Physiologic Specialization of *Phialophora gregata* on Soybean

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ABSTRACT

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Isolates of *Phialophora gregata* were obtained from soybean cultivars BSR 201, PI 437833, Elgin, and Hodgson 78 grown in Illinois, Iowa, Wisconsin, and Minnesota. The isolates were used to inoculate their source cultivars, then reisolated. These four cultivars were used as a differential host set in greenhouse root-dip assays to determine variability among isolates for virulence and aggressiveness, and the experiment was repeated in time. All 27 isolates chosen for detailed study caused defoliation on one or more cultivar. The normally susceptible cultivars Elgin and Hodgson 78 were susceptible to few, but different, isolates. BSR 201 was resistant to all isolates, but PI 437833 was susceptible to four isolates. Isolates derived from BSR 201 were less aggressive on Elgin and Hodgson 78 than were isolates derived from other cultivars. Although the differential host set employed in this study is not conducive for the designation of pathogen races, these data demonstrate a high level of variability for aggressiveness and the first report of physiologic specialization in *P. gregata*.

One of the most commonly occurring diseases of soybean (*Glycine max* (L.) Merr.) in the north central United States is brown stem rot (BSR), caused by *Phialophora gregata* (Allington & Chamberlain) W. Gams (4,15). The pathogen has been broadly categorized into type I isolates that cause both internal stem browning and foliar interveinal necrosis and type II isolates that cause only stem symptoms (5). Yield loss estimates range from 12 to 38% and are associated with cultivars, isolates, and environmental conditions that favor the development of leaf symptoms (6,14).

Mengistu and Grau (9) tested 24 Wisconsin isolates and found they differed widely in the severity of foliar and stem symptoms induced on a single susceptible cultivar. In a cooperative study among four Midwest states, 38 soybean introductions were evaluated to assess their potential as sources of BSR resistance (10). Instability of BSR resistance in several of the lines raised questions about the roles of environment, pathogen variability, and the different methods employed to measure BSR reaction.

Currently, all registered BSR resistant cultivars derive their resistance from PI 84946-2 (11,16,17,19). If virulence against the genes in PI 84946-2 exists, then breeders will need to identify and deploy additional genes in tandem or in combination to control BSR (12).

The objectives of this study were: 1) to measure the variability for virulence (the ability to overcome host genes for resistance) and aggressiveness (severity of symptoms produced) in a set of *P. gregata* type I isolates and 2) to evaluate the effect of location and source cultivar from which isolates were obtained on BSR reactions among a set of differential host cultivars.

MATERIALS AND METHODS

Isolates of *P. gregata* were obtained from stem pieces of 156 plants at growth stage R6–R6.5 (2) that appeared to have BSR symptoms. Diseased material was obtained with the cooperation of L. E. Gray; H. Tachibana, USDA-ARS, Iowa State University; C. R. Grau, Department of Plant Pathology, University of Wisconsin-Madison; and J. H. Orf, Department of Agronomy, University of Minnesota. Isolates were collected from Urbana, Sidney, and DeKalb, Illinois; Ames, Iowa; Madison, Wisconsin; and St. Paul and Rosemount, Minnesota.

Fungal isolates were collected within each site from up to four cultivars: PI 437833, BSR 201, Elgin, and Hodgson 78. PI 437833, maturity group I, has a high level of BSR resistance conditioned by a single dominant gene, Rbs_2 (7). BSR 201, maturity group II, derives its BSR resistance from the same source as L78-4094 that has the Rbs_1 gene (7). Elgin, maturity group II, is intermediate to moderately susceptible to BSR in many environments (10). Hodgson 78, maturity group I, is highly susceptible to BSR (9,10).

Thin slices of vascular tissue from surface-sterilized stem sections (60 sec in 1% NaOCl) were placed on soybean stem agar minimal medium (1) at 22.5 C in the dark. A subset of 64 isolates was selected on the basis of comparison with previous *P. gregata* descriptions (4,9). These isolates were then used in a greenhouse experiment to infect the respective cultivar from which they had been originally isolated. This step was the second cycle of infection on the hosts and was intended to enhance the probability of deriving isolates with different patterns of physiologic specialization.

The greenhouse root-dip assay was conducted as previously described (13) with the following additional details and modifications. From the borders of *P. gregata* colonies, four 1-cm^2 agar plugs were added to flasks containing soybean seed broth (80 g of seed of a susceptible cultivar per liter of water, steamed, strained, and autoclaved). Stationary liquid cultures were incubated 21–24 days at 22.5 C, blended at high speed for 75 sec, and diluted to 1.2×10^6 conidia and mycelial fragments per milliliter. Methylcellulose (400 centipoise viscosity) was added at 0.75% (w/v).

Seeds were germinated in sand in 10-cm-diameter plastic pots. Seedlings at the early unifoliolate stage were removed, roots were rinsed in water, and groups of five uniform healthy plants were selected, blotted with a paper towel, and dipped in 50 ml of inoculum. The excess inoculum was poured into 15-cm-diameter steam-sterilized clay pots containing a steam-treated sand:topsoil mixture (1:1), and inoculated seedlings were transplanted.

Plants were maintained under a 15-hr photoperiod at an average of 600 $\mu E \cdot m^{-2} \cdot s^{-1}$ at midday and 18–24 C. Each pot received 150 ml of water twice daily. Weekly fertilization was with 150 ml of a solution containing 1.56 g of Ra-pid-gro plant food (23% N, 19% P_2O_5, 17% K_2O) and 1.54 g of Peter's fertilizer (W. R.

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Grace & Co.) (20% N, 20% P_2O_5, 20% K_2O) per liter of water providing 0.098 g of N; 0.089 g of P_2O_5; 0.085 g of K_2O; 0.00012 g of chelated Cu, Mn, and Zn; 0.00005 g of B; and 0.00024 g of chelated Fe per liter.

Disease measurements were made after 5–5.5 wk on total node number per plant (*NN*), the node up to which leaf or stem symptoms progressed (*LSN* or *SSN*), and a leaf or stem symptom intensity score on a 0–100 scale for percent tissue discoloration within the affected area of the plant (*LSI* or *SSI*). Separate leaf and stem symptom severity scores were then calculated as: leaf symptom severity = $(LSN \times LSI)/NN$ and stem symptom severity = $(SSN \times SSI)/NN$.

From this initial assay, treatment reactions were grouped in five linear leaf symptom severity classes, with 0 = no leaf discoloration to 5 = over 60% discoloration (Fig. 1). Twenty-seven isolates were selected for detailed study on the basis they induced the highest levels of disease (especially foliar symptoms) among the 64 isolates available from each host cultivar from each site. The fungi were then reisolated from stem sections, and single conidial cultures were used to initiate liquid cultures for the differential host assay. At the same time, four mycelial plugs were placed in vials containing 4 ml of 40% glycerol in water and frozen at −70 C. The subsequent differential host experiment was repeated in time with inocula initiated from these vials in order to avoid sectoring of virulence factors multiple transfers.

Each differential host set experiment, as repeated in time, consisted of two replications except those treatments that in the first experiment showed means over one standard deviation above the across-isolate mean for the respective host cultivar. These atypical treatments were given four replications in the second experiment in order to gain a more precise estimate of host responses to aberrant isolates.

Experiments in time were treated as random and isolates and hosts were treated as fixed effects in the combined analysis (Table 1). Single degree of freedom orthogonal contrasts were made between a priori groupings as indicated (Table 2).

The across-isolate reactions of PI 437833 and Hodgson 78 were chosen, a priori, as population reference scales for resistant and susceptible BSR reactions, respectively, based on past field and greenhouse evaluations of leaf symptoms. Confidence intervals (95%) were calculated about their across-isolate greenhouse reaction means (Fig. 2). Treatments were classified as resistant (R) if the 99% confidence interval about their mean leaf or stem symptom severity score intersected the PI 437833 mean across isolates or as resistant-to-intermediate (R-I) if the treatment interval intersected the upper confidence limit but not the mean. Treatments were classified as intermediate (I) if neither limit intersected the resistant or susceptible reference cultivar limits. Susceptible (S), highly susceptible (HS), and highly resistant (HR) classifications were made similarly (Table 3).

RESULTS AND DISCUSSION

The combined analysis of variance of the differential host set experiments (Table 1) revealed highly significant isolate, cultivar, and isolate × cultivar effects. These effects were consistent over the experiments in time. Only isolates 21 and 24 (Table 3) decreased significantly in aggressiveness, averaged over host cultivars, between experiments.

By orthogonal contrasts, PI 437833 had higher leaf symptom scores than BSR 201, and Elgin had higher stem symptom scores than Hodgson 78 (Table 2, contrasts 1 and 2, respectively). These comparisons were the reverse of those expected based on levels of resistance encountered in the field (10). The high disease pressure and aggressiveness of the isolates chosen may account for these

Table 1. Combined analysis of variance *F* tests[a] of differential host set experiments

Source of variation	df	Symptom severity[b] Leaf	Stem
Experiment (E)	1	NS	NS
Reps/E[c]	4	...	...
Isolate (I)	26	**	**
E × I	26	NS	NS
Host (H)	3	**	**
E × H	3	NS	NS
I × H	78	**	**
E × I × H	78	NS	NS

[a] Significant at $P<0.01$ (**) or not significant (NS).
[b] Expressed as percent of leaf or stem tissue discolored.
[c] Replications within experiment df calculated as sum of df for replicates based on maximum number of replications within each experiment.

Table 2. Single degree of freedom orthogonal contrasts[a] for brown stem rot symptom severity reactions in the greenhouse

Contrast	Symptom severity[b] Leaf	Stem
1. PI 437833 vs. BSR 201	*	NS
2. Elgin vs. Hodgson 78	NS	**
3. BSR 201 reaction to isolates derived from BSR 201 vs. other isolates	NS	NS
4. Hodgson 78 and Elgin reaction to isolates derived from BSR 201 vs. other isolates	*	*
5. Minnesota isolates vs. other isolates	**	**

[a] Significant at $P<0.05$ (*) or 0.01 (**) or not significant (NS).
[b] Calculated as percent leaf or stem tissue discolored.

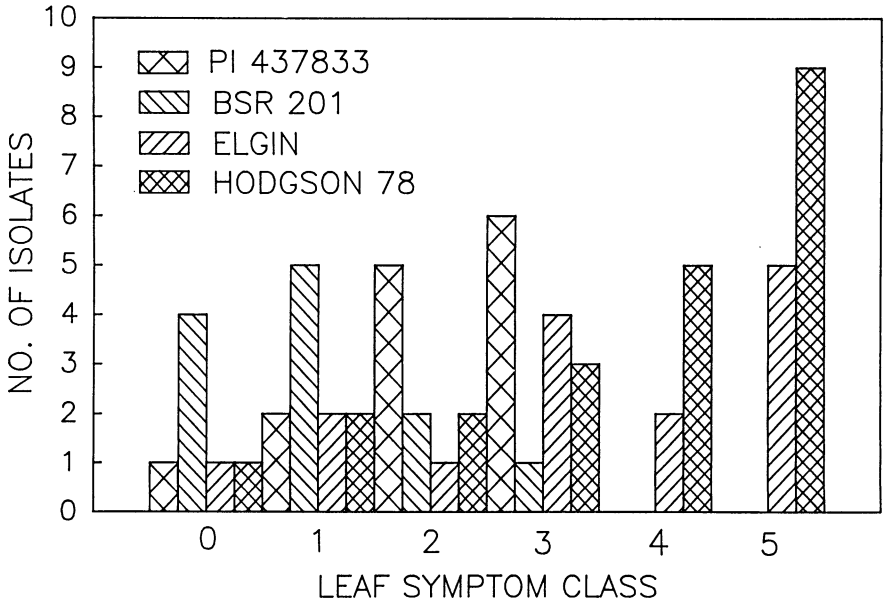

Fig. 1. Distribution of leaf symptom severity classes (0 for no discoloration, 5 for over 60%) after inoculation with 63 isolates of *Phialophora gregata* on one of four soybean cultivars.

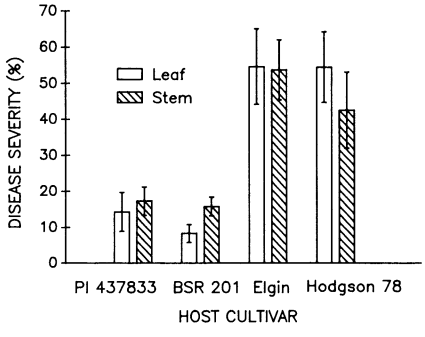

Fig. 2. Mean values of leaf and stem symptom severity scores and 95% confidence intervals for percentage of tissue discolored among four differential soybean host cultivars inoculated with 27 isolates of *Phialophora gregata*.

results. Moreover, the effect of differences in maturity in the field may not be reflected in the response of certain cultivars in a greenhouse assay.

The only identifiable difference due to isolate source location was that Minnesota sites had lower average aggressiveness than the other three states (Table 2, contrast 3). Isolates displayed considerable variability in most sites for both overall aggressiveness and virulence on specific hosts.

All isolates except isolate 22 were virulent on the basis of leaf symptoms on Elgin and/or Hodgson 78 (Table 3). None of the 27 isolates in the differential host set experiments were type II (5). All four cultivars were classified as resistant to isolate 22. It should be considered weakly aggressive rather than a type II isolate, since it did produce leaf

Table 3. Mean scores of leaf and stem symptom severity and brown stem rot reaction classes observed in differential host cultivars inoculated with isolates of *Phialophora gregata* in the greenhouse

Code no.	Origin				Differential host cultivar disease reaction							
					PI 437833		BSR 201		Elgin		Hodgson 78	
	State	Site	Cv.[a]	Symptom	Mean (%)[b]	Class[c]	Mean (%)	Class	Mean (%)	Class	Mean (%)	Class
1	Illinois	Urbana 1	P	Leaf	29	R-I	3	R	46	S	24	R
				Stem	21	R	12	R	50	S	22	R
2	Illinois	Urbana 1	P	Leaf	40	S	11	R	47	S	90	HS
				Stem	30	R	21	R	46	S	41	S
3	Illinois	Urbana 1	P	Leaf	6	R	10	R	72	HS	76	HS
				Stem	11	R	17	R	64	HS	61	HS
4	Illinois	Urbana 1	B	Leaf	12	R	8	R	47	S	44	S
				Stem	14	R	11	R	47	S	36	S
5	Illinois	Urbana 2	E	Leaf	37	I	6	R	34	I	44	S
				Stem	21	R	11	R	40	S	25	R
6	Illinois	Urbana 2	H	Leaf	15	R	6	R	47	S	44	S
				Stem	26	R	12	R	41	S	19	R
7	Illinois	Urbana 3	B	Leaf	15	R	5	R	44	S	45	S
				Stem	23	R	9	R	40	S	29	R-S
8	Illinois	Urbana 3	H	Leaf	11	R	13	R	80	HS	77	HS
				Stem	15	R	19	R	73	HS	61	HS
9	Illinois	Urbana 3	H	Leaf	10	R	7	R	69	S-HS	80	HS
				Stem	11	R	17	R	67	HS	68	HS
10	Illinois	Sidney	H	Leaf	3	R	20	R	82	HS	58	S
				Stem	9	R	28	R	84	HS	63	HS
11	Illinois	DeKalb	H	Leaf	4	R	15	R	96	HS	80	HS
				Stem	8	R	25	R	75	HS	74	HS
12	Iowa	Ames 1	H	Leaf	3	R	9	R	71	HS	71	S-HS
				Stem	7	R	18	R	71	HS	65	HS
13	Iowa	Ames 2	P	Leaf	10	R	8	R	51	S	37	I-S
				Stem	15	R	17	R	47	S	25	R
14	Iowa	Ames 2	B	Leaf	20	R	6	R	32	I	52	S
				Stem	20	R	14	R	35	S	29	R
15	Iowa	Ames 2	E	Leaf	6	R	8	R	78	HS	76	HS
				Stem	5	R-HR	15	R	69	HS	66	HS
16	Iowa	Ames 2	H	Leaf	12	R	8	R	61	S	68	S
				Stem	15	R	13	R	59	HS	77	HS
17	Wisconsin	Madison	P	Leaf	7	R	2	R	42	S	39	I-S
				Stem	15	R	24	R	49	S	21	R
18	Wisconsin	Madison	B	Leaf	6	R	11	R	70	S-HS	67	S
				Stem	10	R	21	R	72	HS	59	HS
19	Wisconsin	Madison	E	Leaf	29	R-I	3	R	73	S-HS	43	S
				Stem	27	R	13	R	69	HS	27	R
20	Wisconsin	Madison	H	Leaf	5	R	7	R	78	HS	67	S
				Stem	7	R	16	R	71	HS	73	HS
21	Minnesota	St. Paul	P	Leaf	30	R	20	R	31	R-I	48	S
				Stem	25	R	15	R	39	S	29	R
22	Minnesota	St. Paul	B	Leaf	12	R	5	R	21	R	21	R
				Stem	26	R	14	R	32	I-S	29	R-S
23	Minnesota	St. Paul	E	Leaf	13	R	12	R	44	S	34	I
				Stem	17	R	18	R	46	S	34	S
24	Minnesota	St. Paul	E	Leaf	15	R	12	R	40	I-S	38	I-S
				Stem	25	R	14	R	44	S	27	R
25	Minnesota	Rosemount	P	Leaf	12	R	5	R	50	S	51	S
				Stem	21	R	11	R	47	S	33	S
26	Minnesota	Rosemount	E	Leaf	18	R	4	R	25	R	50	S
				Stem	28	R	11	R	24	R	24	R
27	Minnesota	Rosemount	H	Leaf	9	R	3	R	45	S	49	S
				Stem	15	R	12	R	48	S	32	R-S
Mean over isolates				Leaf	14	R	8	R	55	S	54	S
				Stem	17	R	16	R	54	S	43	S

[a] Cultivars from which isolates originated were PI 437833 (P), BSR 201 (B), Elgin (E), and Hodgson 78 (H).

[b] Disease reaction as mean percentage of leaf or stem tissue discolored.

[c] Brown stem rot reaction classes defined as highly resistant (HR), resistant (R), intermediate (I), susceptible (S), or highly susceptible (HS) based on comparison of treatment 99% confidence intervals with 95% confidence intervals (over isolates) about PI 437833 and Hodgson 78 as resistant and susceptible host references, respectively.

symptoms, albeit low in severity. Of 64 isolates in the prescreening experiment, seven type II isolates were observed and were not included in the differential host set experiments.

Interestingly, isolate 26 induced a resistant stem reaction on all four host cultivars, even though it was virulent for leaf symptoms on Hodgson 78 (Table 3). In this regard, the leaf symptom means of nine isolates exceeded their stem symptom means on at least one host cultivar. Previous research has generally shown stem symptoms to equal or exceed leaf symptoms (9,14). Again, prescreening of isolates for virulence on the basis of leaf symptoms may account for the frequency of isolates with leaf symptoms that exceed stem symptoms. Pathotoxins have been implicated in leaf symptom development (8). Possibly, these isolates are heavy toxin producers relative to their extent of stem colonization (20).

Researchers have employed different means of rating BSR (9,13,16,18). Correlation coefficients between the rating component scores used in this study were calculated on treatment means to determine the suitability of some more simply measured parameters for BSR evaluation (Table 4). Leaf and stem symptom severity scores are composites of the percentage of the plant affected based on nodes and the intensity of the symptoms within the affected portion. The composite and component ratings are all positively correlated ($P < 0.01$) (Table 4).

Leaf symptom severity was the least variable parameter in the analysis of variance. Moreover, greenhouse leaf symptoms have been found to correlate more strongly than greenhouse stem symptoms with yield losses in the field ($r = 0.42$–0.79 vs. $r = 0.17$–0.41, respectively) (14). For these reasons, emphasis is placed on greenhouse leaf symptom severity scores. High correlations between leaf and stem symptom severity, intensity, and height of discoloration scores suggest that a plant displaying intense foliar necrosis tends to be affected to a large extent. Such severe tissue discoloration results in early defoliation and yield losses in the field (6,14).

Isolates showed widely varying levels of aggressiveness on all of the host cultivars except BSR 201. The resistance in BSR 201 was stable to all isolates in the prescreening and differential host set experiments. Although the genetics of resistance in BSR 201 has not been studied, it derives its resistance from PI 84946-2, which has one major gene and one or more minor genes for resistance (11). If BSR resistance in BSR 201 is due to two or more genes, then its resistance would be expected to be more stable than a cultivar with one gene for resistance.

Isolates derived from BSR 201 were not more aggressive on BSR 201 than isolates derived from other cultivars (Table 2, contrast 4). However, isolates derived from BSR 201 were less aggressive on Elgin and Hodgson 78 than were isolates derived from the other cultivars (Table 2, contrast 5). Possibly, the ability to colonize BSR 201 is associated with a decline in parasitic fitness that is expressed as lower aggressiveness. This would in effect represent selection against the accumulation of genes for virulence in the population.

Susceptible and intermediate leaf symptom severity reactions were induced by isolates 2 and 5 on PI 437833, whereas isolates 1 and 19 induced resistant to intermediate reactions. Of these four, isolates 1 and 2 had been derived from PI 437833; this suggests a positive selection response for physiologic specialization. Isolates 5 and 19 were derived from Elgin. There was no tendency for these aberrant isolates to be virulent on BSR 201. Such specific host-pathogen interactions among resistant cultivars would support Flor's gene-for-gene model (3), since BSR 201 likely has different genes for resistance than PI 437833 (7). Although the differential host set employed in this study does not enable clear designation of races of *P. gregata*, the data show large cultivar × isolate interactions due to physiologic specialization of the pathogen.

Of the aberrant isolates, isolates 1, 5, and 19 were avirulent on Hodgson 78 on the basis of stem symptoms and isolate 1 was avirulent for leaf symptoms as well. This observation is in contrast to the reaction of cultivars to isolates derived from BSR 201, since all four of these isolates maintained considerably higher aggressiveness on Elgin and therefore did not sacrifice overall fitness at the expense of specific virulence. Instead, these isolates revealed gene(s) for resistance in Hodgson 78 whose action is not elicited by other isolates. Subsequent research (20) confirmed the pronounced resistance of Hodgson 78 to isolate 1 in comparison with the three additional cultivars that were susceptible to all isolates.

Of the isolates whose leaf symptom severity means (across cultivars) exceeded the grand mean, seven of eight sectored light orange mycelia on the surface of the seed broth media and had a tangy, tamarindlike smell. However, some other isolates that shared these characteristics were considerably less aggressive.

Aberrant isolates could serve as tools to distinguish different modes of pathogenicity and host resistance mechanisms (20). They may also make it possible to identify genes for resistance in breeding lines. In this way, lines with higher levels or stability of resistance to multiple aberrant isolates could be identified and evaluated. This has not yet been accomplished in view of epistatic effects between dominant genes in response to wild type isolates (7,19). In most breeding line evaluations, however, isolates that are consistently highly aggressive on the basis of leaf symptoms on susceptible standards and that are avirulent on resistant cultivars would be preferred for selection. Such isolates would enable rapid visual selection in 4.5–5 wk after inoculation.

In summary, the *P. gregata* isolates demonstrated a high degree of variability in aggressiveness and a high frequency of aberrant types for physiologic specialization. Information gained from genetic studies of BSR resistance may disclose additional cultivars that could be used as differential hosts to identify races of *P. gregata*. Physiologic specialization might have been revealed in some isolates that appeared as wild types in this study if additional cultivars had been added to the differential host set.

Table 4. Correlation coefficients[a] among rating scores for brown stem rot symptoms induced by 27 isolates of *Phialophora gregata* on PI 437833, BSR 201, Elgin, and Hodgson 78

Plant part	Rating score[b]	Leaf rating		Stem rating		
		Intensity	Height	Severity	Intensity	Height
Leaf	Severity	0.99	0.96	0.84	0.92	0.83
	Intensity		0.97	0.88	0.88	0.88
	Height			0.79	0.92	0.81
Stem	Severity				0.99	0.97
	Intensity					0.99

[a] All r values significant at $P < 0.01$.
[b] Composite scores based on: (height of symptoms in nodes/total nodes) × intensity or percent tissue discolored within affected area.

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