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Resistance to *Phialophora gregata* Is Expressed in the Stems of Resistant Soybeans

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ABSTRACT

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Growth chamber experiments were conducted to determine if resistance to *Phialophora gregata*, the causal agent of brown stem rot (BSR) of soybean, is expressed in the stems of resistant soybean genotypes. Upon introduction of the pathogen into the base of stems of 2-week-old seedlings, the fungus advanced with the growing tips of plants of susceptible genotypes but lagged behind in resistant genotypes. Five weeks after introduction of the pathogen, both mean percent stem length colonized by *P. gregata* and mean percentage of symptomatic trifoliolate leaflets were significantly less for resistant than for susceptible genotypes. These results indicate that resistance can be expressed in the stems of resistant soybean plants and suggest that stem inoculation methods may be useful for assessing resistance to *P. gregata*. Also, in our experiments, internal stem discoloration was not as useful as colonization and foliar symptoms in discriminating resistant from susceptible genotypes.

Additional keywords: BSR, disease resistance

Brown stem rot (BSR) of soybean (*Glycine max* (L.) Merr.), caused by the vascular pathogen *Phialophora gregata* (Allington & D. W. Chamberlain) W. Gams (1), is an economically important disease of soybean in the north-central United States. BSR is prevalent in 68 to 73% of the soybean fields of Illinois, Iowa, and Minnesota (18). The currently recommended BSR management strategy is use of BSR-resistant soybean cultivars combined with rotation to nonhost crops (19).

Selection of BSR-resistant cultivars in breeding relies heavily on artificial inoculation methods; however, there is uncertainty in the literature about which organs of the soybean plant actually express resistance: roots, stems, or both. This uncertainty has hampered the choice and development of appropriate *P. gregata* inoculation methods and resistance assays. Some research suggests that BSR resistance is limited to the roots, whereas other research suggests it also is expressed in the stems. For example, Phillips (13) reported that PI 84946-2, the source of BSR resistance for most commercial cultivars, developed severe BSR symptoms when inoculated in the stem with *P. gregata*, which could be interpreted as indicating that resistance is not expressed in the stems. Bachman and Nickell (2) attempted to

specifically test the location of BSR resistance by inoculating the roots of reciprocal grafts of BSR-susceptible and -resistant soybean genotypes. They reported that resistant scions had more severe BSR symptoms when grafted on susceptible rootstocks than when grafted on resistant rootstocks. Therefore, they concluded that BSR resistance is "root-limited." This conclusion, however, is not consistent with the results of Lewers et al. (9), who detected quantitative trait loci (QTL) associated with *P. gregata* resistance when *P. gregata* was directly introduced into the stems. The main QTL detected using this inoculation technique was the same QTL that was detected when these soybean lines were planted in *P. gregata*-infested fields (9).

The expression of BSR resistance in soybean roots does not seem to be in dispute. The published data, however, are contradictory as to whether resistance also is expressed in the stems. The specific objective of the research reported herein was to determine whether BSR resistance is expressed in the stems of resistant soybean genotypes.

MATERIALS AND METHODS

Three experiments, each conducted twice (1A, 1B, 2A, 2B, 3A, and 3B), assessed the expression of BSR resistance in the stems of BSR-resistant soybean genotypes. Experiments 1A, 1B, 3A, and 3B also determined the reliability of internal stem discoloration in distinguishing resistant from susceptible genotypes when *P. gregata* was introduced directly into the stems. Experiments 4A and 4B assessed the reliability of stem colonization for

differentiating resistant from susceptible soybean genotypes when plants were inoculated with *P. gregata* without wounding the stems.

Soybean genotypes. The soybean genotypes tested in experiments 1A, 1B, 4A, and 4B were the BSR-resistant BSR101 (16) and the BSR-susceptible Sturdy (12). In experiments 2A and 2B, eight genotypes (three susceptible and five resistant) were tested. In experiments 3A and 3B, 17 genotypes (11 resistant and 6 susceptible) were tested. These genotypes either are the original sources of BSR resistance used in most commercial genotypes or have been reported as BSR resistant or BSR susceptible (Table 1).

Seed of each genotype were planted either in an autoclaved mix of sand and soil (1:1; experiments 1A, 1B, 4A, and 4B) or in a pasteurized mix of soil, sand, and perlite (2:1:2; experiments 2A, 2B, 3A, and 3B) in 4-by-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 set at a constant temperature of 22°C; actual mean temperatures ranged from 20 to 24°C depending on the experiment. In all experiments, plants were grown under 16 h of light and were fertilized weekly.

Inoculation or infestation. The *P. gregata* strain (OH2-3) used in the study (9) was a single-spore isolate of strain Oh₂ (6) provided by Cecil Nickell at the University of Illinois. Cultures were started on green bean extract (GBE) medium (ground frozen *Phaseolus vulgaris* L. green pods at 35 g/liter and agar at 20 g/liter) supplemented with ampicillin at 50 mg/liter and were incubated for 44 to 64 days at room temperature (21 to 23°C) in the dark until abundant sporulation was evident.

In experiments 1A, 1B, 2A, 2B, 3A, and 3B, *Phialophora gregata* inoculum was introduced in the stem. Conidia of *P. gregata* were suspended in 0.8% water agar (2.7×10^7 conidia/ml in experiments 1A, 1B, 2A, and 2B and 1.8×10^7 conidia/ml in experiments 3A and 3B). The conidial suspension was mixed thoroughly into a paste. Stems of 2-week-old plants were punctured approximately 2 cm above the soil line with an 18-gauge needle (Becton Dickinson, Franklin Lakes, NJ) with its bevel filled with the inoculum paste. A needle with water agar paste without conidia was stabbed into stems of control plants.

In experiments 4A and 4B, *P. gregata* inoculum was applied to the soil around

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the base of plants without wounding. In experiment 4A, the population density of conidia was adjusted to 3.3×10^6 conidia/ml of sterile deionized water; 3 ml of this suspension (10^7 conidia) was applied to the soil surface around the base of 2-week-old plants. In experiment 4B, the population density of conidia was 1×10^7 conidia/ml of sterile deionized water, and 1 ml of this suspension was applied to the soil around the base of 2-week-old plants. Uninoculated plants of both genotypes were included as controls in both experiments.

Experimental design. In all experiments, plants were arranged in a randomized complete block design; blocks were locations in the growth chamber. There were five blocks in experiment 1A and three in experiment 1B, and each treatment combination (one plant) occurred once in each block. In experiments 2A and 2B, there were four blocks and each treatment combination (one plant) occurred once in each block. There were 10 blocks in experiment 3A and 9 blocks in experiment 3B and each treatment combination (one plant) occurred once in each block. In experiments 4A and 4B, there were four blocks, and each treatment combination occurred once in each block. Each treatment combination comprised two (4A) or three (4B) plants (containers) in a row.

Incidence and severity assays. Incidence and severity of BSR symptoms and *P. gregata* colonization were assessed weekly over a 5-week period in experiments 1A, 1B, 2A, and 2B; once 5 weeks after inoculation in experiments 3A and 3B; and every 1 to 2 weeks over a 12-week period in experiments 4A and 4B.

To determine the extent of *P. gregata* colonization and internal stem discoloration, stems were cut at the soil line and immersed for 3 min in 70% ethanol, followed by 5 min in 10% sodium hypochlorite (Clorox) and a final rinse in sterile, deionized water. Stem length was measured and the stems were cut into 2-cm-long pieces. In experiments in which both discoloration and colonization data were collected, the stem pieces were split in half lengthwise and discoloration was assessed prior to plating of the stem pieces. Incidence of internal stem discoloration was assessed visually. A plant was considered discolored if there was any visible dark brown discoloration on the vascular tissue or the pith on any of the stem pieces. Severity of discoloration (percent stem length discolored) was calculated by adding the discolored lengths of the individual stem pieces. Because collection of discoloration data was subjective, a single investigator collected all discoloration data within a block without knowledge of the treatments.

To determine the percentage of stems colonized (incidence) and maximum height of colonization, one-half of each split stem

piece was plated on GBE agar supplemented with ampicillin. In experiments 2A and 2B, the 2-cm-long stem pieces were not split before plating. The relative position of each stem piece on the plant was recorded, and plates were incubated at 15°C in the dark for 15 days. The emerging fungal mycelia were examined for characteristic *P. gregata* conidia and conidiophore morphology under a microscope. A stem (plant) was considered colonized if *P. gregata* was recovered from any portion of any stem piece. In all instances, stems were colonized starting from the base up to the maximum height colonized without gaps. Severity of colonization was calculated by dividing the maximum height colonized by the total stem height. Colonization data were collected without knowledge of the treatments.

Foliar symptoms were assessed as the proportion of symptomatic trifoliolate leaflets. Each trifoliolate was recorded as healthy, chlorotic, stunted, necrotic, or abscised. If a leaflet was normal in size, not deformed, and not necrotic, it was recorded as healthy or chlorotic depending on the predominant (>50%) color of the leaflet. If a leaflet was abnormally small and deformed but not necrotic, it was recorded as stunted. If there was any necrotic area in the leaf, it was recorded as necrotic, and if the leaflet had fallen off the plant it was recorded as abscised (missing). Severity of foliar symptoms was determined using the formula: (stunted trifoliolate leaflets + necrotic trifoliolate leaflets + abscised trifoliolate leaflets/ total trifoliolate leaflets) \times 100%. Chlorotic leaflets were not included in calculations of symptom severity because many of the noninoculated plants had chlorotic leaflets. Foliar symptoms were assessed without knowledge of the treatments.

Data analyses. All statistical analyses were conducted using the SAS software

package (version 7.0 or 8.2; SAS Institute Inc., Cary, NC). Stem length, stem length colonized, stem length discolored, and percentage of symptomatic trifoliolate leaflets data were analyzed using the GLM procedure. Data were pooled for experiments 1A and 1B because experiment-treatment interaction was not significant ($P > 0.05$). Similar pooling was done for experiments 2A and 2B. Data on incidence of *P. gregata* colonization and discoloration over time from experiments 4A and 4B were analyzed by logistic regression (8) using the GENMOD procedure; χ^2 analysis was used to test the difference between soybean genotypes.

RESULTS

In experiments 1A and 1B, *P. gregata* was isolated from all *P. gregata*-inoculated plants at all sampling times (Fig. 1). The fungus was isolated from all heights (base to apex) of the stems of both susceptible Sturdy and resistant BSR101 at the first sampling time, 1 week after inoculum was introduced directly into the stems. In succeeding weeks, the pathogen advanced with the growing tip more readily ($P < 0.0001$) in Sturdy than in BSR 101. There was no significant difference ($P = 0.38$) in plant height between Sturdy and BSR101 plants. Therefore, the difference in colonization of the pathogen in the stems of the two genotypes was not attributable to a difference in the rate of plant growth between the two genotypes. There was no significant difference ($P = 0.96$) in severity of internal discoloration over 5 weeks between susceptible Sturdy and resistant BSR101.

P. gregata was isolated from 97.5 and 99.4% percent of *P. gregata*-inoculated plants in experiments 2A and 2B, respectively. In both experiments, the fungus was isolated from all heights (base to apex) of

Table 1. Soybean genotypes used in experiments^a

Genotype	Experiment no.	Proposed BSR resistance gene	Reference or source
A95-581022	2,3	None	ISU
Corsoy 79	2,3	None	4
P9233	2,3	None	Pioneer Hi-Bred Intl. Inc.
Parker	3	None	12
Sturdy	1,3,4	None	11
PI84946-2	3	<i>Rbs1</i> and/or <i>Rbs3</i>	7,15
A95-682026 ^b	2,3	<i>Rbs1</i> and/or <i>Rbs3</i>	ISU
A96-597011 ^b	2,3	<i>Rbs1</i> and/or <i>Rbs3</i>	ISU
Archer ^b	3	<i>Rbs1</i> and/or <i>Rbs3</i>	5
BSR101	1,2,3,4	<i>Rbs1</i> and/or <i>Rbs3</i>	16
IA1006 ^b	3	<i>Rbs1</i> and/or <i>Rbs3</i>	ISU
IA2008R ^b	3	<i>Rbs1</i> and/or <i>Rbs3</i>	ISU
IA2050 ^b	3	<i>Rbs1</i> and/or <i>Rbs3</i>	ISU
IA3010	3	None	ISU
PI 437833	3	<i>Rbs2</i>	7
PI 437970	2,3	<i>Rbs3</i>	17
Jack ^c	2,3	Unknown	10

^a BSR = brown stem rot; ISU = experimental genotypes obtained from Department of Agronomy, Iowa State University, Ames.

^b Genotypes with resistance derived from BSR101 or PI 84946-2.

^c BSR resistance derived from unknown source.

the infected stems of both susceptible and resistant genotypes at the first sampling time, 1 week after inoculum was introduced directly into the stems (Fig. 2). However, in succeeding weeks, the pathogen advanced with the growing tip more readily ($P < 0.0001$) in susceptible than in resistant genotypes. There was a difference ($P < 0.001$) in plant height between resistant and susceptible genotypes. However, this height difference was not responsible for the difference in stem length colonized between resistant and susceptible genotypes. When stem length colonized data were converted into percent stem length colonized (stem length colonized/total stem length $\times 100\%$) and analyzed using the GLM procedure, susceptible genotypes had greater ($P < 0.0001$) percent stem length colonized by the fungus than resistant cultivars. Therefore, the difference in colonization of the pathogen in the stems of susceptible and resistant genotypes was not attributable to the difference in rate of plant growth between susceptible and resistant soybeans.

In experiments 3A and 3B, data were collected from a wider range of soybean genotypes than in experiments 2A and 2B; plants were sampled 5 weeks after introduction of the pathogen rather than weekly. *P. gregata* was isolated from 99.4 and 98.8% percent of *P. gregata*-inoculated plants in experiments 3A and 3B,

respectively. In both experiments 3A and 3B, mean percent stem length colonized by *P. gregata* for the susceptible genotypes was significantly ($P < 0.0001$) greater than the mean for the resistant genotypes (Fig. 3A and D). Likewise, mean percentages of symptomatic trifoliolate leaflets for the susceptible genotypes were significantly ($P < 0.0001$) greater than the mean for the resistant genotypes in both experiments (Fig. 3C and F). There was no difference ($P = 0.0867$) in internal stem discoloration between resistant and susceptible soybean genotypes in experiment 3A (Fig. 3B), and resistant genotypes had significantly ($P = 0.0005$) greater discoloration than susceptible genotypes in experiment 3B (Fig. 3E).

To determine whether the reduced colonization of stems of resistant cultivars by *P. gregata* was an artifact of stem inoculation, experiments 4A and 4B were conducted using an inoculation method that did not wound the stem. Specifically, inoculum was applied to the soil around the base of 2-week-old seedlings. The colonization data from experiments 4A and 4B (Fig. 4) were consistent with experiments that used direct stem inoculation. In both experiments, colonization distinguished the resistance and susceptibility of BSR101 and Sturdy. Sturdy and BSR101 differed in incidence (experiment 4A, $P = 0.057$; experiment 4B, $P < 0.0001$) and severity

(experiment 4A, $P = 0.0225$; experiment 4B, $P < 0.0001$) of *P. gregata* colonization over time. Incidence and severity of internal stem discoloration was low for both Sturdy and BSR101 in both experiments even at 12 weeks after inoculation. There was greater variation within treatments, as shown by error bars, for severity of colonization in experiments 4A and 4B, in which *P. gregata* spores were applied to the soil, than in experiments 1A, 1B, 2A, 2B, 3A, and 3B, in which inoculum was introduced directly into the stem.

DISCUSSION

Our results indicate that resistance to *P. gregata* colonization and to BSR foliar symptoms is expressed in the stems of BSR-resistant soybean genotypes. There was reduced *P. gregata* colonization of stems and reduced foliar symptom expression in a wide range of BSR-resistant genotypes, including PI 84946-2 (*Rbs1*), PI 437833 (*Rbs2*), PI 437970 (*Rbs3*), and Jack (unknown gene or genes) when inoculum was introduced directly into the stems. Our results are consistent with those of Lewers et al. (9), who detected QTL controlling BSR resistance using the same stem inoculation technique used in our experiments. Thus, our results and those of Lewers et al. suggest that BSR resistance is expressed in the stems of BSR-resistant soybean genotypes and that stem inoculation methods may be useful in BSR research.

Our results do not support the earlier report by Bachman and Nickell (2) that BSR resistance is root-limited. Bachman and Nickell inoculated the roots of reciprocal grafts of susceptible and resistant genotypes with *P. gregata* and demonstrated that grafts with resistant rootstocks had less severe BSR symptoms than grafts with susceptible rootstocks. Therefore, they concluded that BSR resistance is present in the roots, and that, in addition, it is root-limited in all the BSR-resistance sources they evaluated. In contrast, when we bypassed the roots and inoculated the stems with *P. gregata*, we detected reduced colonization of stems by *P. gregata* and reduced foliar symptoms in a wide range of BSR-resistant genotypes, indicating that resistance also is expressed in the stems of BSR-resistant soybean plants.

There is a possible explanation why Bachman and Nickell were unable to detect BSR resistance in the stem using reciprocal grafts and root inoculation, whereas we were able to detect it using stem inoculation. If a resistant scion is grafted on a susceptible rootstock and the rootstock is inoculated with *P. gregata*, secondary inoculum abundantly produced on the susceptible rootstock could continuously move up to the resistant scion and possibly overwhelm any resistance present in the scion. Our results are consistent with such a hypothesis. In our experiments, the

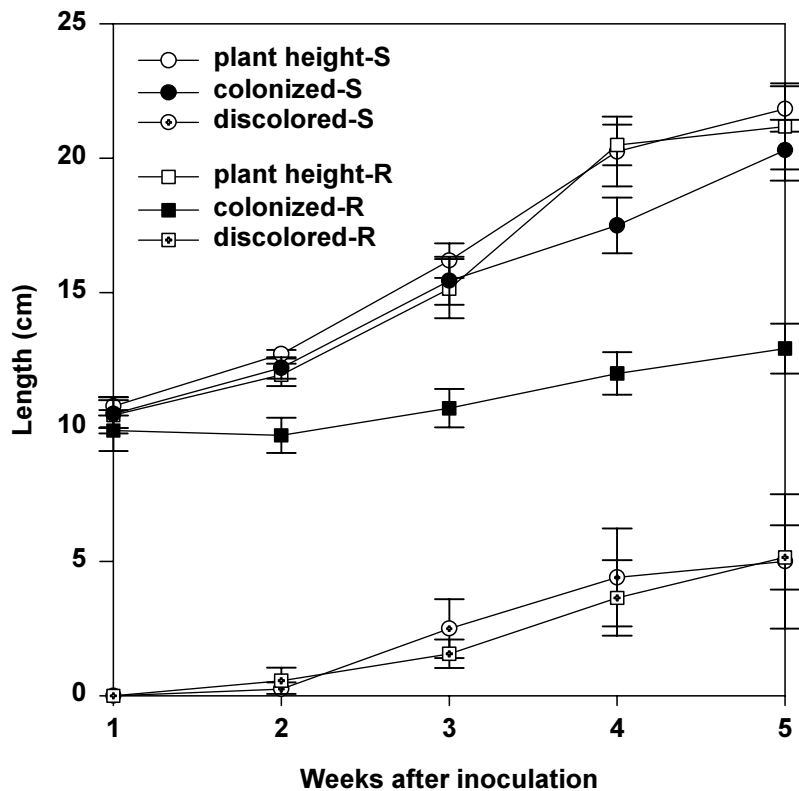


Fig. 1. Stem colonization and internal stem discoloration after introduction of conidia of *Phialophora gregata* into the base of stems of 2-week-old plants of susceptible (S) Sturdy and resistant (R) BSR101 soybean. Each data point represents the combined mean of a total of eight replicates from two experiments (1A and 1B). Each error bar represents the standard error for each week and for each soybean cultivar.

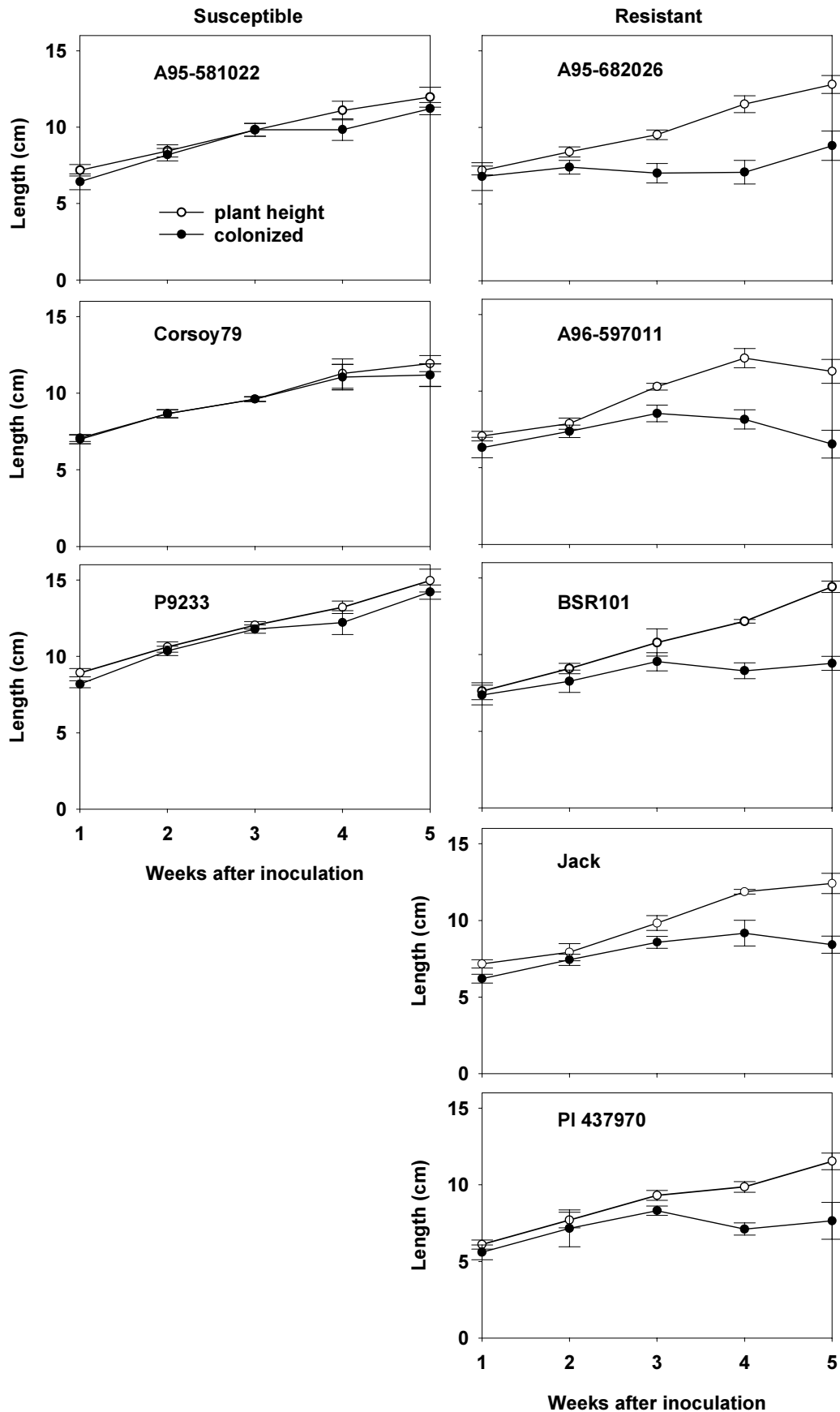


Fig. 2. Severity of stem colonization after introduction of conidia of *Phialophora gregata* into the base of stems of 2-week-old plants of three susceptible and five resistant soybean genotypes. Results presented are for combined experiments 2A and 2B. Each data point represents the mean of eight replicates with one plant each. Each error bar represents standard error for each week and for each soybean genotype.

fungus was isolated from base to apex of both resistant and susceptible genotypes 1 week after inoculum was introduced at the base of the stems (Figs. 1 and 2). This fast advance of the fungus following inocula-

tion of the stem suggests that conidia of *P. gregata* can move readily through the xylem vessels of both BSR-resistant and BSR-susceptible soybean. Our results and previous reports (7,14,17) indicate that

BSR-resistant cultivars generally exhibit some BSR symptoms, suggesting that resistance is incomplete. In addition, our results indicate that the fungus can colonize both resistant and susceptible cultivars

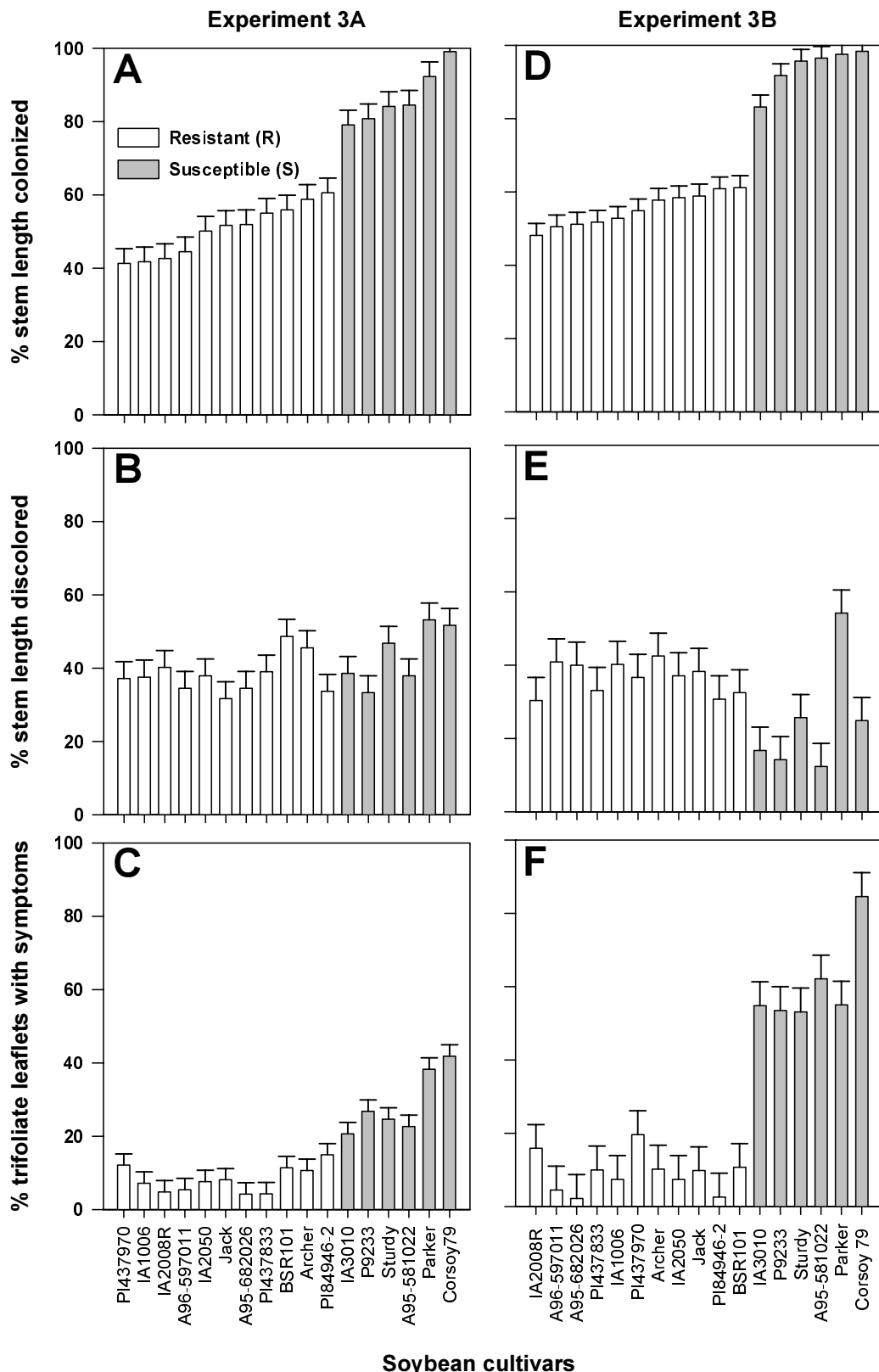


Fig. 3. Severity of stem colonization, stem symptoms, and foliar symptoms 5 weeks after conidia of *Phialophora gregata* were introduced into the base of the stems of 2-week-old resistant and susceptible soybean genotypes. Results presented are for experiments 3A and 3B. Values are means of 10 replicates in experiment 3A and 9 replicates in experiment 3B with one plant each. Error bars represent experiment-wise standard error.

even when inoculum is applied to the soil around the base of plants. Thus, it would not be surprising if BSR-resistant scions grafted on BSR-susceptible rootstocks were overwhelmed by secondary inoculum produced in the susceptible rootstocks and continually transported to the scions.

Our data also suggest that measurements of stem colonization may be more reliable than measurements of internal stem discoloration for distinguishing resistant from susceptible soybean genotypes. Stem colonization was consistently effective and foliar symptoms were generally effective in discriminating resistant from susceptible genotypes in our experiments; however, internal stem discoloration was ineffective in most experiments. In fact, internal stem discoloration was frequently absent in

stems that were heavily colonized by *P. gregata* (Fig. 4). This lack of internal stem discoloration in infected soybean could contribute to the reported difficulty in distinguishing resistant from susceptible genotypes in commonly used screening procedures, which usually rely, at least in part, on measurements of internal stem discoloration (3,15). In our experiments, stem colonization more consistently distinguished resistant from susceptible genotypes than did foliar symptoms (note the low ability of foliar symptoms to distinguish resistant from susceptible genotypes; Fig. 3C). However, it is not known whether this difference between stem colonization and foliar symptoms occurs commonly. The variable effectiveness of foliar symptoms for distinguishing resistant from

susceptible soybean genotypes has been noted previously; Sebastian et al (15) reported that the heritability of BSR resistance based on foliar symptoms was 0.0 and 0.38 in two greenhouse experiments.

Our demonstration that resistance is expressed in soybean stems suggests a novel method for assaying BSR resistance that does not depend on assessment of stem or foliar symptoms. When *P. gregata* was introduced into the stem, the pathogen rapidly moved to the stem apices of both susceptible and resistant plants. However, in the subsequent weeks, the pathogen advanced with the growing tip in the susceptible genotypes but lagged behind in the resistant genotypes (Figs. 1, 2, and 3). Such a difference between resistant and susceptible plants has the potential to be

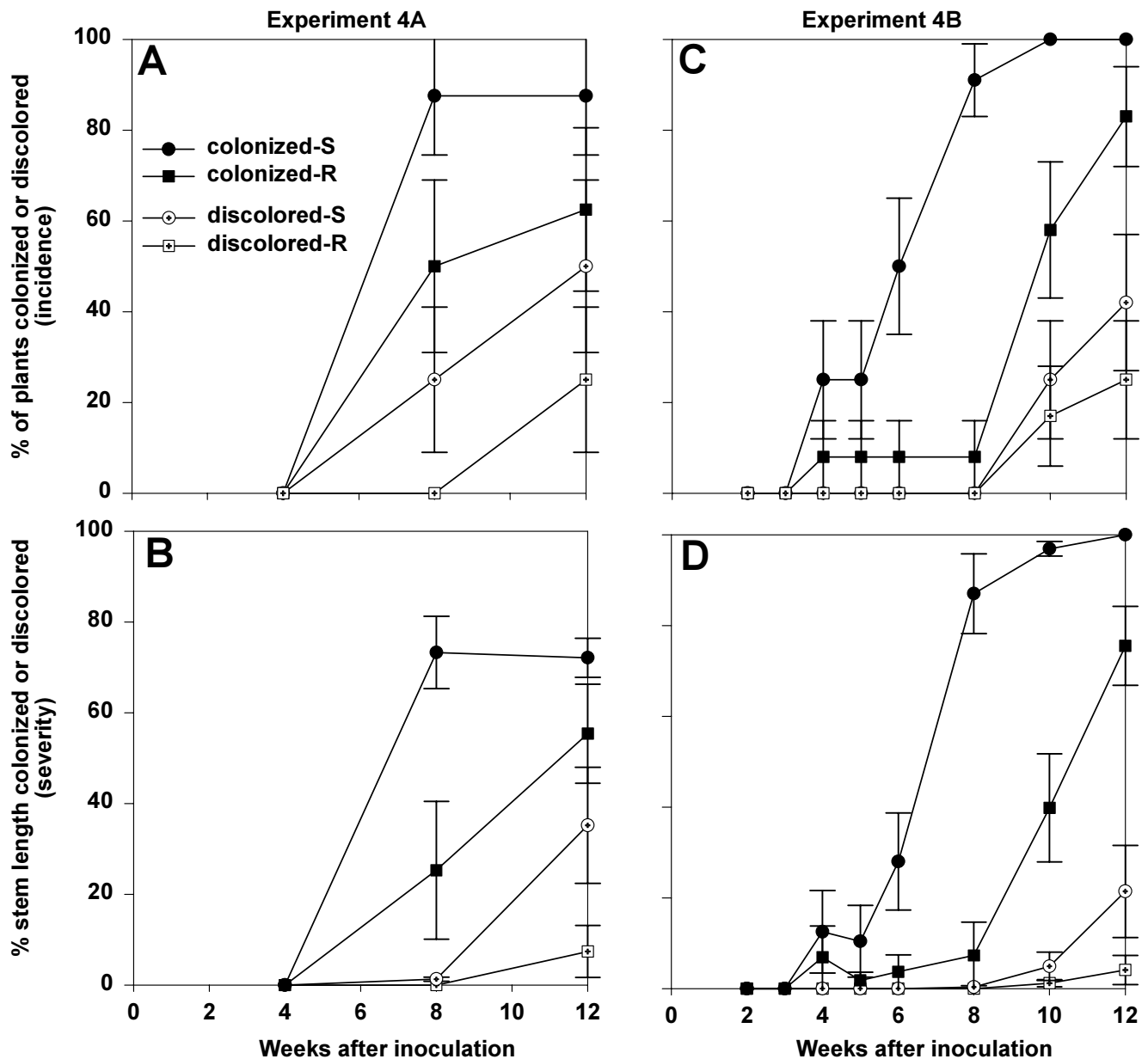


Fig. 4. Incidence and severity of stem colonization and stem discoloration in the stems of susceptible (S) Sturdy and resistant (R) BSR101 soybeans at various times after conidia of *Phialophora gregata* were applied to the soil around the base of the plants. Each data point represents the percentage incidence or severity for 8 plants in experiment 4A and 12 plants in experiment 4B. Each error bar represents standard error for each week and for each soybean genotype.

developed into a resistance screening technique based on inoculation of stems followed by assessment of the top sections of the stems for the presence of the pathogen. The potential for such a screening technique warrants further investigation.

The results reported herein have implications for researchers and breeders who need to reliably assay BSR resistance in soybean genotypes. However, in applying any of the results reported here, it is important to note that they are from experiments that employed limited temperature regimes. Further research will be necessary to determine whether the results are similar under other environmental conditions.

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