Toxic Culture Filtrates Produced by *Calonectria ilicicola*, Causal Agent of Red Crown Rot of Soybean

K.D. Kim,[1,2] J.S. Russin,[1,3] J.P. Snow[1] and K.E. Damann, Jr.[1]

Eleven soybean cultivars with different levels of susceptibility to virulent isolate SG915 of *Calonectria ilicicola* were examined for reaction to metabolites produced by the isolate. When the culture filtrate from isolate SG915 was applied to trifoliates from 11 cultivars, cvs. 'Cajun' and 'Asgrow 7986' exhibited reduced wilting severity. However, there was no correlation between sensitivity to culture filtrate and susceptibility to the fungal isolate. Wilting severity on cv. 'Riverside 699' was greatest when trifoliates were treated with culture filtrates from isolates SG915 (highly virulent) and C31 (less virulent). The dilution end-point for culture filtrates of virulent isolate SG915 was determined to be 1:8. Nonautoclaved culture filtrates caused complete wilt of soybean trifoliates after 36 h, but autoclaved culture filtrates demonstrated a reduced ability to wilt leaves. Electrolyte leakage from treated leaf tissues increased over time regardless of the concentrations of culture filtrate tested. The greatest electrolyte losses were observed during the initial 30 min incubation of leaf tissues. The highest concentration of culture filtrate (50%, v/v) induced more electrolyte loss than the low concentration (10%, v/v) or control. These results suggest that toxic metabolites of *C. ilicicola* may be involved in disease development with leaf symptom expression.

KEY WORDS: *Calonectria crotalariae; C. ilicicola*; red crown rot; soybean; toxin.

INTRODUCTION

Red crown rot of soybean (*Glycine max* (L.) Merr.) is caused by the soilborne fungus *Calonectria ilicicola* Boedijn & Reitsma [anamorph: *Cylindrocladium parasiticum* Crous, Wingfield & Alfenas (6), syn. *C. crotalariae* (Loos) Bell & Sobers (1)]. This disease was first found in the United States in 1972 (17), and in Louisiana in 1976 (3). Yield losses of soybean caused by the fungus have been difficult to determine, but Berner *et al.* (4) predicted a 50% yield loss for affected fields. Roy *et al.* (18) reported the disease on soybean in Mississippi and estimated yield losses to be 25–30% in affected fields.

The symptoms of red crown rot include leaf chlorosis, interveinal necrosis, defoliation, and wilting (2). Roots are discolored and stems show reddish discoloration alone or in conjunction with reddish-orange perithecia. Reddish discoloration and perithecia generally are restricted to stems, up to 8–10 cm above the soil line. Leaf symptoms along with these stem symptoms appear frequently during late growth stages (R_3-R_4) (9). *C. ilicicola* was not isolated from symptomatic leaf tissues in our repeated attempts, suggesting the

Received Jan. 26, 2000; revised ms. received Oct. 19, 2000; http://www.phytoparasitica.org posting Jan. 25, 2001.

[1]Dept. of Plant Pathology and Crop Physiology, Louisiana State University, Baton Rouge, LA 70803, USA [2,3]Present addresses: [2]Dept. of Agricultural Biology, Korea University, Seoul 136-701, Korea [Fax: +82-2-9251970; e-mail: kidkim@korea.ac.kr]; and [3]Dept. of Plant, Soil and General Agriculture, Southern Illinois University, Carbondale, IL 62901, USA.

involvement of toxic fungal metabolite(s) in disease development. Involvement of toxic metabolites produced by *C. ilicicola* in Cylindrocladium black rot of peanut was also suspected by several researchers who observed necrosis on hypocotyls (11) and tap roots (10) prior to fungal invasion.

Toxins produced by plant pathogens are involved, to various degrees, in pathogenesis in numerous host–parasite systems (15,21,23). The role of toxins in pathogenesis has been divided by Yoder (24) into 'pathogenicity factors' and 'virulence factors'. Toxins that are required for inducing disease are classified as pathogenicity factors, whereas toxins whose level of production relates to virulence are termed virulence factors. This system works well to identify host-selective toxins such as those produced by *Helminthosporium victoriae* on oats, *H. sacchari* on sugarcane, and *Alternaria kikuchiana* on Japanese pear (21). In these systems, disease symptom development is induced entirely by the toxin. However, the system fails to classify the toxin produced by *Diaporthe phaseolorum* var. *caulivora*, causal agent of stem canker of soybean (14). This fungus produces a toxin which induces foliar symptoms but apparently plays no direct role in the canker production on soybean stems (14). A second approach classifies toxins as primary determinants necessary to produce disease and secondary determinants which are not required for disease development (7,22). The toxin produced by *D. phaseolorum* var. *caulivora* may fit well into the latter category.

We reported previously that isolates of *C. ilicicola* exhibit a wide range of virulence on soybean (12). In those studies, virulence of *C. ilicicola* was expressed as the ability to induce not only stem lesions but also chlorosis and wilting of leaves far removed from the site of the fungal infection. Therefore, this study was conducted to examine the possible involvement and role of a toxic fungal metabolite(s) in the *C. ilicicola*–soybean system.

MATERIALS AND METHODS

Soybean cultivars, culture filtrates, and trifoliate bioassays Susceptibility of 11 soybean cultivars (Table 1) to virulent isolate SG915 of *C. ilicicola* was determined previously in greenhouse tests (13). Cultivars such as 'Riverside 699', 'Hartz 7126' and 'Riverside 677' were highly susceptible, whereas 'Forrest', 'Cajun', 'Braxton' and 'Asgrow 7986' were less susceptible; cvs. 'Bedford', 'Deltapine 726', 'Centennial' and 'Hartz 6200' had intermediate susceptibility (13).

An agar disc containing mycelium (5 mm in diameter) from the actively growing edge of a culture (1 week old) of *C. ilicicola* was transferred to 50 ml of potato dextrose broth (PDB, Difco Laboratories, Detroit, MI, USA) in a 250-ml Erlenmeyer flask. The inoculated flasks were incubated, without shaking, in darkness for 4 weeks at 25°C. Mycelial mats were removed by filtration. Cell-free culture filtrates were obtained after the medium was passed through 0.2-μm Nalgene filters (Nalge Company, Rochester, NY, USA) under vacuum. Culture filtrates were stored at -15°C. Dry weights of mycelia from each flask were determined after 3 days at 60°C.

The same 11 cultivars (Table 1) were examined for reaction to culture filtrate produced by virulent isolate SG915 of *C. ilicicola*. Plants of each cultivar were grown for 6 weeks in a greenhouse prior to use. Completely expanded second trifoliates from the top of each plant were cut under water using a razor blade. Half-strength culture filtrates (50%, v/v) in PDB diluted with sterile distilled water were used throughout this bioassay. Sterile distilled water and half-strength PDB served as controls. Preliminary results indicated that undiluted

PDB was phytotoxic to soybean after 48 h but that half-strength PDB was not (unpublished results). The cut petiole (5 cm in length) immediately was immersed in 4.5 ml of culture filtrate or control liquids in a vial (45 × 15 mm) under fluorescent light (16 h/day) at room temperature (25°C). Wilt reactions of trifoliates were rated 36 h after treatment, as follows: 0=no wilting; 1=leaves flaccid; 2=slightly wilted leaves; 3=completely wilted leaves. This experiment was conducted twice with five replicates each.

Fungal isolates and bioassays Virulence of nine isolates (Table 2) of *C. ilicicola* was determined in a previous study on six soybean cultivars, Asgrow 7986, Braxton, Cajun, Centennial, Forrest and Hartz 7126, using *in vitro* inoculation (12). Isolates such as SG915, BH2 and 2PN exhibited high virulence, whereas S44, J2 and S44 had low virulence, and isolates 323, BH1 and C31 were moderately virulent (12).

Susceptible cv. Riverside 699 was used to test the reaction to culture filtrates of the nine isolates (Table 2) of *C. ilicicola*. Culture filtrates of these nine isolates were prepared and dry weights of mycelia were determined as described above. Plants were grown in a greenhouse, and procedures for production of trifoliates, bioassay, and wilt evaluation were as described above. This experiment was conducted twice with four replicates each.

Dilution and stability of culture filtrates Culture filtrates from virulent isolate SG915 of *C. ilicicola* on PDB were diluted with sterile distilled water to concentrations of 50, 25, 12.5, 6.25 and 0% (v/v), or not diluted. Similar concentrations of PDB served as controls. These culture filtrate concentrations were tested against trifoliates of susceptible cv. Riverside 699. Wilt reactions of trifoliates were rated 36 h after treatment, as described previously. This experiment was conducted twice with four replicates for each treatment.

Half-strength culture filtrates from virulent isolate SG915 on PDB were autoclaved (121°C, 1.05 kg/cm^2) for 15 min and cooled to room temperature (25°C). Autoclaved culture filtrates were bioassayed against trifoliates from susceptible cv. Riverside 699 to test for heat stability of the toxic fungal metabolites. Half-strength PDB and sterilized distilled water were used for controls. Wilt reactions of trifoliates were rated 36 h after treatment, as described previously. This experiment was conducted twice with five and four replicates, respectively.

Electrolyte leakage Electrolyte leakage from leaf tissues was determined as described previously (8). Soybean cvs. Cajun (less susceptible) and Riverside 699 (highly susceptible), were grown for 6 weeks in a greenhouse as described in the trifoliate assay. Leaf tissue (1 g) from a completely expanded second trifoliate from the top of each plant was placed in cheesecloth and soaked in deionized water. The tissue samples, after removal of excess water, were placed in beakers containing 100 ml of culture filtrates (10% and 50%, v/v) from virulent isolate SG915 or deionized water, and incubated for 1 h on a shaker (170 rpm) at room temperature (25°C). After incubation, these tissues were washed three times with deionized water (200 ml). The tissue samples were then placed in 50 ml of deionized water (<1 μmho) and incubated on a shaker (170 rpm) for 4 h at room temperature (25°C). Conductance of the sample solution was measured at 0, 0.5, 1, 2, 3 and 4 h after incubation using a conductivity meter (Model 31, Yellow Springs Instrument Co., Inc., Yellow Springs, OH, USA). This experiment was conducted twice with three replicates of each treatment.

Experimental design and analysis of data All tests in this study were established in a completely random design. Statistical analyses were conducted with pooled data from

repeated experiments using the Statistical Analysis System (20) when no significant difference was found at P=0.05 between the tests. Analysis of variance was conducted using the general linear models procedure and means were separated using the least significant difference (LSD). Relationships among variables were examined using the correlation procedure.

RESULTS

A range of wilt responses (Table 1) was recognized among the cultivars when tested against culture filtrates from *C. ilicicola* isolate SG915 in trifoliate assays (Fig.1). Cvs. Cajun and Asgrow 7986 exhibited reduced wilting severity (Table 1). However, disease susceptibility and wilting severity among all tested cultivars did not correlate in tests 1 (r=0.15, P=0.27) and 2 (r=0.03, P=0.83).

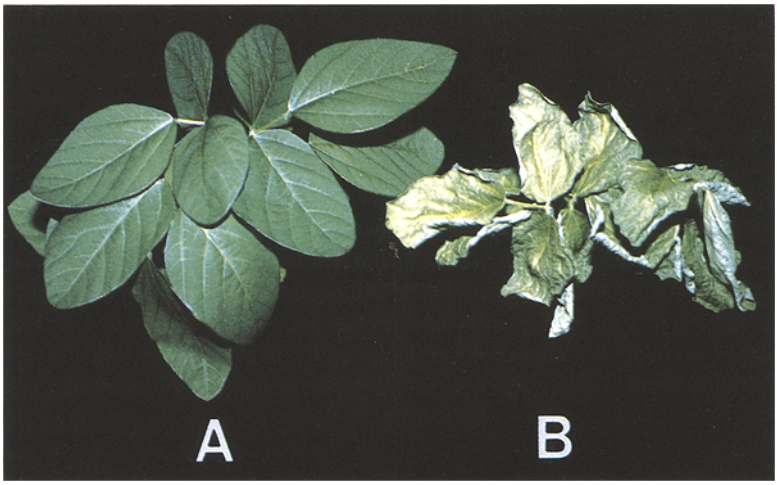

Fig. 1. Healthy (A) and wilted (B) trifoliates of highly susceptible soybean cv. Riverside 699 at 36 h after treatment with half-strength potato dextrose broth (PDB) and half-strength culture filtrates from virulent isolate SG915 of *Calonectria ilicicola* grown on PDB for 4 weeks, respectively. No trifoliate wilting was observed after treatment with sterile distilled water.

Wilting severity on susceptible cv. Riverside 699 was greatest when trifoliates were treated with culture filtrates from isolates SG915 (highly virulent) and C31 (less virulent) (Table 2). Isolates SG915, BH2 and S38 produced a high level of mycelial weight while the others had less mycelial growth (Table 2). Virulence and wilting severity among all tested isolates did not correlate (r=0.21, P=0.08). Wilting severity among isolates against susceptible cv. Riverside 699 also did not correlate with dry mycelial weights of *C. ilicicola* isolates (r=0.23, P=0.09).

Soybean (cv. Riverside 699) trifoliate wilting severity increased as the concentration of culture filtrate from SG915 increased (Fig. 2). Slightly wilted or flaccid leaves of trifoliates appeared at lower concentrations, but complete wilting occurred at higher concentrations ($\geq$50%). The dilution end-point for culture filtrates was determined to be 1:8 (12.5% culture filtrates, v/v) (Fig. 2). No wilting was observed using half-strength PDB or sterile distilled water.

TABLE 1. Wilting severity of trifoliates of 11 soybean cultivars with treatment of half-strength culture filtrates from virulent isolate SG915 of *Calonectria ilicicola* grown on potato dextrose broth for 4 weeks

Cultivar	Reaction to fungus[z]	Wilting severity[y]	
		Test 1	Test 2
Riverside 699	3.9	3.0	3.0
Hartz 7126	3.5	2.6	2.6
Riverside 677	3.3	3.0	3.0
Bedford	3.0	3.0	3.0
Deltapine 726	3.0	2.8	3.0
Centennial	2.9	3.0	2.4
Hartz 6200	2.6	3.0	3.0
Asgrow 7986	2.2	2.8	1.0
Braxton	2.1	3.0	2.8
Cajun	2.0	1.4	1.8
Forrest	2.0	3.0	2.4
$LSD_{0.05}$		0.6	0.6

[z]Reaction of soybean cultivars to fungus, expressed as disease severity caused by SG915 of *C. ilicicola*, was determined previously on a scale of 0 (no visible symptoms) to 5 (dead plants) in greenhouse tests (13).

[y]Wilting was evaluated on a scale of 0 (no wilting) to 3 (complete wilting of leaves) 36 h after treatment of trifoliate leaves. Values are means of five observations.

TABLE 2. Mycelial weight and wilting severity of trifoliates of soybean cv. Riverside 699 with treatment of half-strength culture filtrates from nine isolates of *Calonectria ilicicola* grown on potato dextrose broth for 4 weeks

Isolate[z]	Wilting severity[y]	Mycelial weight (mg)[x]
SG915	2.9	405
BH2	1.8	403
2PN	1.1	365
323	1.6	368
BH1	0.3	368
C31	2.5	357
S38	1.6	398
J2	2.0	362
S44	0.8	373
$LSD_{0.05}$	0.7	16

[z]Virulence of isolates expressed as disease severity caused by the isolates of *C. ilicicola* was determined previously on six soybean cultivars on a scale of 0 (no visible symptoms) to 5 (dead plants) in *in vitro* seedling tests (12). Virulence of isolates was found to be as follows: SG915=3.4, BH2=3.3, 2PN=2.8, 323=1.3, BH1=1.0, C31=0.6, S38=0.3, J2=0.1, and S44=0.1 (12).

[y]Wilting was evaluated on a scale of 0 (no wilting) to 3 (complete wilting of leaves) 36 h after treatment of trifoliate leaves. Values are means of eight observations.

[x]Values are means of six observations.

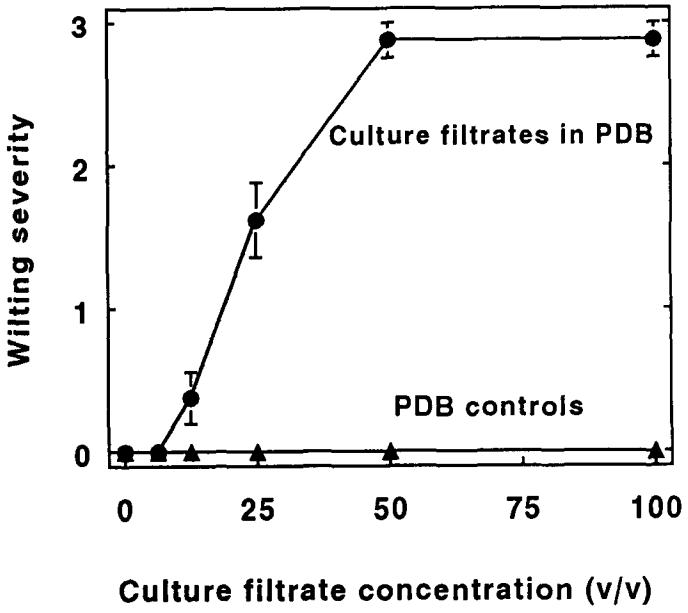

Fig. 2. Trifoliate wilting in highly susceptible soybean cv. Riverside 699 produced by various concentrations (0, 6.25, 12.5, 25, 50 and 100%, v/v) of culture filtrates from virulent isolate SG915 of *Calonectria ilicicola* grown on potato dextrose broth (PDB) for 4 weeks. Wilting was evaluated on a scale of 0 (no wilting) to 3 (complete wilting of leaves) 36 h after treatment. Values are means of eight observations; bars = SEM.

Autoclaved culture filtrates of virulent isolate SG915 exhibited a significantly (P=0.05) reduced ability to wilt leaves of highly susceptible cv. Riverside 699 (mean wilt severity = 2.0 and 1.2 for tests 1 and 2, respectively), compared with nonautoclaved culture filtrates (mean wilt severity = 3.0 and 2.8 for tests 1 and 2, respectively). Culture filtrate that was not autoclaved caused complete wilt of soybean trifoliates after 36 h. Wilting was not observed with half-strength PDB or sterile distilled water.

Electrolyte leakage from leaf tissues of less susceptible cv. Cajun and highly susceptible cv. Riverside 699 increased over time regardless of the concentrations of culture filtrate from isolate SG915 (Fig. 3). The greatest electrolyte losses were observed during the initial 30 min incubation of leaf tissues. The highest concentration of culture filtrates (50%, v/v) induced significantly (P<0.001) more electrolyte loss than the low concentration (10%, v/v) and control in the cultivars over time (Fig. 3). No differences in electrolyte loss between the two cultivars were observed over time with concentrations of 50% (P = 0.83) and 10% (P = 0.07). However, significant differences (P = 0.004) in the electrolyte losses over time were found between two cultivars in the controls (0% culture filtrates).

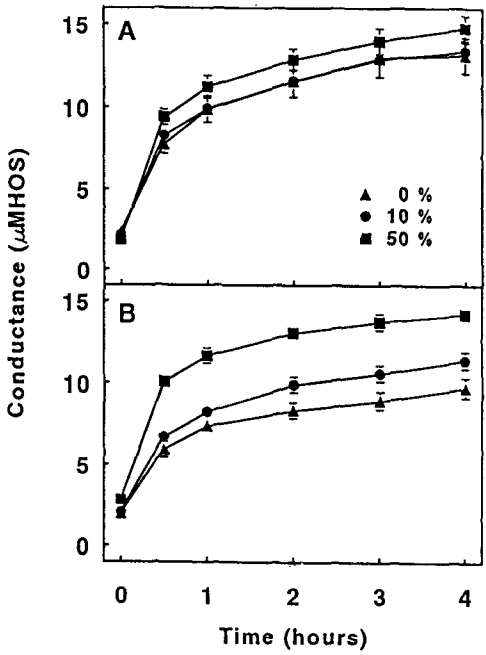

Fig. 3. Rates of electrolyte loss over time from highly susceptible (A, cv. Riverside 699) and less susceptible (B, cv. Cajun) soybean leaves induced by various concentrations (0, 10 and 50%, v/v) of culture filtrates from virulent isolate SG915 of *Calonectria ilicicola* grown on potato dextrose broth for 4 weeks. Values are means of six observations; bars = SEM.

DISCUSSION

Toxins produced by fungal plant pathogens are considered one of the determinants in disease development (21). However, responses of plants to such toxins sometimes are not related to virulence of their pathogens, but rather may result from other determinant factors such as metabolites and enzymes involved in the host–parasite systems (21). Nevertheless, a search for the role of a toxin in disease development is still useful for identifying a factor for pathogenicity or virulence. Although toxin-related symptoms on leaves in the *C. ilicicola*–soybean system were suspected, there have been no reports of involvement of toxin in red crown rot of soybean. However, several authors have proposed the involvement of toxic metabolites produced by *C. ilicicola* in pathogenesis (10,11). In histological studies, necrosis on peanut hypocotyls (11) and tap roots (10) was observed prior to fungal invasion, and consequently toxic metabolites were suspected.

In this study we evaluated the role of metabolites produced by *C. ilicicola in vitro* to elucidate disease development on soybean. The results suggested the presence of toxic metabolite(s) in culture filtrate of *C. ilicicola*, capable of inducing electrolyte loss in soybean leaf tissue. The phenomenon of damage in host cells by toxins has been documented in other host–parasite systems (5). These compounds were produced on PDB but not on the semiselective medium of Phipps *et al.* (16 and our unpublished data). The lack of correlation between wilting severity produced by isolates and their dry mycelial weights

in a liquid medium also indicates that the toxin production by individual isolates may not be related to the growth of the isolates in the medium. Evidently, production of toxic metabolites was affected by media composition, a phenomenon which has been observed in other studies (19). Because disease symptoms caused by the toxic metabolites occur on leaves of soybean, a trifoliate assay with half-strength culture filtrates on PDB was adopted in the current study.

In the present work, the general lack of correlation between sensitivity to *C. ilicicola* culture filtrates and disease reaction of soybean cultivars indicates that this toxin may not fit into either 'pathogenicity factors' or 'virulence factors' (24). Daly (7) proposed that toxins can be secondary determinants of virulence – which are not critically important to produce disease, or as primary determinants which are necessary to produce disease (22). A toxin which acts as a secondary determinant of virulence is that produced by *D. phaseolorum* var. *caulivora*, causal fungus of stem canker of soybean. Lalitha *et al.* (14) tested culture filtrates from four isolates of *D. phaseolorum* var. *caulivora* on five soybean cultivars that exhibited different levels of disease susceptibility in the field. They found that the toxin was not associated with susceptibility to the pathogen in the field (14). In the present study, susceptibility of soybean cultivars to *C. ilicicola* was not correlated with sensitivity to culture filtrates and virulence of isolates of *C. ilicicola* was not correlated with production of toxic metabolites *in vitro*. These results suggest that the toxic metabolites produced by *C. ilicicola* are secondary determinants of virulence and are not critical for disease development.

The present work showed that toxic metabolites of *C. ilicicola* may be responsible, at least in part, for leaf symptom development. However, trifoliate assays did not reproduce the typical interveinal chlorosis and necrosis which frequently are observed in the field. Rudolph (19) suggested that most toxins will not act alone but rather in combination with other products of the pathogen, such as metabolites and enzymes. This may explain the incomplete production of disease symptoms in the assay as well as the limitations for detecting fungal toxin with *in vitro* assays. Further studies focusing on host range tests, purification, and characterization of the metabolites will be needed.

ACKNOWLEDGMENT

This research was supported in part by the Louisiana Soybean and Small Grain Research and Promotion Board. Approved for publication by the Director of the Louisiana Agricultural Experiment Station as manuscript 00-38-0634.

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