Phytotoxin Production by *Diaporthe phaseolorum* var. *caulivora*, the Causal Organism of Stem Canker of Soybean

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ABSTRACT

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Diaporthe phaseolorum var. *caulivora* isolated from soybean plants infected with stem canker produces a toxin that caused symptoms characteristic of stem canker when introduced into the plant. The amount of toxin and the degree of symptoms were linearly related. Four fungal isolates produced the toxin in culture in significantly different amounts, when measured in terms of the degree of symptoms and the dilution end point. The amount of toxin produced by the isolates correlated with the length of cankers produced by the fungus upon inoculation. Of the 12 plant species evaluated, only soybean and lima bean were sensitive to the toxin and also susceptible to the fungus. The purified toxin produced symptoms very similar to those of stem canker, and some of its chemical properties are similar to those of a phytotoxin suggested to have a role in pine wilt caused by *Phomopsis* sp. The *D. p. caulivora* toxin appears to play a role in stem canker of soybean.

Additional keywords: pathogenicity factor, virulence factor.

Stem canker of soybean (*Glycine max* (L.) Merr.) is caused by *Diaporthe phaseolorum* (Cke. & Ell.) Sacc. var. *caulivora* Ath. & Cald. The disease was originally described in Iowa in the 1940s. In the past decade, stem canker has caused severe losses in the southeastern United States, and yield losses of 20–50% have been reported in individual counties of some states (12). Stem canker was found in 24 soybean-producing parishes in Louisiana in 1983, with 16 parishes reporting severe losses (20). The disease is characterized by long brown lesions on stems, usually beginning at the nodes and spreading to the internodes and petioles. The lesions enlarge, become sunken, and may eventually girdle the stem. Leaf symptoms appear as interveinal chlorosis and necrosis. These symptoms are followed by plant death in highly susceptible cultivars.

D. p. caulivora has been isolated from mature soybean plants that were asymptomatic throughout the growing season and also from resistant and susceptible cultivars apparently infected too late in the season to develop symptoms (1,18). In some cases the fungus could not be isolated from parts of plants showing symptoms, which suggested the possible involvement of a metabolite in disease expression, advancing before the fungus and producing symptoms.

Several phytotoxins have been reported to have a role in disease and act as either pathogenicity or virulence factors (23), and some are involved in other stem canker diseases. A host-specific toxin is produced by *Alternaria alternata* f. sp. *lycopersici*, causing stem canker of tomato (6). *Phomopsis* sp., the anamorph of *Diaporthe* sp., produces the toxic metabolites cytochalasin H and cytochalasin B (17,22), which have not been implicated in any plant diseases. Recently, culture filtrates of *D. phaseolorum* var. *sojae* were reported to contain a toxic metabolite that caused wilting of soybean seedlings (14). In this study, we investigated the possibility of the involvement of a toxin in stem canker and evaluated some of the fundamental chemical characteristics of the toxin.

MATERIALS AND METHODS

Isolates of *D. p. caulivora*. Cultures of *D. p. caulivora* were obtained from cankers on soybean plants collected from four locations in Louisiana: Opelousas, St. Joseph, Slaughter, and Lawtell. Single-spored cultures were derived from field isolates and maintained on potato-dextrose agar (PDA). The soybean cultivar Ring Around 606 (RA 606), which is susceptible to stem canker, was used in all studies.

Culture conditions. The four isolates were separately introduced into 50 ml of sterile potato-dextrose broth (PDB) in 250-ml flasks with cotton plugs, which were incubated in darkness for 3 wk at 24 C as stationary cultures. The inoculum consisted of 10-mm plugs of the fungus from 10-day-old PDA cultures. The medium was filtered through Whatman #1 filter paper (W and R Balston Ltd., England) and used for bioassay.

The optimum temperature for toxin production was determined by inoculating the medium with the Opelousas isolate and incubating it at 12, 16, 20, 24, and 28 C for 23–25 days. The culture medium was filtered through a Whatman #1 filter paper and retained for bioassay. The mycelium was dried in an oven at 45 C for 3 days. The dry weight of the mycelium in three replications of each treatment was recorded.

Bioassay. Cell-free culture filtrate was obtained by passing the medium through a sterile 0.45-μm Millipore filter (Millipore Filter Corporation, Bedford, MA) under vacuum. Twofold dilutions of the culture filtrate from 1:10 to 1:160 in sterile distilled water were used for the assay. Similar dilutions of sterile PDB were used as controls. Soybean plants were grown in the greenhouse, maintained at 22–30 C, in autoclaved 12.5-cm clay pots containing a mixture of silt, peat moss, and perlite (3:2:1); 2 g of Osmocote plant food 14-14-14 (Sierra Chemical Co., Milpitas, CA) was added to each clay pot after seedlings emerged. Trifoliates of nearly uniform size from 30- to 50-day-old plants were excised under water with a razor blade. The cut end of each trifoliate petiole was immersed immediately in 3 ml of the toxin solution in a vial (4.5×1.0 cm). Symptoms were rated on a 0–5 scale after 36 hr (0 = no symptoms; 1 = 1–25% of the petiole and the midrib and veins discolored; 2 = 26–50% of the petiole and the midrib and veins discolored; 3 = 51–75% of the petiole and the midrib and veins discolored; 4 = 76–100% of the petiole and the midrib and veins discolored; 5 = midrib and veins discolored, forming a fine network, with interveinal chlorosis, leaves turning brittle, and 76–100% of the petiole discolored). Three or more replications were used to overcome the

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slight differences in the sensitivity of leaves of different ages.

Stability of the toxin. The stability of the toxin under changes in temperature and pH was tested. Test tubes containing 10 ml of the culture filtrate of the Opelousas isolate were heated individually to 40, 60, 80, and 95 C in a hot-water bath. The temperature of the culture filtrate in the test tube was increased through the required range, and the upper temperature maintained for 15 min. The culture filtrate (10 ml) was also autoclaved for 15 min. The tubes were cooled to room temperature, and the filtrates assayed. The pH of 10-ml aliquots of the culture filtrate was adjusted with 1 N HCl to pH 3 and pH 5 and with 0.5 M sodium bicarbonate to pH 9.0. The bioassays were conducted as described above.

Specificity of the toxin. The pathogenicity of the four *D. p. caulivora* isolates was determined by the toothpick inoculation technique (10) on 30-day-old greenhouse-grown plants. Toothpicks were boiled, air-dried, and saturated with PDB; they were then autoclaved for 20 min, inoculated with the four isolates separately, and incubated for 3 wk at 24 C. The pointed ends of the toothpicks were cut to a length of 5–6 mm with a razor blade and inserted into a puncture made with a sterile needle at the lowest plant node. Five plants per treatment were inoculated, and the length of the cankers above and below the point of inoculation was measured after 4 wk. A bioassay of the culture filtrates of the four isolates was conducted simultaneously.

The Opelousas isolate was used to test several plant species for sensitivity to the toxin. The plant species listed in Table 1 were grown in the greenhouse at 22–30 C in autoclaved pots as described previously, and leaves of 30- to 50-day-old plants were used for bioassay. Some of the plant species were also tested for susceptibility to the Opelousas isolate by the toothpick inoculation technique.

Varietal reaction to toxin treatment. The Opelousas, St. Joseph, Slaughter, and Lawtell isolates were grown under conditions described previously, and culture filtrates of each bioassayed. Five cultivars were grown in the greenhouse: Bragg (susceptible); RA 606 (moderately susceptible); Davis and Asgrow 5474 (moderately resistant); and Tracy M (resistant) (2,3). Symptoms produced by 1:10 dilutions of the culture filtrates were rated, and the dilution end point (the highest dilution at which symptoms were produced) was noted in all cultivars. The reaction of the cultivars to the culture filtrates of the four isolates was compared to their stem canker reaction in the field (2,3).

Purification of the toxin. Culture filtrate (2–3 L) was acidified to pH 5.0 with 1 N HCl and extracted with equal amounts of ethyl acetate. The combined organic phase was dried by passing through anhydrous sodium sulfate and evaporated to dryness under vacuum at 40 C in a Rotary Flash Evaporator (Buchi, Switzerland). Sephadex LH20 (Pharmacia Fine Chemicals, Piscataway, NJ) was allowed to swell in methanol for 24 hr and packed in a column (1 × 30.5 cm). The column was washed with 200 ml of methanol before the sample was loaded. The residue was dissolved in a small quantity of methanol, placed on the column, and eluted with the same solvent. Fractions (10 ml) were collected and dried under vacuum. The residue was dissolved in acetone and bioassayed. The fractions showing activity were pooled and further purified by preparatory thin-layer chromatography (TLC) using silica gel GF 254 (J. T. Baker Chemical Company, Phillipsburg, NJ) and a mixture of chloroform and methanol (9:1) as the solvent system. The three major compounds that were visualized under UV light as purple spots were scraped off the plate separately, eluted with 100 ml of a mixture of chloroform and methanol (1:1), and bioassayed. The toxin was purified by repeated separation and elution in TLC. The purity of the compound was assessed by separating it with various solvent mixtures in TLC. The crude residue and the purified toxin spot on the TLC plate were sprayed separately with various reagents (13), and the color reaction was observed (Table 2).

A modified bioassay was used for routine detection of the toxin. Trifoliates were excised under distilled water and placed over moist filter paper in a petri plate. The toxin residue was dissolved in 5 ml of acetone, and a 10-μl aliquot was placed on a puncture made with a sterile needle at the base of the midrib of each leaflet. The presence of the toxin was indicated by a reddish discoloration of the midrib and veins within 24 hr. Trifoliates treated in a similar manner with acetone served as controls.

The purified toxin was dissolved in spectrophotometric-grade methanol for the UV absorption spectrum, which was recorded by a Gilford Response UV-Vis spectrophotometer (CIBA-Corning Diagnostic Corp., Oberlin, OH), and in acetone for the IR spectrum, recorded by an IBM IR44 spectrophotometer.

Reaction of soybean trifoliates to other toxic metabolites. Pure toxins from *Phomopsis* sp.—cytochalasin H and cytochalasin B (17,22)—and a toxin produced by *Phomopsis* sp. from wilted pine trees (16) were dissolved in acetone (100 μg of toxin in 100 μl of solvent) and made up to 3 ml with distilled water. The cut end of each excised trifoliate was immersed in the solution. The compounds (100 μg) were also assayed by the leaf puncture method. Controls containing solutions of acetone in water were tested simultaneously.

RESULTS

Toxic activity of the culture filtrate. Excised trifoliates treated with the culture filtrate developed symptoms similar to stem canker symptoms observed in the field. Initial symptoms appeared on the petiole within 24–36 hr as small reddish brown lesions, which gradually increased in length. On the leaflets, the midrib and veins became reddish brown. The leaf lamina began to exhibit prominent interveinal chlorosis and subsequent necrosis 48–72 hr after treatment with a 1:10 dilution of the culture filtrate. No wilting was seen after 4–5 days at dilutions above 1:10. At a dilution of 1:10 the leaflets became brittle and dry. The mean disease rating ranged from 3.83 at 1:10 to 0.16 at 1:160, showing an

TABLE 1. Reaction of various plant species to *Diaporthe phaseolorum* var. *caulivora* and a toxin extracted from a culture filtrate of the fungus

Plant species	Reaction to fungus[x,y]	Reaction to toxin[y,z]
Soybean (*Glycine max*)	+	+
Lima bean (*Phaseolus lunatus*)	+	+
Cowpea (*Vigna unguiculata*)	−	−
Snap bean (*Phaseolus vulgaris*)	−	−
Runner bean (*Phaseolus vulgaris*)	NT	−
Cotton (*Gossypium hirsutum*)	−	−
Okra (*Hibiscus esculentus*)	−	−
Sorghum (*Sorghum vulgare*)	−	−
Cabbage (*Brassica oleracea*)	−	−
Eggplant (*Solanum melongena*)	NT	−
Tomato (*Lycopersicon esculentum*)	NT	−
Bell pepper (*Capsicum annuum*)	NT	−

[x]Plants were inoculated with the fungus by the toothpick inoculation technique.
[y]+ = Symptoms produced; − = no symptoms; NT = not tested.
[z]Assays were conducted with a 1:10 dilution of culture filtrate and partially purified toxin.

TABLE 2. Effect of various reagents on the toxigenic spot separated from a culture filtrate of *Diaporthe phaseolorum* var. *caulivora* by thin-layer chromatography (TLC)

Reagent	Color[z]	Test for
50% ethanolic sulfuric acid	Gray*	Organic compound
5% phosphomolybdic acid in ethanol	Bluish green*	Reducing compound
1% aqueous $KMnO_4$ in 5% aqueous $NaHCO_3$	Yellow	Aromatic carboxylic and reducing compound
0.05% aqueous $KMnO_4$	Yellow*	Oxidizability
Pauly's reagent	Yellow	Phenolic groups
Folin-Ciocalteu reagent	Blue*	Phenolic groups
Ferric chloride in 0.5 N HCl	Blue	Hydroxy groups
Ninhydrin	None*	Protein or free amino acids
Iodoplatinate	None*	Nitrogen-containing groups

[z]Asterisk indicates that the TLC plate was heated to 100 C for 5–10 min.

inverse linear relationship ($r = -0.9$) with dilution (Fig. 1). The pH of the culture filtrate in all cases ranged from 7.6 to 7.9. The control samples treated with similar dilutions of uninoculated PDB filtrate did not show any symptoms.

All four isolates produced the toxin, though they differed significantly in the amounts of toxin produced, based on the severity of symptom expression. The Opelousas isolate produced greater amounts than the other isolates and was therefore used for further studies. The dilution end point was 1:160 for the Opelousas isolate and 1:20 for the other three isolates. The symptom rating also gave similar results, the Opelousas isolate differing significantly from the other three (Table 3). Mycelial dry weight and toxin production were greatest at 20 C. Fungal growth was similar at 20 and 28 C, but toxin production measured in terms of symptom severity sharply declined at the higher temperature (Fig. 2), indicating that greater fungal growth is not necessarily associated with greater toxin production.

Specificity of the toxin. Among the different plant species tested, only soybean and lima bean were sensitive to the toxin and susceptible to the fungus (Table 1). The symptoms of toxin treatment of lima bean were similar to those of soybean, with reddish lesions on the petiole, midrib, and veins and interveinal chlorosis. Many of the other plant species were neither sensitive to the toxin nor susceptible to the fungus.

RA 606 plants inoculated with the four *D. p. caulivora* isolates developed typical stem canker symptoms after 4 wk. The length of the cankers produced by the four isolates varied significantly. The Opelousas isolate produced the longest cankers and also caused chlorosis of the leaves and death of the plants. It differed significantly from the other three isolates in the length of the cankers produced and also in the symptom severity produced by the culture filtrate (Table 3). Three out of five plants inoculated with each fungus isolate developed cankers, and only plants showing symptoms were included in analyses. A correlation of $r = 0.82$ was obtained between the length of the cankers and the amount of toxin produced in vitro by the four isolates (Table 3).

Varietal reaction to the toxin. The dilution end point for all five cultivars treated with the culture filtrate from the Opelousas isolate was 1:160. The differences in dilution end point were not definitive enough to distinguish differences in the sensitivity of the cultivars to the culture filtrates of the isolates. The disease rating on the 0–5 scale at the 1:10 dilution was therefore used for comparison of the cultivars (Table 4). The Opelousas isolate produced significantly more severe symptoms than the other isolates tested. However, the cultivars did not show any differences in symptom rating in response to the culture filtrate of the Opelousas isolate. A similar trend was observed at other dilutions. Tracy M and Asgrow 5474, which are resistant and moderately resistant, respectively, to stem canker in the field (2,3), had significantly higher symptom ratings than the other cultivars when treated with the toxic culture filtrates (Table 4).

Stability of the toxin. The dilution end point of the culture filtrates subjected to heating was 1:160 at all temperatures, except for that of the autoclaved toxin, which was reduced to 1:40. However, symptoms produced by the culture filtrate at the 1:10 dilution were different in samples heated to 95 C or autoclaved. Interveinal chlorosis and necrosis were not produced by these samples, but the discoloration of the midrib and veins and the production of lesions were similar to symptoms caused by samples heated to other temperatures. The dilution end point at pH 5 was 1:160, the same as that of the original culture filtrate, which had a pH of 7.6–7.9. At pH 3.0 and pH 9.0 the dilution end point was reduced to 1:40. Control solutions consisting of uninoculated PDB with pH adjusted in a similar manner did not produce any discoloration or chlorosis.

Characteristics of the toxin. Dialysis with distilled water for 24 hr removed all detectable toxic components from the culture

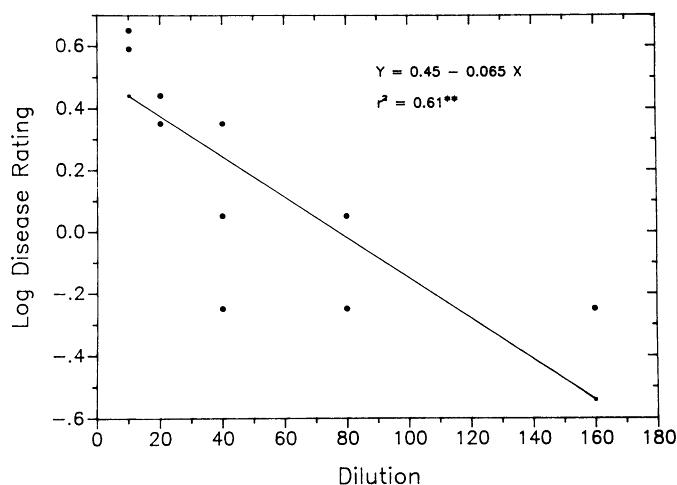

Fig. 1. Relationship between the dose of the toxin produced by *Diaporthe phaseolorum* var. *caulivora* and disease severity in excised soybean trifoliates. Cut ends of excised trifoliates were dipped in dilutions of a culture filtrate (dilution series of 1:10, 1:20, 1:40, 1:80, and 1:160), and symptoms were rated on a 0–5 scale (0 = no symptoms; 5 = 100% of the midrib and veins discolored, forming a fine red network in the lamina, interveinal chlorosis, and 76–100% of the petiole discolored) after 36 hr. The data points at each dilution represent replications. The experiment was conducted with the Opelousas isolate and was repeated at least twice with similar results.

TABLE 3. Reaction of the soybean cultivar Ring Around 606 to *Diaporthe phaseolorum* var. *caulivora* and culture filtrates of four isolates of the fungus

	Culture filtrate[x]		Fungal inoculation
Isolate	Dilution end point	Symptom rating[y]	Canker length[z]
Opelousas	160	3.83 a	22.1 a
St. Joseph	20	0.98 b	5.4 b
Slaughter	20	1.83 b	12.2 b
Lawtell	20	1.66 b	11.4 b

[x]Culture filtrate diluted to 1:10.

[y]Symptoms rated on a 0–5 scale 36 hr after treatment of trifoliates (0 = no symptoms; 5 = midrib and all veins discolored, forming a fine network, interveinal chlorosis, and 76–100% of the petiole showing reddish lesions). Ratings followed by the same letter are not significantly different ($LSD_{0.05}$ = 1.48).

[z]Length of canker measured 4 wk after inoculation. Measurements followed by the same letter are not significantly different ($LSD_{0.05}$ = 9.39).

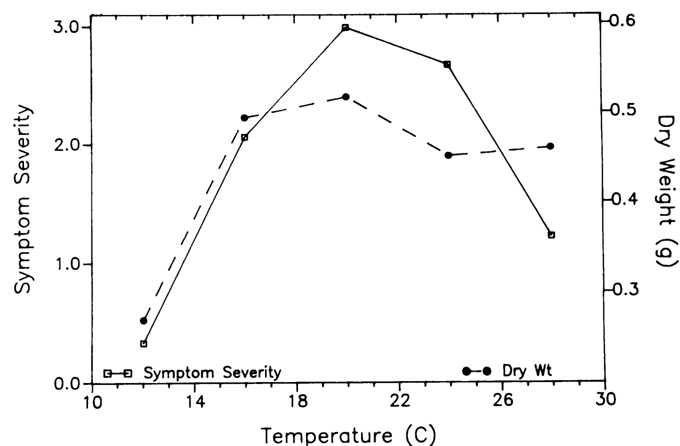

Fig. 2. Effect of incubation temperature on the growth of *Diaporthe phaseolorum* var. *caulivora* and toxin production. The amount of toxin produced was measured in terms of symptom severity rated on a 0–5 scale (0 = no symptoms; 5 = 100% of the midrib and veins discolored, forming a fine red network in the lamina, interveinal chlorosis, and 76–100% of the petiole discolored) 36 hr after treatment of soybean trifoliates with a 1:10 dilution of a culture filtrate. Dry weight was obtained by drying the mycelium at 45 C for 3 days. The experiment was conducted with the Opelousas isolate and was repeated twice with similar results.

filtrate, indicating that the molecular weight of the compound is less than 1,000. Ethyl acetate extracted most of the toxic component from the culture filtrate, and the residue obtained by drying the extract produced symptoms typical of stem canker. When the crude extract was separated by TLC, three major compounds appeared as purple quenched spots under UV light. All three spots reacted with ethanolic sulfuric acid (brown). The compounds also reacted with phosphomolybdic acid (bluish green), alkaline potassium permanganate (yellow), neutral potassium permanganate (yellow), Pauly's reagent (yellow), Folin-Ciocalteu reagent (bluish gray), and acidic ferric chloride (blue). No reaction was seen with either ninhydrin or iodoplatinate, indicating that the toxin is not a protein and does not have a primary or secondary amine (Table 2). When each spot was eluted and assayed, the majority of the toxic activity was detected in the spot with R_f 0.4. The purified compound migrated as a single spot in TLC with various solvent systems. No other spot was detected when the compound was sprayed with various reagents. The fractions eluted from the column and the purified compound produced lesions on petioles, a discoloration of midribs and veins, and interveinal chlorosis; control plants did not exhibit symptoms. The toxin produced reddish brown lesions in 2–3 days on stems, petioles, and midribs when applied to a puncture at the node of intact plants. Lesions formed both above and below the point of toxin application. The dried TLC-purified toxin was redissolved in acetone, and small aliquots of the solution containing the required amount of the compound were assayed by the leaf puncture technique. The minimum amount of toxin required to produce reddish veinal discoloration in 36 hr was 4 μg. The UV spectrum in methanol showed maximum absorption at 210 nm, and the IR spectrum showed peaks at 3,339, 2,928, 2,855, 1,711, 1,662, and 1,599 nm (Fig. 3).

TABLE 4. Response of soybean cultivars to culture filtrates of four isolates of *Diaporthe phaseolorum* var. *caulivora*

	Cultivar[x]					
	Tracy M (R)	Asgrow 5474 (MR)	Davis (MR)	Bragg (S)	Ring Around 606 (MS)	LS mean[y]
Opelousas isolate	4.50	4.50[z]	4.50	4.00	3.50	4.20 a
Lawtell isolate	3.83	3.75[z]	1.66	1.00	1.66	3.05 b
Slaughter isolate	3.83	4.25	2.00	2.33	2.16	2.91 b
St. Joseph isolate	3.83	3.00	2.83	3.25	2.33	2.38 c
LS mean[y]	4.00 a	3.80 a	2.75 b	2.64 b	2.41 b	

[x]Symptoms were rated on a 0–5 scale 36 hr after treatment of the trifoliates with a 1:10 dilution of the culture filtrate (0 = no symptoms; 5 = midrib and veins discolored, forming a fine network, interveinal chlorosis, and 76–100% of the petiole discolored). Reaction of cultivars to stem canker: R = resistant; MR = moderately resistant; MS = moderately susceptible; S = susceptible.

[y]LS = least squares. LS means followed by the same letter are not significantly different ($P = 0.05$) according to least significant difference tests performed on individual least squares means.

[z]Standard error 0.40. For all other isolate-cultivar interactions, the standard error is 0.32.

Reaction of soybean to *Phomopsis* toxins. No symptoms were produced by 100 μg of cytochalasin H or cytochalasin B after 72 hr. The toxin from *Phomopsis* sp. isolated from wilted pine trees, however, produced a red discoloration of the midrib and veins after 72 hr. The intensity of the discoloration was very low. In comparison, 100 μg of the *D. p. caulivora* toxin produced severe discoloration in 24–36 hr (Table 5).

DISCUSSION

D. p. caulivora produces a toxin in vitro that caused symptoms typical of stem canker on excised trifoliates and intact plants. The severity of symptoms was linearly related to the toxin dose, which may indicate a role in symptom production. The variation in disease rating and dilution end point in the two experiments (Tables 3 and 4) is probably due to differences in the capacity of the

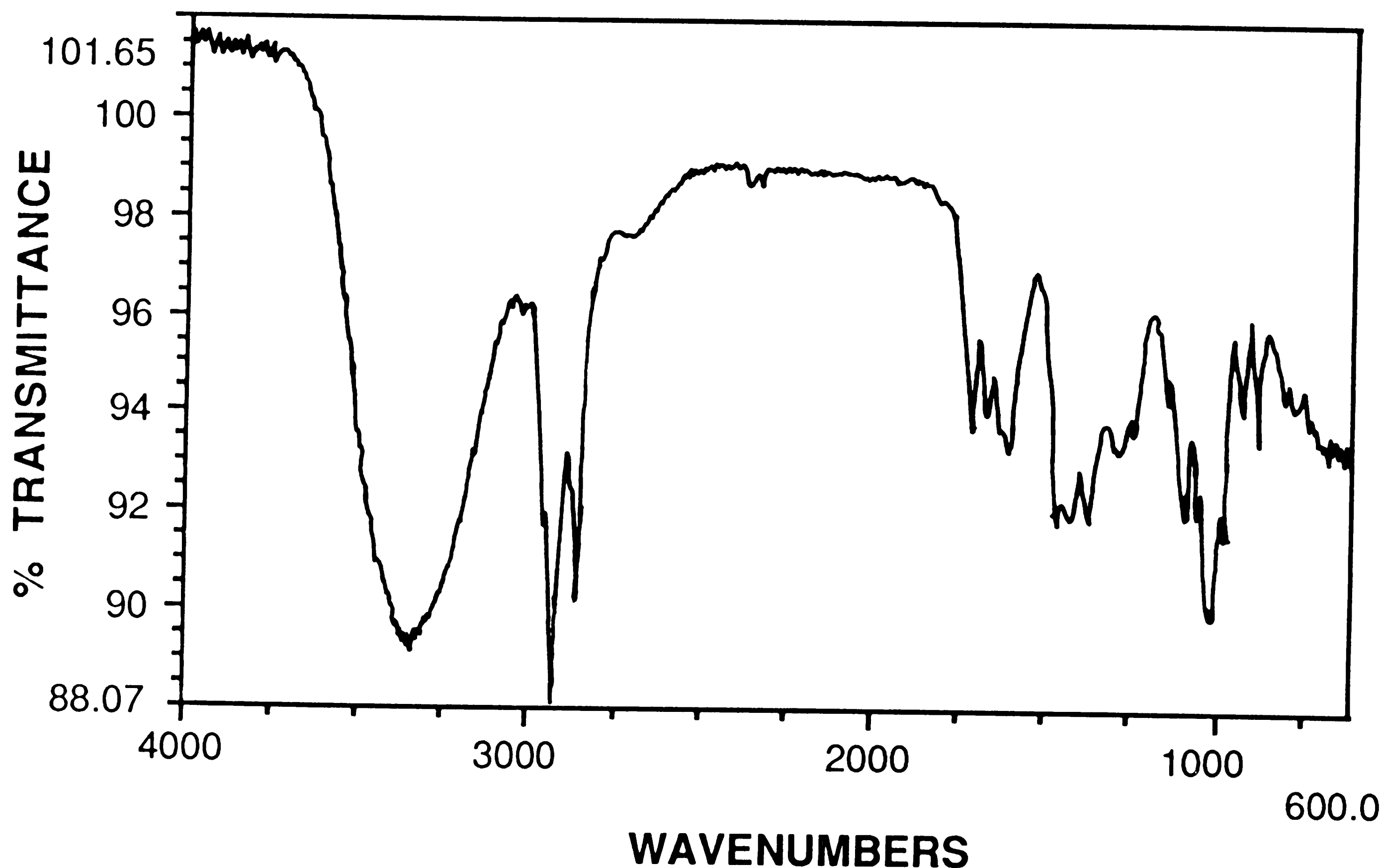

Fig. 3. Infrared spectrum of purified toxic metabolite produced by *Diaporthe phaseolorum* var. *caulivora*.

trifoliates to translocate the toxin. Assay by the uptake of toxin solution by petioles, though not very efficient and sensitive, does provide valuable information and worked satisfactorily. The two commonly used assays, which are the inhibition of seed germination and the increase in electrolyte leakage, could not be used in this study. Preliminary studies using the culture filtrates did not induce electrolyte leakage of soybean trifoliates or petioles and did not inhibit seed germination. However, a reddish blue discoloration was observed when the seed was treated with the partially purified toxin. A more sensitive assay based on the site of action or viability tests using protoplasts (5) would probably resolve the differences more effectively. The minimum amount of toxic metabolite required for symptom production by the leaf puncture assay was 4 μg. It was concluded that the toxin acts on the vascular system, since inoculation of any part of the lamina produced symptoms on the midrib and veins before any chlorosis was seen. The reproduction of many of the disease symptoms by the culture filtrate and also by the partially and completely purified toxic metabolite, though not an indication of a specific role of the toxin in disease, does suggest a role in symptom production.

The correlation between the amount of toxin and the length of cankers produced by the isolates is also evidence for the involvement of the toxin in symptom expression. A similar correlation of virulence with the quantity of toxin produced has been reported in other cases (19). However, a nontoxigenic isolate of *D. p. caulivora*, if shown to be nonpathogenic, would give evidence that might determine the role of the toxin as a pathogenicity or virulence factor. The sensitivity of lima bean to the toxin gives an indication of specificity, because lima bean is also a host of *D. p. caulivora* (9). *D. phaseolorum* is also reported to cause fruit rot of pepper and tomato (9), but both were insensitive to the *D. p. caulivora* toxin. Eggplant, which has been reported to be a host of *D. vexans* (9), was also insensitive to the toxin. However, for a toxin to be host-specific, varieties susceptible to the fungus should also be sensitive to the toxin, and in addition, the toxin or its metabolite should be produced in the host in quantities sufficient to cause symptoms.

In preliminary studies, resistant and susceptible soybean cultivars reacted similarly to treatment with 1:10 dilutions of the culture filtrates. Reaction to the toxin did not reflect the resistance of the cultivar to the pathogen. Tracy M, which has been reported to be highly resistant to the pathogen (21), developed severe symptoms when treated with 1:10 dilutions of culture filtrates of all isolates and was as sensitive as the susceptible cultivar Bragg to the Opelousas culture filtrate. Similar trends were observed for other dilutions. Davis, which is moderately resistant in the field, was not significantly different from the susceptible cultivar Bragg or the moderately susceptible RA 606 in its sensitivity to the culture filtrates of all four isolates. One possibility for the lack of correlation of the effects produced by *D. p. caulivora* and the toxin is that the toxin may not be a pathogenicity factor. Another possibility is based on earlier reports of the physiologic specialization of *D. p. caulivora* isolates. Significant differences were observed between some cultivars treated with culture filtrates from the St. Joseph, Slaughter, and Lawtell isolates, but the differences did not correspond to their reaction to the fungus. The differences in the reaction may be due to physiologic differences in the isolates, as observed in the case of Tracy M, which was reported

TABLE 5. Reaction of soybean trifoliates of the cultivar Ring Around 606 to toxic metabolites

Metabolite[x]	Symptoms[y]	Organism
Cytochalasin B	−	*Phomopsis* sp.
Cytochalasin H	−	*Phomopsis* sp.
Phomopsis toxin[z]	+	*Phomopsis* sp. from pine wilt
Diaporthe phaseolorum var. *caulivora* toxin	+++	*D. p. caulivora* from soybean

[x] 100 μg of compound tested by leaf puncture technique.
[y] Symptoms appear as red discoloration of midrib and veins: − = no symptoms; + = slight discoloration; +++ = intense discoloration.
[z] 6α,7β,9α-Trihydroxy-18(14),15-isopimaradiene-20,6γ-lactone.

to be susceptible to one pathogenic isolate from Iowa, resistant to isolates from Mississippi (7), but susceptible to some isolates from Mississippi (11). However, these results do not give any conclusive evidence on the role of the toxin as a pathogenicity factor. Further studies using a larger number of isolates, including a nonpathogenic isolate, and cultivars simultaneously inoculated with the fungus might lead to conclusions on the role of the toxin in the disease.

The role of a toxin in stem canker expression may explain many of the observations of the disease in nature. The presence of the fungus in symptomless plants could be explained by a nontoxigenic isolate, and conversely, the presence of symptoms without an association with the fungus could be due to the translocation of the metabolite ahead of the fungus. The stem canker pathogen in the southern United States has been reported to be more aggressive than the pathogen in the midwestern United States (1). Recent reports also suggest that there are differences in the pathogens causing stem canker in the midwestern and the southeastern states (1,8,15). The involvement of a toxin in the disease could explain these differences in terms of the varying toxigenic potential of the pathogen.

Structural information on the toxin is necessary for mode-of-action studies and also to relate the presence of the toxin in vivo. The purification procedure was satisfactory for obtaining quantities sufficient for experimental studies. Though there are no reports of the production of a toxic metabolite by *D. p. caulivora*, the imperfect stage, *Phomopsis* sp., produces a class of compounds known as cytochalasins (17,22). These compounds, however, have not been implicated in plant diseases. Purified cytochalasin H and cytochalasin B were assayed on soybean trifoliates, and no symptoms resembling those of stem canker were observed.

The passage of the metabolite through the dialysis membrane indicates a low molecular weight, and the solubility and mobility of the compound in polar solvents suggest a lipophilic nature. The color reaction to spray reagents indicates that the toxic metabolite could have a reducing nature (phosphomolybdic acid and alkaline potassium permanganate), aromatic carboxylic groups (alkaline potassium permanganate), one or more phenolic groups (Pauly's reagent and Folin-Ciocalteu reagent), hydroxy groups (ferric chloride), and a heterocyclic ring (Pauly's reagent) (13). However, the absence of nitrogen and a nonproteinaceous nature are indicated by the negative reactions with iodoplatinate and ninhydrin, respectively (13). The presence of one or more $-OH$ groups is also indicated by the peak at 3,339 nm; $-CH_3$ groups, by the peaks at 2,928 and 2,855 nm; and C=C, by the peaks at 1,711, 1,662, and 1,599 nm. A species of *Phomopsis* isolated from wilted pine trees produces a metabolite that causes a reddish discoloration of pine callus (17). The chemical properties of the *D. p. caulivora* toxin, which include the maximum absorbance in the UV spectrum, color reaction to spray reagents, and the presence of $-OH$ and $-CH_3$ groups, resemble those of the *Phomopsis* toxin. The reddish discoloration of the midrib and veins and lesions on the petiole caused by the *Phomopsis* toxin after 72 hr at about 10 times the concentration of the *D. p. caulivora* toxin indicate a lower activity than that of the *D. p. caulivora* toxin but the possibility of a relationship. There are reports of closely related molecules that show differences in biological activity. *Alternaria tenuis* produces dihydroxytentoxin, which has two fewer protons than tentoxin and activity at 100 μg/ml, compared with 0.4–0.8 μg/ml for tentoxin (4). Further characterization of the *D. p. caulivora* toxin is in progress, with an improved extraction procedure to obtain the metabolite in larger quantities.

Our results indicate that a toxin may be involved in stem canker of soybean. This may answer questions about the sudden increase of stem canker in the southern United States, which is probably due to the appearance of toxigenic strains. The variation in the severity of stem canker may be due to variation in the occurrence of toxigenic strains of *D. p. caulivora*. Many outbreaks of epidemics involved toxins (23). In a preliminary study (*unpublished*), negligible amounts of the toxin were found in culture filtrates of *D. p. caulivora* isolates from Iowa. This observation in

conjunction with reports of the greater aggressiveness of the southern isolates (1) may indicate that the increased severity of the disease in the southeastern states could be caused by a toxin. The *D. p. caulivora* toxin may be related to the toxin produced by *Phomopsis* sp. Further studies on the possible role of a toxin in the disease are in progress. Toxins acting as pathogenicity factors are required for pathogenesis and hence can be used to select for plants with a high degree of resistance. However, toxins acting as virulence factors enhance the severity of symptoms produced by the causal organism and may be used to select for lower levels of resistance (23). The information regarding the presence and involvement of a phytotoxin in the host-parasite interaction may be helpful in the development of breeding programs to select for disease resistance on the basis of the reaction of the host to the toxin.

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