low population density of *H. glycines* (Fig. 1B and Table 2). *H. glycines*-induced earlier infection by *C. gregata* may increase yield loss caused by the fungus.

Our data suggest that for the two *H. glycines*-susceptible cultivars, BSR101 and Sturdy, the incidence and/or severity of colonization of stems by both genotypes of *C. gregata* increases as the population density of *H. glycines* increases (Table 1). In contrast, colonization of the *H. glycines*-resistant cultivar, Jack, by both genotypes of *C. gregata* is not affected by the presence of *H. glycines* at low or high population densities. This suggests that, to manage BSR disease, it might be necessary to plant soybean cultivars with dual resistance to *H. glycines* and both genotypes of *C. gregata*.

The mechanism by which low or high *H. glycines* population densities affect the progression of colonization of soybean stems by both genotypes of *C. gregata* was not investigated in our experiments. Tabor et al. (19) has suggested mechanical wounds created by nematode activities such as movement, feeding, growth, and reproduction on soybean roots may provide a direct route for entry into the root for the fungus. Additionally, infection of soybean by *H. glycines* is known to alter

photosynthesis, respiration, and nodulation caused by nitrogen-fixed bacteria (15), any of which may affect infection of the plant by the fungus. In addition, the disease defense mechanism(s) of the roots in the vicinity of wounds created by the nematode might be compromised, thus facilitating colonization of the plants by the fungus. Likewise, *H. glycines* infection might compromise the general health of the plants and adversely affect resistance to colonization by the fungus.

Genotype specificity was evident in the significant soybean cultivar $\times$ pathogen genotype interactions for incidence ($P = 0.0471$) and severity ($P < 0.0001$) of colonization. Two of the three soybean cultivars did not have constant rankings when ordered according to the degree of colonization by the two genotypes of *C. gregata* (Table 3). That is, genotype B is more aggressive on Jack than on Sturdy, and genotype A is more aggressive on Sturdy than on Jack. Jack was more severely ($P = 0.0200$) colonized by genotype B than by genotype A. Conversely, Sturdy was more frequently ($P = 0.0110$) and severely ($P < 0.0001$) colonized by genotype A than by genotype B. For BSR101, there was no difference either in incidence or severity of colonization between genotype A and B. It has been reported that, under field conditions, soybean varieties derived from the same source of BSR resistance as Jack are colonized more frequently by genotype B than by genotype A (11). One such cultivar is Bell; of 87 stem samples collected from plants grown on soil naturally infested with both genotypes A and B, 99% of the stem samples from Bell tested positive for genotype B and 1% for genotype A. The corresponding numbers for sturdy were 57% genotype A and 43% genotype B (11).

Our results have implications for both growers and BSR researchers. The sources of BSR resistance in the soybean genotypes tested in our experiments are similar to the sources of BSR resistance in most currently available commercial cultivars. Consequently, cultivars with resistance

Table 3. Reaction of soybean cultivars to genotypes A and B of *Cadophora gregata* in growth chamber experiments

Soybean cultivar	AUDPC ^{w,x}				Rank ^y			
	Incidence ^z		Severity ^z		Incidence ^y		Severity ^y	
	Genotype A	Genotype B	Genotype A	Genotype B	Genotype A	Genotype B	Genotype A	Genotype B
BSR101	83 a	63 a	50 a	48 a	1	1	1	1
Jack	217 a	275 a	116 a	201 b	2	3	2	3
Sturdy	409 a	204 b	338 a	156 b	3	2	3	2

^w Area under the disease progress curve. $AUDPC = \sum_{i=1}^{n-1} [(X_i + X_{i+1})/2][t_{i+1} - t_i]$ where X_i = disease incidence or severity expressed as a percentage of the i th observation, t_i = time (weeks after planting) at the i th observation, and n = total number of observations.

^x AUDPC data for each cultivar were analyzed using mixed model analysis of fixed effects (Mixed Procedure) considering experiment and blocks as random effects. Treatments were compared using orthogonal comparisons. Each number is derived from a total of 84 plants, three experiments each with four replications and one plant per replication.

^y Rankings of three cultivars (1 = least colonized and 3 = most colonized) based on AUDPC values for incidence and severity of colonization by each genotype of *C. gregata* (without *H. glycines*) presented in left-hand side of this table.

^z Numbers with the same letter within a row are not significantly ($P < 0.05$) different from each other.

only to *C. gregata* may be ineffective for management of brown stem rot disease in fields infested with *C. gregata* and high population densities of *H. glycines*. It is important, however, to note that our data are from growth chamber experiments with a limited range of soybean cultivars and pathogen populations and population densities. Thus, whether *H. glycines*, at any population density level, results in earlier infection by *C. gregata* under field conditions is yet to be tested.

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