Optimizing Conditions of a Cell-Free Toxic Filtrate Stem Cutting Assay to Evaluate Soybean Genotype Responses to *Fusarium* Species that Cause Sudden Death Syndrome

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Abstract

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Cell-free toxic culture filtrates from *Fusarium virguliforme*, the causal fungus of soybean sudden death syndrome (SDS), cause foliar symptoms on soybean stem cuttings similar to those obtained from root inoculations in whole plants and those observed in production fields. The objectives of this study were to (i) optimize the production conditions for *F. virguliforme* cell-free toxic culture filtrates and the incubation conditions of the stem cutting assay used to test the toxicity of the cell-free toxic culture filtrates, and (ii) use the optimized assay and a whole plant root inoculation assay to compare four SDS-causing isolates on a panel of selected

soybean genotypes. Area under the disease progress curve (AUDPC) values were highest ($P = 0.05$) when cuttings were immersed in culture filtrate of fungus grown in soybean dextrose broth, in filtrate produced from the fungus grown for 18 or 22 days, and when stem cuttings were incubated at 30°C. AUDPC values and shoot dry weights from the whole plant root inoculations and the AUDPC values from the stem cutting assay differed ($P < 0.05$) among nine soybean genotypes tested with *F. virguliforme* and *F. tucumaniae* isolates, and the AUDPC values from the two assays were positively correlated ($r = 0.44$ at $P < 0.0001$).

Fusarium virguliforme O'Donnell & T. Aoki causes sudden death syndrome (SDS) on soybean [*Glycine max* (L.) Merr.] (2). Sudden death syndrome was first observed in Arkansas in 1971, and by the 1980s and 1990s, SDS was reported in most soybean-producing states throughout the United States (21,22). In addition, *F. brasiliense*, *F. crassiseptatum*, *F. cuneirostrum*, and *F. tucumaniae* have been reported to cause SDS in South America along with *F. virguliforme* (2–4). Yield losses from SDS range from slight to nearly 100% on individual plants and are dependent on plant age at disease onset and on disease severity. Infected plants often have increased flower and pod abortion, reduced seed size, and prematurely defoliate (21–23).

Foliar symptoms observed in the field are most readily apparent in the mid to late reproductive growth stages. The leaves show scattered interveinal chlorotic spots, which may expand and become necrotic. Later, leaves become necrotic with only the midvein and major lateral veins remaining green. Severely affected leaflets detach from the petioles, while the petioles remain attached to the stem. Infected plants are easily pulled from the ground (21).

Phytotoxins are mostly low molecular weight secondary metabolites, which interfere with plant metabolism or cause plant cell death (5). Many organisms including bacteria, fungi, and insects produce phytotoxins, with phytopathogenic fungi being the largest group of phytotoxin producers (8). Phytotoxins produced by *Fusarium* species include fumonisins, fusaric acid, moniliformin, and trichothecenes (1,17), but these toxins have not been reported in cell-free toxic filtrates of *F. virguliforme* (6,13).

The components of the cell-free toxic filtrates of *F. virguliforme* have been partially characterized. The amino-terminal sequence of 15 amino acids was determined for a phytotoxic polypeptide identified in culture filtrates of *F. virguliforme*; the purified protein with an estimated molecular weight of 17 kDa caused browning of soybean calli (13). Recently, a protein of approximately 13.5 kDa, named FvToxin1, was purified from the culture filtrates of *F. virguliforme* and shown to induce chlorosis and necrosis in soybean leaves (6).

Different bioassays have been developed to assess the toxicity of *F. virguliforme* culture filtrates. One of the first reports showed that cell-free culture filtrate of *F. virguliforme* was toxic to calli of 14 soybean cultivars, causing them to turn brown, and there was a significant positive correlation between calli browning and SDS severity obtained from inoculated plants (14). Another bioassay used soybean cell suspension cultures and a viability stain to assess toxicity of cell-free culture filtrate of *F. virguliforme* (15). In the same study, when a soybean stem cutting assay was used as another method to test toxicity of cell-free culture filtrates of *F. virguliforme*, plants developed marginal leaf curling, mottling, and mosaic, and later interveinal chlorosis and necrosis of foliage (15) similar to leaf symptoms on infected whole plants. A significant positive correlation was reported between soybean foliar symptom severity and the percentage of cell death in soybean suspension-culture (16).

A variety of approaches have been used to increase phytotoxin production by fungi in culture, and a recent review detailed how altering nutrient or pH composition of growth media, aeration, and light and temperature conditions during growth affected phytotoxin production (5). For example, the quantity of fusaric acid produced by *F. oxysporum* f. sp. *lycopersici* was shown to be greater when the fungus was grown in 100 g/liter potato infusion and 10 g/liter sucrose compared with other concentrations of potato infusion and sucrose (19). In addition to the type of medium, aeration and light conditions also affected phytotoxin production. Optimized conditions for *Mycosphaerella fijiensis* phytotoxin production occurred when the fungus was grown in V8 medium in shake cultures at a 12:12 h photoperiod compared with other media, aeration, and photoperiod conditions (20). Phytotoxin production of *F. virguliforme*

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isolates may be similarly affected by environmental conditions. Cell-free culture filtrates of *F. virguliforme* originating from cultures grown at 15, 20, and 25°C caused greater foliar disease severity on soybean stem cuttings than cell-free culture filtrates prepared from fungal cultures grown at 30°C (9). In the same study, cell-free culture filtrates from cultures grown on modified Septoria medium (MSM) and Czapek Dox broth resulted in greater foliar disease severity than culture filtrates produced in potato dextrose broth (PDB) (9). Another study, which did not measure toxicity of cell-free culture filtrates, found a differential growth response among of 18 isolates of *F. virguliforme* tested for their carbon source utilization profiles (28).

Using cell-free culture filtrates of *F. virguliforme* in a stem cutting assay could be an improved or alternative method to evaluate soybean genotypes for resistance, and also would provide the highly regulated environmental conditions that are often needed to detect small quantitative differences in disease severity. For an effective assay, the isolate should be cultured in conditions that favor toxin production and the stem cutting assay itself should be conducted under conditions that favor development of foliar symptoms. Specifically, conditions that increase toxicity of cell-free culture filtrates of *F. virguliforme* and the development of foliar symptoms in leaves of stem cuttings need further optimization to determine if changes in conditions alter the effectiveness of the assay.

The goal of this research was to determine if a filtrate assay could be optimized for toxin production and then used to compare species of *Fusarium* on soybean genotypes for the purpose of characterizing isolates as well as evaluating reaction of genotypes. The objectives of this study were to (i) optimize the production conditions for *F. virguliforme* cell-free toxic culture filtrates and the incubation conditions of the stem cuttings assay used to test the toxicity of the cell-free toxic culture filtrates, and (ii) to use the optimized assay and a whole plant root inoculation assay to compare four SDS-causing isolates on a panel of selected soybean genotypes as well as to compare the assay methods. We are not aware of any reports of testing *F. tucumaniae* culture filtrates for toxicity; however, it may be presumed that filtrates of *F. tucumaniae* contain toxin(s) similar to those found in filtrates of *F. virguliforme* since root inoculation tests of *F. tucumaniae* and *F. virguliforme* produce similar foliar symptoms (24).

Materials and Methods

A series of nine experiments were completed and all independently repeated once with new randomizations to address the objectives. Experiments 1 to 7 were stem cutting assays, which altered the growing conditions of *F. virguliforme* in liquid culture or incubation conditions of the stem cutting assay (Table 1). Experiments 8 and 9 used root inoculation of whole plants and the optimized stem cutting assay, respectively, to evaluate the foliar response of soybean genotypes from Argentina and the United States to isolates of *F. virguliforme* and *F. tucumaniae* and to compare the assay methods.

Preparation of plant material and fungal isolates for optimizing foliar symptoms in the stem cutting assay. Seeds of an SDS-susceptible soybean cultivar, Spencer (10), were sown in Sunshine Mix LC1 (Sun Gro Horticulture, Canada) in 27 × 54 × 6 cm flats at a rate of 72 seeds per flat. Seedlings were grown in a growth chamber at 30°C under a 12-h photoperiod at a light intensity of 436 $\mu\text{E}/\text{m}^2/\text{s}$ and watered daily. Soybean plants with fully expanded trifoliolate leaves (2- to 3-week-old plants) were selected for the assay. Mont-1 isolate of *F. virguliforme* was used in all the experiments based on its performance in previous culture filtrate assay studies (9,11,16). The fungus was grown on potato dextrose agar (PDA) in the dark at 25°C in a growth chamber for 2 weeks before use.

Preparation of cell-free culture filtrates and stem cutting assays. Soy dextrose broth (SDB) was used to culture the isolate, as previously reported (29), to produce cell-free culture filtrates for all experiments except experiment 8 where plants were inoculated with the fungus.

Five 4-mm diameter mycelial plugs from the outer edge of 2-week-old cultures grown on PDA were transferred into SDB and incubated at 25°C in the dark for 14 days or incubated under treatment conditions described for altering fungal growth. At 14 days or the duration outlined in the treatment regime, the liquid culture medium

was filtered through miracloth (Chicopee Mills, Inc., Milltown, NJ) and 0.22 μm Millipore membrane to make cell-free culture filtrate, which was stored at 4°C if not used immediately.

For each stem cutting assay, cell-free culture filtrate was diluted 1:25 with sterilized distilled water. The diluted culture filtrate was decanted (40 ml) into 50-ml centrifuge tubes (11 cm long × 3 cm diameter) and covered with Parafilm to prevent evaporation. Plant stems were cut with a sharp razor blade between the soil line and the first node. Cotyledons and unifoliolate leaves were removed and the stem cuttings with first trifoliolates were placed through a small slit in the Parafilm into tubes containing the diluted cell-free culture filtrate or a control of 1:25 SDB.

Alteration of growing conditions for *F. virguliforme* in liquid culture. Experiments 1 to 5 altered media, lighting, duration of incubation, aeration, and temperature when growing *F. virguliforme* for subsequent testing of toxicity of cell-free culture filtrate using the stem cutting assay. Filtrates obtained from the fungus grown under varying conditions in each experiment were then tested concurrently in a stem cutting assay as previously described. Treatments in experiments 1 to 5 were arranged in a randomized complete block design (RCBD) with three blocks using a total of nine cut seedlings per treatment. The stem cutting assays for experiments 1 and 2 were conducted at 25°C with a light intensity of 436 $\mu\text{E}/\text{m}^2/\text{s}$ and a 14-h photoperiod; experiments 3 through 5 were performed at 30°C with a light intensity of 203 $\mu\text{E}/\text{m}^2/\text{s}$ and a 12-h photoperiod.

Two growth media, PDB and SDB, were compared in experiment 1. PDB was prepared by adding 24 g of dehydrated PDB (Difco Laboratories, Sparks, MD) to 1 liter of distilled water, and SDB was prepared as described (29). The fungus was grown in and cell-free filtrate was prepared from each medium as previously described.

Two lighting conditions were tested in experiment 2. The fungus was grown under a light intensity of 436 $\mu\text{E}/\text{m}^2/\text{s}$, for 12 h per day or in the dark, in flasks completely covered with aluminum foil.

Five incubation periods were tested in experiment 3. Cell-free culture filtrates were collected at 6, 10, 14, 18, and 22 days and were kept at 4°C until used.

Two aeration conditions were tested in experiment 4. Fungal cultures were incubated in anaerobic or aerobic conditions. For the anaerobic treatment, five 4-mm diameter mycelial plugs from the outer edge of 2-week-old cultures grown on PDA were transferred into SDB in 250-ml glass bottles with air-proof screw caps (Schott North America, Inc., Elmsford, NY) and bottles were treated inside an O_2 control glove box as described by the manufacturer (Coy Manufacturing, Grass Lake, MI). The atmosphere inside the O_2 control glove box was composed of 90% nitrogen, 5% hydrogen, and 5% carbon dioxide. For the aerobic treatment, cell-free cultures were prepared as previously described.

Four temperature conditions were tested in experiment 5. The fungus was grown at 15, 20, 25, and 30°C.

Alteration of the stem cutting assay conditions. Experiments 6 and 7 altered the incubation conditions of the stem cutting assay to investigate the effect of temperature and photoperiod. For both experiments, the cell-free culture filtrate was prepared and a stem cutting assay was conducted as previously described. Experiment 6 incubated the stem cuttings at 15, 20, 25, and 30°C under a 12-h photoperiod, while experiment 7 used photoperiods of 4, 8, 12, or 16 h per day at 30°C. Both experiments were performed under a light intensity of 203 $\mu\text{E}/\text{m}^2/\text{s}$ and, and were arranged in a completely randomized design (CRD) using four cut seedlings per each of four replications.

Preparation of plant material, fungal isolates, and inoculum for comparing whole plant root inoculations and the stem cutting assay. Nine soybean genotypes were selected for experiments 8 and 9 based on geographic origin and level of susceptibility to SDS in previous tests: 299 N, DM3100, DM4870, DM3070, DM9800, Essex, Forrest, Spencer, and PI567374. The DM (Don Mario Seeds, Chacabuco, Argentina) genotypes and the remaining soybean genotypes have been extensively tested in a number of different studies, with PI567374 often designated as a resistant check (10,11).

Four fungal isolates, Mont-1 and M5 of *F. virguliforme* and Puj and 8-1 of *F. tucumaniae* were selected for the two experiments.

Mont-1 was obtained from the Laboratory for Soybean Disease Research at the University of Illinois and the remaining isolates were received from the Laboratorio Agrícola Río Paraná, San Pedro, Buenos Aires, Argentina, in 2012 under a restricted use APHIS permit (Table 1). Isolates were subcultured on PDA and incubated at 25°C in the dark for 2 weeks before use.

Sorghum grain from a commercial elevator in Teutopolis, IL, was used to make fungal inoculum for each isolate. Dry seeds (2.5 liters) were soaked overnight in tap water. Floating seeds and debris were removed and the grain was washed three times. Excess water was drained and 500 ml of clean sorghum seed was added to each of five 1-liter flasks. The mouth of each flask was blocked with a foam plug and wrapped with aluminum foil. The sorghum seeds were autoclaved for 40 min at 121°C, left to cool overnight, and then autoclaved and cooled again similarly. The next day, cultures of each isolate growing on 9-cm-diameter culture plates were sliced and approximately five 1-cm² square pieces were added to the sterile sorghum seeds in each flask. The flasks were incubated at room temperature (24°C) in ambient light for 14 days. The inoculum was used fresh. Based on observations of toxicity to soybean of autoclaved sorghum (unpublished), noninoculated, nonautoclaved sorghum seeds were used as control.

Evaluation of nine soybean genotypes with four isolates using a whole plant root inoculation assay. Experiment 8 tested nine soybean genotypes against the four fungal isolates (Table 1) in a whole plant root inoculation assay in the greenhouse following previously reported methodology (10,15). Soybean seeds of the nine genotypes were sown in 3.8 × 21 cm Cone-Tainers (Ray Leach Cone-Tainers, #SC10, Hummert International, Earth City, MO) in a double-steamed soil mix consisting of equal parts soil, quartz sand, and soil-less mix (Sunshine Mix, LC1, Sun Gro Horticulture Inc., Bellevue, WA). Five cubic centimeters of the inoculum or control were placed 2 cm below the soybean seed. Treatments were arranged in a factorial (genotype × isolate) in a CRD in Cone-Tainer racks with five replications (each plant was an experiment unit its one Cone-Tainer) for each isolate × genotype combination treatment. Racks were placed on the same greenhouse bench under supplemental lighting using a 12-h photoperiod with a light intensity of 436 µE/m²/s, day and night temperatures of 25 ± 1°C and 20 ± 1°C, respectively, and watered daily. Four weeks after planting, all plants were cut at the soil line to collect shoots. Shoots were dried for 48 h at 38°C and then weighed. The shoot weight of each plant was calculated as a percentage of the noninoculated control plant.

In addition, plants of genotypes Essex and PI567374, noninoculated and inoculated with M5 and Puj for all five replications, were soaked in tap water for 1 h, uprooted, and washed to remove soil. Roots were dried for 48 h at 38°C and then weighed. The dry root weight of each plant was calculated as a percentage of the noninoculated control plant for each of five replications.

Evaluation of nine soybean genotypes with four isolates using a stem cutting assay. Experiment 9 tested the response of nine soybean genotypes for toxicity to cell-free culture filtrate of four fungal isolates (Table 2) in a stem cutting assay. Stem cuttings from each of the nine genotypes were tested in cell-free culture filtrate produced from each of the four isolates and in one SDB-only control. Isolates were grown, filtrate was produced, stem cuttings were obtained, and

the assay was conducted as previously described in the section on preparation of cell-free culture filtrates and stem cutting assays. The tubes with cut stems were incubated in a growth chamber set at 30 ± 1°C under 12-h photoperiod with a light intensity of 436 µE/m²/s. Treatments were arranged in a factorial (genotype × isolate) in a CRD. Each treatment was replicated three times in one trial, and the experiment was repeated once.

Foliar ratings. The following 1 to 8 rating scale applied to all experiments: 1 = no symptoms; 2 = 1 to 9% foliage affected (appearance of yellow blotches); 3 = 10 to 29% foliage affected (interveinal chlorosis); 4 = 30 to 49% foliage affected (interveinal chlorosis and necrosis with leaf cupping); 5 = 50 to 79% foliage affected (interveinal chlorosis and necrosis with necrosis along the entire margin of cupped leaves); 6 = 80 to 90% foliage affected (interveinal necrosis with only green main vein); 7 = 91 to 99% foliage affected (interveinal necrosis and defoliation); and 8 = 100% defoliation with stem only. In experiments 1 to 7, plants were observed for 7 to 10 days and rated from three to five times for each experiment. Experiments 8 and 9 were rated, respectively, 14, 21, and 28 days after planting and 5, 9, and 13 days after the stems were immersed in the culture filtrate.

Data analysis. Data from repeated experiments were combined if error variances were homogeneous in Bartlett's unequal variance test and there were no interactions between trials and treatments. The area under the disease progress curves (AUDPC) were calculated using disease severity ratings recorded over time (25). The AUDPC values, shoot dry weight, and root dry weight were analyzed using ANOVA with the aid of JMP 9.0 (JMP 9.0.2, SAS Institute, Cary, NC). If the model was significant, the means were compared and separated by Student's *t* test at *P* = 0.05.

Correlation coefficients between the following variables were calculated: AUDPC values and shoot dry weights from the inoculation of whole plants in the greenhouse (experiment 8); AUDPC values from the inoculation of whole plants in the greenhouse and AUDPC values from the stem cutting assay (experiments 8 and 9); and AUDPC, shoot dry weights, and root dry weights of genotypes PI567374 and Essex inoculated with isolates M5 and Puj from the seedling assay (experiment 8). Pairwise correlation were calculated at *P* = 0.05.

Results

The calculated AUDPC values for trials within each experiment were combined based on the test of equal variances (*P* > 0.05) and the lack of a trial × treatment interaction (*P* > 0.05).

Alteration of growing conditions for *F. virguliforme* in liquid culture. There was a significant (*P* < 0.0001) effect of media on AUDPC values (Table 1). Higher (*P* = 0.05) AUDPC values were recorded when using filtrate from fungus grown in SDB (AUDPC = 23) compared with that grown in PDB (AUDPC = 13) (Table 3).

There was no significant (*P* > 0.05) effect of light on AUDPC values (AUDPC = 27) when the fungus was grown in 12 h of light or in the dark.

There was a significant (*P* < 0.0001) effect of incubation duration on AUDPC values (Table 1). Mean separation showed that higher (*P* = 0.05) AUDPC values occurred when the fungus was grown for 18 and 22 days compared with when it was grown for 14 and 6 days, and that AUDPC when the fungus was grown for 10 days did not differ from when the fungus was grown for 22 days (Table 3).

Table 1. Significance of media, incubation period, and temperature on the area under disease progress curve (AUDPC) values for foliar severity of sudden death syndrome in stem cutting assays

Experiment	Treatment	df	<i>P</i> > <i>F</i>
Fungal growth conditions			
1. Media	Potato or soymilk dextrose broth	1	<0.0001
2. Light	0 or 12 h per day	1	NS
3. Incubation period	6, 10, 14, 18, or 22 days	4	<0.0001
4. Aeration	Aerobic or anaerobic	1	NS
5. Temperature	15, 20, 25, or 30°C	3	NS
Stem cutting assay conditions			
6. Temperature	15, 20, 25, or 30°C	3	0.0004
7. Photoperiod	4, 8, 12, or 16 h per day	3	0.008

Table 2. Isolate of *Fusarium* species and their geographic origins evaluated for pathogenicity and cell-free culture filtrate toxicity

Species	NRRL ^z	Isolates	Geographic origin	Source
<i>F. virguliforme</i>	34552	M5	Argentina, Santa Fe, Serodino	(24)
<i>F. virguliforme</i>	22292	Mont-1	Illinois, U.S.	(15)
<i>F. tucumaniae</i>	34547	8-1	Argentina, Santa Fe, Las Parejas	(24)
<i>F. tucumaniae</i>	34550	Puj	Argentina, Santa Fe, Pujato	(24)

^z NRRL = The Agriculture Research Service Culture Collection, National Center for Agricultural Utilization Research, USDA/ARS, Peoria, IL.

AUDPC values on stem cuttings were not significantly different ($P > 0.05$) when the fungus was grown in anaerobic or aerobic conditions (AUDPC = 26), or when the fungus was grown at four temperatures (Table 1).

Alteration of the stem cutting assay conditions. AUDPC was significantly affected ($P < 0.0004$) by incubation temperature during the stem cutting assay (Table 1). Mean separation showed that higher ($P = 0.05$) AUDPC values occurred on stem cuttings in culture filtrates incubated at 30°C than on those incubated at 25°C, and on stem cuttings incubated at 25°C than on those incubated at 15 and 20°C (Table 3).

There was a significant ($P < 0.008$) effect of photoperiod on AUDPC values (Table 1). Mean separation showed that higher ($P = 0.05$) AUDPC values occurred on stem cuttings that were exposed to 8-, 12-, and 16-h photoperiods compared with stem cuttings that were exposed to 4 h of light (Table 3).

Evaluation of nine soybean genotypes and four isolates using a whole plant root inoculation assay. Soybean seedlings started to show SDS foliar symptoms 2 weeks after planting. There was no significant ($P > 0.05$) genotype $\times$ isolate interaction for the AUDPC values, but there were main effect differences among the nine genotypes ($P < 0.02$) and among the four isolates ($P < 0.0001$) (Table 4). Essex had an AUDPC value of 28, which was higher ($P = 0.05$) than Forrest, DM9800, PI567374, and 4870 (Table 5). *F. virguliforme*

isolates M5 and Mont-1 and *F. tucumaniae* isolate Puj caused higher ($P = 0.05$) AUDPC values than *F. tucumaniae* isolate 8-1.

There was a significant ($P < 0.004$) genotype $\times$ isolate interaction (Table 4) for shoot dry weight which, as a percentage of the noninoculated control, ranged from 8 to 59% (Fig. 1). The percentage shoot dry weight for PI567374, the resistant check, was 59% when inoculated with Mont-1 and this did not differ ($P = 0.05$) from 43, 40, and 40% when inoculated with M5, 8-1, and Puj, respectively (Fig. 1). Soybean genotype 3100, which had the lowest value for percentage shoot dry weight, 8% with isolate M5, was not significantly ($P = 0.05$) different at 12, 14, and 17% when inoculated with Puj, 8-1, and Mont-1, respectively. The percentage shoot dry weight for Spencer, the susceptible check, was not significantly ($P = 0.05$) different from genotype 3100 for two of the isolates, M5 and Mont-1, and was not significantly different from the resistant check PI 567374 for isolate Puj.

Subsamples of two genotypes, Essex and PI567374, noninoculated and inoculated with M5 and Puj, showed no significant ($P > 0.05$) difference in percentage of noninoculated control plant root dry weight values between genotypes, isolates, or their interaction.

Evaluation of nine soybean genotypes and four isolates with the stem cutting assay. There was no significant ($P > 0.05$) genotype $\times$ isolate interaction while there were significant differences among isolates and genotypes ($P < 0.0001$) (Table 4). M5 and Mont-1 had the highest AUDPC values ($P = 0.05$); 8-1 and Puj were lower (Table 5). Among the nine genotypes, PI567374 had the lowest ($P = 0.05$) AUDPC value compared with all the other genotypes, but was not significantly different from DM9800 (Table 5). Genotype 3100 had the highest AUDPC, which differed ($P = 0.05$) from DM9800, PI567374, and 4870, that were all lower.

Correlations. AUDPC values in the whole plant root inoculation assay were negatively correlated ($r = -0.63$, $P < 0.0001$) with shoot dry weight but positively correlated with AUDPC values from the stem cutting assay ($r = 0.44$, $P < 0.0001$). For the subsamples of the two genotypes inoculated with either Puj or M5, root dry weight was negatively correlated with AUDPC ($r = -0.36$, $P = 0.00271$), while it was positively correlated with shoot dry weight ($r = 0.84$, $P < 0.0001$).

Discussion

The analysis of cell-free toxic filtrates from plant pathogens, including defining and isolating the active components of the filtrate and determining the structure of the toxin(s), is important for understanding the mechanisms of toxin activity in plants. A modified Septoria medium (MSM) was used to produce cell-free culture filtrate of *F. virguliforme* in previous studies (9,14,16,26). Our preliminary experiments using MSM showed that soybean stem cuttings immersed in cell-free toxic filtrate of the fungus grown in SDB produced significantly more disease symptoms than those grown in MSM. In this study, foliar disease severity ratings of stem cuttings immersed in culture filtrates of the fungus grown in SDB were higher than severity ratings for cuttings immersed in PDB, and higher when

Table 3. Mean area under disease progress curve (AUDPC) values of foliar disease severity ratings on soybean cultivar Spencer stem cuttings immersed in cell-free culture filtrates

Type	Experiment	AUDPC ^z
Fungal growth conditions	Media	
	Potato dextrose broth	13 b
	Soy dextrose broth	23 a
Fungal growth conditions	Incubation period (days)	
	6	12 d
	10	21 bc
	14	18 c
	18	26 a
Stem cutting assay conditions	22	22 ab
	Temperature (°C)	
	15	5 c
	20	6 c
	25	8 b
Stem cutting assay conditions	30	11 a
	Photoperiod (h per day)	
	4	8 b
	8	10 a
	12	10 a
	16	11 a

^z Means within each experiment were separated by Student's *t* test at $P = 0.05$, and means followed by the same letter are not significantly different.

Table 4. Analysis of variance for the area under disease progress curves (AUDPC) and shoot dry weight for the seedling assay, and AUDPC for the stem cutting assay, evaluated on nine soybean genotypes with *F. virguliforme* and *F. tucumaniae*

Experiment	Variable	Source of variation	df	$P > F$
Seedling assay ^x	AUDPC	Genotype	8	0.0173
		Isolate	3	<0.0001
		Genotype $\times$ isolate	24	0.1792
	Shoot dry weight ^z	Genotype	8	<0.0001
		Isolate	3	0.0422
		Genotype $\times$ isolate	24	0.0042
Stem cutting assay ^y	AUDPC	Genotype	8	<0.0001
		Isolate	3	<0.0001
		Genotype $\times$ isolate	24	0.0907

^x Analysis based on two trials each with 5 replications using sorghum inoculum in Cone-Tainers under greenhouse conditions (experiment 8).

^y Analysis based on two trials each with 3 replications using cell-free culture filtrates in the growth chamber (experiment 9).

^z The shoot dry weight of each plant was calculated as the percentage of the noninoculated control plant.

the fungus was grown for 18 and 22 days compared with when grown for fewer days.

A previous study (9) reported that altering light conditions for fungal growth did not change the toxicity of cell-free culture filtrates based on the soybean stem cutting assay. However, in our study, there was an effect of photoperiod during the stem cutting assay on disease severity, with photoperiods longer than 8 h resulting in more severe SDS foliar symptoms than at a 4-h photoperiod. The presence of light is essential for SDS symptom development, and it has been shown that SDS symptoms are due to the programmed cell death initiated by degradation of the RuBisCo (ribulose-1,5-bisphosphate carboxylase oxygenase) large subunit in diseased leaves (12). Similarly, light was shown to be important for the FvTox1 to induce necrosis symptoms, presumably by causing the production of free radicals that interrupt photosynthesis (6). Along with the presence or duration

of light being important for symptom development, it is likely that light quality would be important, although this has not been thoroughly studied.

SDS symptom severity was greater when stem cuttings were incubated at 30°C than at lower temperatures; fungal growth at 15, 20, 25, or 30°C did not alter the toxicity of cell-free culture filtrates in our study. Although transpiration rates were not measured, their interaction with temperature and humidity may be a key factor in foliar symptom severity. How these in vitro experiments that alter factors like photoperiod and temperature relate to SDS development in the field is not clear. Additional research is needed to determine if light quality or quantity interact with temperature and/or humidity to cause an increase in foliar field symptoms.

When *F. virguliforme* was grown in aerobic and anaerobic conditions in our study, the toxicity of cell-free culture filtrate was not

Table 5. Area under disease progress curves (AUDPC) of nine soybean genotypes inoculated with four *Fusarium* isolates in a whole plant root inoculation assay using sorghum inoculum in Cone-Tainers under greenhouse conditions and in a stem cutting assay using cell-free culture filtrates in the growth chamber

Genotype ^w	AUDPC		Isolate ^z	AUDPC	
	Whole plant ^x	Stem cutting ^y		Whole plant ^x	Stem cutting ^y
Essex	28 a	11 a-c	Mont-1	28 a	15 b
DM3100	27 ab	13 a	M5	26 a	17 a
DM3070	24 a-c	11 ab	Puj	25 a	7 d
299N	23 a-d	11 bc	8-1	18 b	9 c
Spencer	22 a-d	11 ab			
Forrest	19 b-d	11 ab			
DM9800	17 cd	9 cd			
PI567374	16 d	7 d			
DM4870	16 d	11 bc			

^w Genotypes Essex, 299 N, Spencer, Forrest, and PI567374 were from the United States; all other genotypes were from Argentina.

^x Means based on main effects of genotype and isolate in two trials each with five replications. Means followed by the same letter are not significantly different.

^y Means based main effects of genotype and isolate two trials each with three replications. Means followed by the same letter are not significantly different ($P = 0.05$).

^z Mont-1 and M5 are *F. virguliforme* (United States and Argentina, respectively), and Puj and 8-1 are *F. tucumaniae* (both from Argentina).

Fig. 1. Shoot dry weight measured as percentage of the noninoculated control of nine soybean genotypes inoculated with four *Fusarium* isolates (Mont-1 and M5 = *F. virguliforme* and Puj and 8-1 = *F. tucumaniae*) based on two trials with five replications each. Means followed by the same letter are not significantly different ($P = 0.05$).

altered. Several studies have reported anaerobic growth of other species, including *F. solani* and *F. oxysporum*, which not only were capable of surviving in nearly complete anaerobic conditions, but which grew at higher rates under these conditions (27). Compared with normal soil aeration, low soil oxygen between 0 and 3% also caused certain dry bean cultivars to be more susceptible to *Fusarium* root rot (*F. solani* f. sp. *phaseoli*) (18). There are no reports about the association of anaerobic growth of fungal pathogens and toxin production, but from our results, it appears that toxins produced by the fungus were not affected by the lack of oxygen, which may or may not be an important factor for SDS occurrence under field conditions.

The primary methods used to evaluate soybean genotypes for SDS resistance have been greenhouse and field-based (7,10,15). Cell-free toxic culture filtrates of *F. virguliforme* have not been extensively used. The stem cutting assay has some advantages, including minimized space and simplified logistical demands, compared with evaluating whole plants in the field or greenhouse. As such, the assay may be carried out in an environmental chamber, eliminating certain variables like photoperiod, temperature, and watering differences that may not remain constant in field or greenhouse assays. The stem cutting assay has the disadvantage of bypassing any potential root resistance, though this is an advantage if only the toxin sensitivity of genotypes is of interest.

This is the first study to test the activity of cell-free culture filtrates produced by *F. tucumaniae*. Both isolates of *F. tucumaniae* produced foliar symptoms in the stem cutting assay, but with lower severity than the two *F. virguliforme* isolates. However, in the whole plant root inoculation assay, one of the *F. tucumaniae* isolates (Puj) produced foliar symptoms equal to the two *F. virguliforme* isolates tested. Since the phytotoxin(s) in the cell-free culture filtrates were not purified, it is not known if the four isolates used in this study were producing the same or equal quantities of phytotoxin(s). It has not been reported that the different species that cause SDS would have a differential reaction to soybean genotypes. We did not observe this in our study that examined two isolates each of *F. tucumaniae* and *F. virguliforme*. More research is needed to determine if all SDS-causing *Fusarium* species produce the same phytotoxins, and if isolates within one species produce the same array of toxins. To evaluate the potential array of toxic metabolites and secretory toxic proteins in a number of isolates, traditional biochemical approaches and analysis of transcriptomes may provide additional information about the toxin array. In addition to the stem cutting bioassay, other approaches may include the use vector expression systems developed to confirm toxicity on soybean plants.

Foliar severity of the nine soybean genotypes tested against two *F. virguliforme* isolates and two *F. tucumaniae* isolates in the whole plant assay was similar to that in the stem cutting assay, indicating that root resistance appears not to be a critical factor in the foliar disease severity ratings for the genotypes used in our study. Although the correlation coefficient value between the two methods in our study was low, the correlation was significant, indicating a positive association between the two assay methods in our study. These results agree with those of a previous study (11), and indicates that foliar severity was dependent on toxin translocation to leaves, whether it originates in infected roots or culture filtrate. The variation between the two assays might be further reduced by the use of growth chambers to achieve stricter environmental controls. More research is needed to determine the specific toxin(s) produced by the other *Fusarium* spp. that have been reported to cause SDS and whether the optimized conditions used here are effective for their evaluation.

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