Factors Influencing Infection of Soybean Seedlings by Southern *Diaporthe phaseolorum*

R. C. Ploetz and F. M. Shokes

University of Florida, NFREC, Route 3, Box 4370, Quincy 32351. Current address of the first author: TREC, 18905 S.W. 280 St., Homestead, FL 33031.

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ABSTRACT

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The influences of tissue type, genotype, temperature, and inoculum density on infection of soybean seedlings by ascospores and α-conidia (spores) of the fungus causing soybean stem canker in the southeastern United States (southern *Diaporthe phaseolorum*) were studied under high moisture conditions (free water maintained on plant surfaces for 48 hr). Leaf laminae were the least frequently infected tissues of those assayed for infection. Significantly higher levels of infection were observed for petioles, petiole bases, and stem tissue ($P < 0.01$). No relationship was found between the field susceptibility or resistance of 12 genotypes tested and the frequency with which a genotype was infected. Events responsible for resistance in soybean to southern stem canker apparently occur after infection has taken place. Maximum levels of infection occurred at 28 and 34 C; lower levels of infection occurred at 10, 16, and 22 C. Infection did not occur at 40 C. Frequency of infection and inoculum density ($\log_{10}$) were positively correlated ($P < 0.001$; $r^2 = 0.97$) within a range of 1×10^3 to 1×10^6 spores per milliliter.

Additional key word: *Diaporthe phaseolorum* var. *caulivora*.

Stem canker of soybean (*Glycine max* (L.) Merr.) (incited by southern *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. (24) or SDP) has been an important disease in the southeastern United States for about 10 yr (4,23). In recent years, the disease has devastated fields of susceptible cultivars in soybean-production areas throughout the region (34). In 1983, the Southern Soybean Disease Workers estimated a combined loss of $59 million due to this disease for the 16 soybean-producing states in the Southeast (20).

In spite of the importance of stem canker in the Southeast, little is known about factors that influence its occurrence and recurrence once it is found in an area. One of the more perplexing aspects of this disease has been its inconsistent development in a given area from one year to the next. For example, the incidence and severity of stem canker in several counties in the Florida panhandle were very high during 1983, but the disease was almost unnoticed in the same areas during 1984 and 1985 (26). Reduced incidence and severity of stem canker detected in some areas may be partially explained by a reduction in the use of susceptible cultivars (26), but the variable occurrence of stem canker also has been noted in areas planted to susceptible cultivars in consecutive years (26; Ploetz, *unpublished*). Although factors other than cultivar susceptibility apparently play a role in the development of this disease in the field, the identity and importance of these factors in most cases are not known.

The stem canker disease cycle is not thoroughly understood, but it is clear that when the soybean host is infected during early vegetative growth stages, symptoms of the disease may develop after reproductive growth begins (4,24). Incubation periods of ≥ 50 days have been reported previously (24). Recent work suggests that infections that occur after the soybean growth stage V9 (7) may not result in disease development (2). However, infection that occurs before V9 may cause significant disease and crop loss in susceptible cultivars.

Not much is known about factors that influence infection of soybean seedlings by SDP. In a report on work conducted with the closely related soybean pathogen *D. phaseolorum* var. *caulivora* Athow & Caldwell, Athow (1) suggested that infection occurs primarily via leaves based on the lack of disease development after removal of the six lowest trifoliates in the plant canopy. This conclusion is subject to other interpretations (e.g., the removal of leaves in this work may have retarded disease development by inducing resistance mechanisms in the host or altering the microclimate required for infection and/or symptom development). Although similar work has not been conducted for SDP, Backman et al (3) have implied the same route of infection for southern stem canker. Other than two preliminary reports regarding the effects of high moisture environments (24,31) and temperatures of 21–27 C (31), no objective work has been published on factors that affect infection of the soybean host by SDP. Work identifying these factors is needed.

Host infection may be observed directly with histological techniques (9,11,21,29). Unfortunately, these methods are time-consuming and impractical when many tissue samples are monitored for infection. Alternatively, infection can be indirectly monitored by isolating the organisms under study from host tissue on artificial media (10,13,28).

Cultivar susceptibility (11,18,21,27,30), temperature (5,10,33), and inoculum density (6,13) are factors known to influence host infection. The present work was conducted to determine the influence of these factors on the infection of soybean seedlings by SDP. In addition, work is presented demonstrating the relative frequency with which different parts of the soybean host (seedlings) are infected by SDP. Portions of this work have been reported previously (26).

MATERIALS AND METHODS

Soybean seedlings were grown in plastic pots 10 cm in diameter (three seedlings per pot) containing Metro Mix 220 or Metro Mix 300 potting medium (Grace Horticultural Products, W. R. Grace & Co., Cambridge, MA). Plants were given 20-20-20 and chelated Fe fertilizers as needed and were grown in a greenhouse under day lengths lengthened to 16 hr with fluorescent light. The growth stages of seedlings used in this work ranged from V2 to V5, but all plants used in a given experiment were at the same stage of growth.

Inoculum was produced by culturing an isolate of SDP (No. 8) on mature soybean stems, autoclaved for 40 min on each of two consecutive days, and placed on Difco potato-dextrose agar in

petri plates 9 cm in diameter. In preliminary toothpick assays (14), isolate No. 8 was consistently virulent on seedlings of several different soybean cultivars (Snell and Shokes, *unpublished*). Cultures were incubated for >3 wk in closed Ziploc storage bags on a laboratory bench. After 3 wk, perithecia and pycnidia were scraped from stems with a scalpel into tap water in watch glasses; harvested sporocarps were macerated with a glass pestle. Inoculum preparations were then passed through cheesecloth to remove mycelia and stem debris. Inocula contained ascospores and α-conidia of the pathogen (spores). In previous work with soybean seedlings, both types of spore were shown to be infective and pathogenic (24). Because no pathogenic differences were noted between ascospores and α-conidia in the above work (24), no effort was made to characterize the composition of inocula used in the present studies with regard to ratios of the respective types of spore. Spores were diluted with tap water and quantified with a hemacytometer. Unless specified otherwise, inoculum for an experiment contained 1×10^5 spores per milliliter. For each experiment, the viability of spores was determined after 48 hr of incubation on 1.5% Difco water agar. Spores with germ tubes longer than spore length were considered to be viable; about 300 spores were assayed for each experiment.

Plant surfaces were moistened to runoff with tap water before inoculation. Spore suspensions were then sprinkled to runoff on plants, with shakers made of 2-L Erlenmeyer flasks capped with perforated aluminum foil. Plants in temperature studies were then covered with plastic bags and placed in an incubator set at one of six temperatures (10, 16, 22, 28, 34, or 40 C). Plants in all other experiments were placed in a mist chamber in a greenhouse. Extreme temperatures for the latter experiments were 16 and 38 C; however, for most of these experiments temperatures ranged from 22 to 32 C. Plants in all experiments were incubated for 48 hr, and plant surfaces remained wet for the duration of an incubation period.

Infection of soybean seedlings was determined by isolating SDP from tissue of inoculated plants. Tissue was harvested immediately after an incubation period, and unless specified otherwise, consisted of petiole bases as defined below. Noninfective propagules and (epiphytic) growth of the pathogen in these tests were distinguished from infective (endophytic) growth by disinfestation of tissue surfaces with 1.05% NaClO for 2 min. After disinfestation, tissues were rinsed with sterile tap water and blotted dry on sterile paper towels before placement on Phillips' (22) medium, which is selective for the growth of SDP. Tissue was then incubated without light at ambient temperatures (20–28 C) on a laboratory bench for recovery of SDP. Tissue was observed for growth of SDP 6, 10, and 12 days after incubation on Phillips' medium began. The characteristic spidery and closely appressed growth of SDP on this medium was used to identify the pathogen (Fig. 1).

Tissue assays. Four types of seedling (cultivar Hutton) tissue were assayed for infection: stem, petiole base, petiole, and leaf (Fig. 2). Stem tissue was excised from internodal sections of stem and was 1.3 cm in length. Petiole bases included a portion of the base of a petiole 0.7 cm in length plus an attached portion of stem 0.8 cm in length. Petiole tissue was 2.0 cm in length, and leaf tissue was recovered from the center of the middle leaflet in a trifoliate with a cork borer 1.3 cm in diameter. None of the tissues were contiguous. Based on calculations made with 5-wk-old Hutton seedlings (V4), surface areas of each of the types of tissue that were exposed to inoculum were equal (about 135 mm^2). Each of the four tissues was recovered from 16 plants in each of two experiments. All tissue above the cotyledonary node was used. A total of at least 110 pieces of a respective tissue were assayed for infection during the two tests. Percent infection data (mean recovery of SDP) were arcsin square-root transformed before analyses of variance were performed (Table 1).

Infection of soybean genotypes. Seedlings of 12 soybean genotypes were assayed for infection by SDP. The 12 genotypes included nine soybean cultivars (maturity groups IV to VIII; susceptible to resistant) and a moderately resistant and two very susceptible breeding lines (S-100, maturity group V, and GA81-2057 and J77-339, maturity groups VII and VI, respectively). Six of the 12 genotypes used (Arksoy, Centennial, J77-339, Kingwa, S-100, and Tracy-M) are differentials used by Keeling (15) in work on pathogenic variation in the pathogen population. Reactions of the genotypes to stem canker were based on ratings by Hiebsch (12) and Ploetz (*unpublished data*).

With one exception, the genotypes were replicated three times (three plants per replicate) in a randomized complete block design in each of seven experiments; Bragg was used in six experiments. A total of about 250 petiole bases was assayed for each genotype. Percent infection data were arcsin square-root tranformed before analyses of variance were performed. Infection data were analyzed for individual genotypes and for groups of these genotypes segregated on the basis of field reaction to stem canker (Table 2).

Temperature studies. The effect of six temperatures (10, 16, 22, 28, 34, and 40 C) on the infection of GA81-2057 seedlings by SDP was tested in a growth chamber study. The effect of each temperature was evaluated three times in a random sequence over time. For each experiment, an inoculated and uninoculated

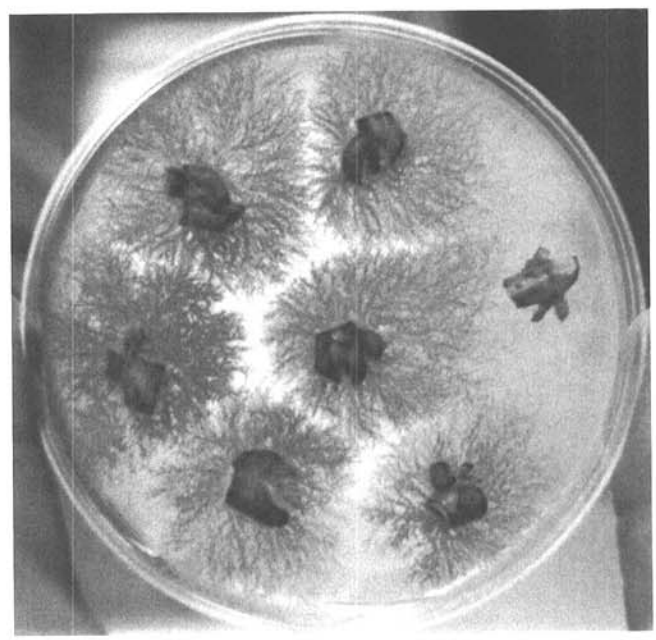

Fig. 1. Growth of southern *Diaporthe phaseolorum* from infected petiole bases after 10 days' incubation on Phillips' (22) medium.

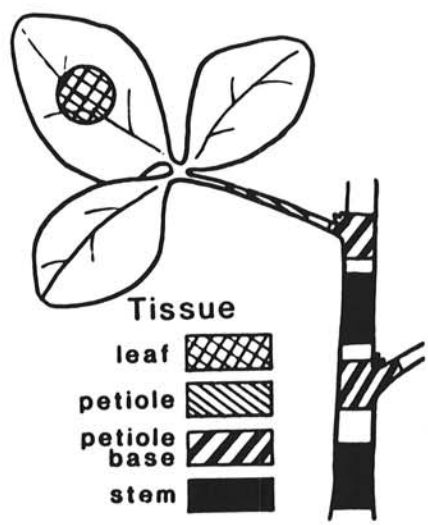

Fig. 2. Location of soybean seedling tissue assayed for infection by southern *Diaporthe phaseolorum*.

control treatment were replicated five times (three plants per replicate). Infection data were arcsin square-root transformed before calculating standard deviations used in Figure 3.

Inoculum density studies. The effect of inoculum density of SDP on infection of Hutton seedlings was tested in a series of four experiments; seven inoculum densities (1×10^3, 5×10^3, 1×10^4, 5×10^4, 1×10^5, 5×10^5, and 1×10^6 spores per milliliter) and an uninoculated control treatment were evaluated in each experiment. Treatments were replicated four times (three plants per replicate) in a randomized complete block design. Linear regressions were computed with transformed (percent infection:arcsin square-root; inoculum density:$\log_{10}$) or nontransformed data.

RESULTS

The viability of spores used in the present work was uniformly high, ranging from 92 to 99% for a given experiment. SDP was not recovered from uninoculated control plants in most experiments. Less than 1% of the petiole bases from control plants were infected in one inoculum density experiment and two temperature experiments. In these instances, infected control plants may have been inadvertently inoculated by splashed spores or during the handling of inoculated plants. No infection of uninoculated control plants was detected in any other experiments.

Tissue assays. In each of two experiments, leaf laminae were the least frequently infected tissues of those assayed for infection ($P < 0.01$; Table 1). Petioles, petiole bases, and stem tissue were each infected approximately three times as often as leaf tissue. No significant differences were detected among infection frequencies for the three former tissues.

Infection of soybean genotypes. Four groups of soybean genotypes (very susceptible, susceptible, moderately resistant, and resistant to stem canker) were analyzed for infection by SDP. As a group, resistant genotypes were less frequently infected than more susceptible genotypes ($P < 0.05$; Table 2). However, when individual genotypes were considered, it was apparent that infection frequency was not consistently related to field reaction to stem canker. For example, Braxton and Tracy-M, two cultivars with a high level of resistance to stem canker, were as frequently infected as Bragg, a susceptible cultivar.

Temperature studies. Maximum levels of infection occurred at 28 and 34 C (Fig. 3). Lower levels of infection occurred at 10, 16, and 22 C, and no infection was observed at 40 C.

Inoculum density studies. A linear relationship was observed between infection frequency and inoculum densities between 1×10^3 and 1×10^6 spores per milliliter. The linear regression with nontransformed infection and transformed ($\log_{10}$) inoculum density data was highly significant ($P < 0.001$; $r^2 = 0.97$; Fig. 4).

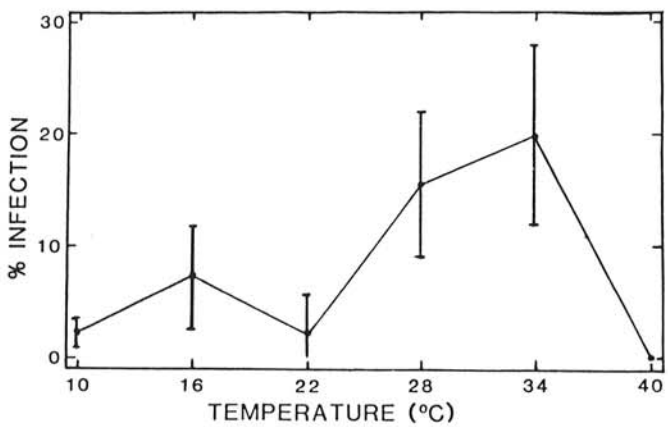

Fig. 3. Effect of temperature on the infection of GA81-2057 soybean seedlings by southern *Diaporthe phaseolorum*.

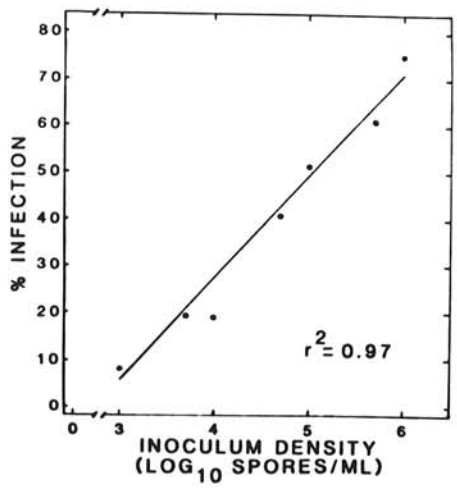

Fig. 4. Relationship between inoculum density ($\log_{10}$) of southern *Diaporthe phaseolorum* and frequency of infection of Hutton soybean seedlings.

TABLE 1. Influence of type of soybean seedling tissue on infection by southern *Diaporthe phaseolorum* (SDP)[v]

Tissue[w]	% Infection[x] Experiment 1	Experiment 2	Mean infection[y]
Leaf	18.0	14.9	16.5 b[z]
Petiole	43.9	45.6	44.8 a
Petiole base	55.6	46.3	51.0 a
Stem	48.7	52.3	50.5 a

[v] Infection of Hutton soybean seedlings (V3 [7]) was monitored after inoculation with ascospores and α-conidia of SDP and a 48-hr incubation in a high moisture environment.
[w] Tissues according to Figure 2. A piece of each of the types of tissue had approximately the same surface area (135 mm^2).
[x] Percentages of pieces of tissue from which SDP was recovered on Phillips' (22) medium.
[y] Mean infection for experiments 1 and 2.
[z] Means followed by the same letter are not significantly different from each other according to Duncan's new multiple range test ($P < 0.01$).

TABLE 2. Effect of cultivar susceptibility on infection of soybean seedlings by southern *Diaporthe phaseolorum* (SDP)

Genotype	Maturity group	Field reaction[v] to soybean stem canker	% Infection[w,x]	Mean[x,y] infection
GA81-2057	VII	VS	24.9 ab	
J77-339[z]	VI	VS	27.1 b	26.0 a
Bragg	VII	S	19.7 ab	
Hutton	VIII	S	29.1 b	
RA 604	VI	S	32.7 b	27.2 a
Centennial[z]	VI	MR	19.9 ab	
RA 680	VI	MR	31.2 b	
S-100[z]	V	MR	30.2 b	27.1 a
Arksoy[z]	VI	R	11.3 a	
Braxton	VII	R	19.4 ab	
Kingwa[z]	IV	R	22.6 ab	
Tracy-M[z]	VI	R	18.7 ab	18.0 b

[v] Susceptibility/resistance to soybean stem canker: VS = very susceptible; S = susceptible; MR = moderately resistant; R = resistant. Ratings according to Hiebsch (12) and Ploetz (*unpublished data*).
[w] Percentage of petiole bases infected by SDP. Results are means from seven experiments except for Bragg which was used in six experiments; about 250 petiole bases were assayed for each genotype.
[x] Numbers followed by the same letter within a column are not significantly different from each other according to Duncan's new multiple range test ($P < 0.05$).
[y] Mean infection for genotypes for a given field reaction to soybean stem canker.
[z] Differentials used by Keeling (15).

DISCUSSION

In the present study, leaf laminae of soybean seedlings were infected less frequently by SDP than petioles, petiole bases, and stem tissue. These results corroborate field observations made during the 1984 and 1985 seasons in Florida, in which soybean leaves were infrequently infected relative to petiole bases and stem tissue (26; Ploetz, *unpublished*). In a previous report, Athow (1) suggested that *D. phaseolorum* var. *caulivora* infects soybeans primarily via leaves. From our results, it is apparent that SDP is capable of infecting soybean leaves, but other tissues are infected much more frequently. Also, in recent work, symptom development has been observed in soybean seedlings in which only nonleaf tissues were inoculated and infected by SDP (Ploetz, *unpublished*). Leaf infection probably does not play as important a role in the development of southern stem canker of soybeans (caused by SDP) as has been assumed (1) for soybean stem canker in the midwestern United States (caused by *D. phaseolorum* var. *caulivora*) or demonstrated for the *D. helianthi* Munt.-Cvet. et al/sunflower (*Helianthus annuus* L.) pathosystem (19).

In studies on the infection of seedlings of different soybean genotypes by SDP, resistant genotypes, as a group, were less frequently infected by SDP than more susceptible genotypes (Table 2). However, this was not a consistent relationship. Seedlings of some resistant or moderately resistant cultivars were as frequently infected as susceptible cultivars. Consequently, it would appear that seedling infection frequency is not a reliable indicator of the field reaction of soybean genotypes to stem canker. Also, it is unlikely that the magnitude of differences in infection frequencies evident between some susceptible and resistant genotypes would explain differences in their field reactions to stem canker. Braxton and Tracy-M are almost immune to the effects of stem canker (34), whereas J77-339 and GA81-2057 are very susceptible lines that consistently develop severe symptoms of the disease in the field (Ploetz, *unpublished*). It is doubtful that the subtle (and nonsignificant) differences in infection frequencies observed in the present work would result in the great differences in symptom development observed on these genotypes in the field.

Also, it is unlikely that the variable infection data in Table 2 could be the result of reactions of differential genotypes of soybean to a physiologic race of SDP (all experiments on cultivar infection were conducted with one isolate of SDP). Keeling (15,16) has described variation in virulence among isolates of the stem canker pathogen. He has identified seven pathotypes of SDP on six differentials also used in the present work (15; Table 2). If frequency of infection by SDP was a factor determining the susceptibility of soybean genotypes to stem canker, and the above genotypes reacted as differentials to infection by the isolate used in our tests, one would expect greater differences among infection data than those recorded in Table 2.

The susceptibility of cultivars in different pathosystems may (11,18,27) or may not (9) be determined by the frequency with which cultivars are infected. Marshall and Rush (18) detected a significant positive correlation between disease severity ratings for several cultivars of rice (*Oryza sativa* L.) and the frequency with which culms of those cultivars were invaded by *Rhizoctonia solani* Kühn ($P < 0.01$; $r = 0.935$). Over eight times as many infection structures (infection cushions and lobate appressoria) formed on susceptible as on resistant cultivars. Coincidently, in response to inoculation with *Phytophthora infestans* (Mont.) de Bary, eight times as many papillae (pathogen-induced structures in the host that restrict penetration by the pathogen) were formed in an incompatible cultivar of potato (*Solanum tuberosum* L.) as in a compatible cultivar (11). In contrast, Gray and Sackston (9) detected no differences in the invasion of sunflower seedlings by compatible or incompatible races of *Plasmopara halstedii* (Farl.) Berl. & de Toni. Resistance in this pathosystem "... was expressed only some days after infection. ... "

Results of the present study indicate that it is probable that resistance in soybean to stem canker is not expressed before infection has taken place. Events that are responsible for resistance to this disease apparently occur after the host is infected. Work identifying and describing these events is needed.

Temperature is an important factor influencing infection of hosts in many pathosystems (5,10,33). Ranges of temperatures conducive to infection are usually broad (5,10,33).

In the present work, infection of soybean seedlings by SDP also occurred within a wide range of temperatures (10–34 C). Also, it is apparent that extremely high temperatures, which occur infrequently in the soybean canopy ($\geq$40 C), would stop infection by this pathogen from occurring.

Recently, Backman et al (2) have proposed a model for the management of soybean stem canker with fungicides. Their model incorporates critical host (infection before V9) and pathogen (occurrence of mature perithecia) factors when predicting periods during which application of fungicides will be beneficial. "Fine tuning" this model or others simulating the development of stem canker may depend on knowledge of factors that are critical to infection. In light of the present results, temperature alone would probably not be a useful term in such models.

A factor in need of additional study, with regard to its role in the development of stem canker or inclusion in models simulating disease development, is inoculum density. In our work, a significant linear relationship was observed between inoculum density (within a range of 1×10^3 to 1×10^6 spores per milliliter) and the incidence of infection (Fig. 4). However, because the levels of inoculum that occur naturally in the field are not known, it is difficult to relate the present results with what one might expect in the field. Development of systems to forecast maturation of spores and spore dose in the field similar to those constructed by MacHardy and Gadoury (8,17) for *Venturia inaequalis* (Cke.) Wint. would be of obvious benefit in studies on the epidemiology of soybean stem canker.

The relationship between inoculum density and the ultimate expression of stem canker symptoms has been studied only incidentally. In work describing the infectivity and pathogenicity of ascospores and α-conidia of SDP, Ploetz and Shokes (24) detected a higher incidence of disease with increasing inoculum densities. Obviously, the inoculum densities that occur in a given field may influence the development of stem canker in that field. Still, research is needed to determine the effect of given inoculum densities and infection frequencies on the development of soybean stem canker. Through the study of these and other factors (24,31,32) that may influence the development of this disease, plant pathologists may more fully understand this unpredictable disease.

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