Phytotoxicity of *Discula destructiva* Culture Filtrates to *Cornus* spp. and the Relationship to Disease Symptomology

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ABSTRACT

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Discula destructiva culture filtrates and partially purified culture filtrates (PPCF) inhibited radish (*Raphanus sativus*) and dogwood (*Cornus*) species in a seedling root bioassay. Noninoculated potato-dextrose broth (PDB) extracted and separated in a similar manner also inhibited seedling growth. Reverse phase high performance liquid chromatography separated this inhibitory activity into two fractions, with one associated with the inhibitory action observed with PDB controls. The active fraction without interference with PDB, determined by bioassays, was extracted from cultures grown on Murashige-Skoog (MS) medium, which had no inhibitory activity associated with noninoculated controls. The active fraction was tested in a leaf overlay technique using 10 *Cornus* spp. All dogwood species were sensitive to the fraction and exhibited necrotic lesions bounded by a red margin, typical of dogwood anthracnose. The active fraction was translocated in *Cornus alba* to the leaf margin. *C. canadensis* showed minimal primary lesion formation but developed leaf curling and necrosis on leaf margins of newly emerging leaves, indicating apical translocation of the fraction from the application site. Comparison of three *D. destructiva* (Type 1) isolates and a *Discula* sp. (Type 2) isolate for production of the active fraction showed that the Type 2 isolate did not produce detectable amounts of the active component.

Additional keyword: toxins

Since dogwood anthracnose was identified in the Northeastern United States (3) and its causal agent, *Discula destructiva*, was described (12), it has caused a serious decline in flowering dogwoods (*Cornus florida*) in the Appalachian mountains of the United States (13,22,23). The severity and impact of this disease has been well documented (4,5). In studies of dogwood anthracnose, two types of fungal isolates were isolated from diseased leaves and twigs. These isolates were distinguished by their ability to utilize gallic acid (17) and by DNA amplification fingerprinting (1,16) and were designated Type 1 and Type 2 (21). Type 1 is *D. destructiva*, the described pathogen. The relevance of the Type 2 isolates is not known.

Elucidation of the pathogenic mechanism of *D. destructiva* in the progression of disease symptoms would contribute significantly to our knowledge of the physiology of this disease. Necrosis of cells preceding hyphal proliferation (19) and identification of four phytotoxic phenols from culture filtrates (18) suggest an association between toxin production and symptom development in dogwood anthracnose. Venkatasubbaiah and Chilton (18) previously isolated phytotoxic phenols from a *Discula* sp. isolated from diseased dogwood tissue. The involvement of these phytotoxins in symptom development has not been investigated and no comparisons have been made between isolates of *D. destructiva* and the unidentified Type 2 *Discula* species.

The objectives of our research were to (i) extract fungal toxins from culture filtrates of *Discula* spp. (*D. destructiva*, Type 1, and *Discula* sp., Type 2), (ii) demonstrate the involvement of fungal toxin production in symptom expression, and (iii) determine the sensitivity of various *Cornus* spp. to toxins.

MATERIALS AND METHODS

Fungal isolates and biomass production. Four *Discula* isolates were obtained from cooperators; Type-1 isolates (*D. destructiva*) from S. D. McElreath, Department of Forest Resources, Clemson Uni-

versity (isolate 100); M. L. Daughtrey, Long Island Horticultural Research Laboratory, Cornell University (isolates 123 and 125); and a Type-2 isolate (*Discula* sp.) from R. L. Anderson, USDA Forest Service, Asheville, NC (isolate 99). Two of these isolates were used in previously published DNA amplification fingerprinting studies of *D. destructiva* and *Discula* sp. Type 2 isolates (1,16). Isolates 99 and 123 were identified as NC-2 and NY-329, respectively, in these studies. Cultures were initiated from mycelial plugs, grown on potato-dextrose agar (PDA) for 21 days at $23 \pm 2^\circ\text{C}$, and transferred to oak sporulation medium (9) for conidial production. Potato-dextrose broth (PDB, 100 ml) was inoculated with 1.0×10^6 conidial suspensions prepared in sterile distilled water for all four isolates. Murashige-Skoog (MS) medium (10) containing 30 g/liter of sucrose was inoculated with isolate 100. Stationary cultures were maintained at $23 \pm 2^\circ\text{C}$ under ambient laboratory lighting ($3 \pm 1 \mu\text{mol}$) with cool white fluorescent lights and 12-h photoperiods. PDB cultures were grown for 3 months and MS cultures were grown for 10 months.

Extract preparation. Extraction and separation procedures were a modification of procedures used to study toxins associated with *Pythium* spp. (6). Cultures were initially filtered through Whatman #2 and #42 filter papers and a GF/F 0.7- μ glass microfibril filter to remove hyphae. Culture filtrates were run through YM100 ($>100,000$ Da) and YM3 ($>3,000$ Da) ultrafiltration membranes (Amicon Inc., Beverly, MA). The final low-molecular-weight filtrate was stored overnight (-80°C) and then lyophilized. Lyophilate was reconstituted with 50% methanol: water (vol/vol), stored overnight at 5°C , filtered to remove precipitates, and rotary evaporated (60°C) under 600 to 700 mm Hg vacuum to dryness. Residue was reconstituted with 80% methanol:water (vol/vol) and final volume was adjusted to 2% (vol/vol) of the original culture filtrate. This partially purified culture filtrate (PPCF) resulted in a 50-fold concentration of toxic metabolites. Samples were obtained at each stage of the purification procedure and evaluated for activity using the radish seedling root bioassay (7,11).

High performance liquid chromatography (HPLC) was used to further separate the PPCF using a Hewlett-Packard 1090

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HPLC (Hewlett-Packard Inc. Avondale, PA) equipped with a diode array detector set at 206, 240, and 254 nm, and reverse phase C₁₈ column (Rexchrom, 3 µ, 10 cm × 4.6 mm i.d.; Regis Chemical Co., Morton Grove, IL). The solvent was 2:98 acetonitrile:water for 10 min, then increased to 15:85 acetonitrile:water over the next 10 min, followed by 100% acetonitrile for 10 min. Flow rates were 0.4 ml/min for 10 min and then 0.5 ml/min for 20 min with 250 µl of sample injected. HPLC fractions were collected at 5-, 1-, and 0.5-min intervals in different analysis runs. Fractions were dried at 50°C, resuspended in HPLC grade water, and subjected to bioassay tests.

In vitro toxin bioassay. Toxicity of *Discula* spp. culture filtrates was tested using modifications of seedling root growth bioassays which have been used extensively as indicators of allelopathic and phytotoxic compounds (7,11,14,20). *Cornus* spp. have very complex dormancy requirements and are not good candidates for seedling bioassays; therefore, seeds from three *Cornus* spp. were compared with radish (*Raphanus sativus*) seeds in a seedling bioassay to determine if a radish seedling bioassay could replace *Cornus* spp. seedlings in initial testing. Seedling root bioassays were conducted in sterilized 90-mm Petri plates containing a piece of Whatman #2 filter paper to which 8 ml of culture filtrate or equivalent control solution (diluted 1:10 with sterile, distilled-deionized water) were added. A total of 10 *Cornus* spp. or radish seeds were added to each plate. Stones from *Cornus* spp. were harvested from stratification medium when cracked naturally by the enlarging embryos. Fleshy embryos were removed by hand and disinfested with 0.525% sodium hypochlorite for 10 min, followed by three sterile, distilled-water rinses. Petri dishes were placed on a countertop under room lighting with cool white fluorescent lights and a 12-h photoperiod at 22 ± 2°C. Root growth was measured 4 days after treatment (DAT) for radish and 7 DAT for *Cor-*

nus spp. A completely randomized design was used for the seedling root bioassay using three Petri plate replicates for each treatment, with each replicate containing 10 seeds.

In vivo bioassay. A modification of the leaf-puncture bioassay (8,15) was used to study the effects of extracts on *Cornus* spp. leaves. Samples obtained from two chromatographic runs of the isolate 100 MS culture filtrate at 23.5 to 24.5 min were used to inoculate *Cornus* spp. MS broth without fungal inoculum and subjected to the same lyophilization, evaporation, reconstitution, and HPLC collection procedures was used as the control. Serial dilutions (1:2, 1:4, and 1:8) of the samples were tested in a dose-response format. Each leaf in the second leaf pair was wounded twice approximately 1 cm apart with a 26-gauge tuberculin needle. The two wound sites on each leaf divided the leaf into approximately three equal portions. Each puncture site was then overlaid with 10 µl of serial-diluted HPLC culture filtrate samples or sterile water. Control plants were inoculated in the same manner using the HPLC-prepared MS control (serial dilutions of 1:2, 1:4, and 1:8) and sterile water.

A total of 10 *Cornus* spp. were grown in GA7 magenta jars (Sigma Chemical Co., St. Louis) containing 80 cm³ of sand and 25 ml of Hoagland's #2 basal salt mixture (Sigma Chemical Co.). Seedlings (6 to 8 cm in height; average seedling age, 2 to 3 months) were used to test the response of *Cornus* spp. to HPLC samples of PPCF. Experiments were repeated twice with three replicates of each species used for the PPCF treatments and one replicate for the control treatment.

Analysis of variance (ANOVA) and the post hoc Tukey's honestly significant difference (Tukey's HSD) were used for analysis, and post hoc procedures were used to test significance of treatment effects. Duncan's multiple-range test was used for comparison of necrotic lesion size

in different *Cornus* spp. Pearson's correlation coefficient (*r*) was used to evaluate the strength of the relationship between species. An overall experiment-wide level of a type 1 error was set at 0.5. Statistical analysis was performed using PC-SAS (release 6.04; SAS Institute Inc., Cary, NC).

RESULTS

Results from *C. racemosa*, *C. amomum*, *C. florida*, and *R. sativus* seedling bioassays using culture filtrates of Type 1 *D. destructiva* (isolates 100, 123, and 125), Type 2 *Discula* sp. (isolate 99), and controls showed a high correlation between the results of *Cornus* spp. and radish root growth bioassays (Table 1). Pearson correlation coefficients (*r*) by species, replicate, and individual seedlings were significant (*P* < 0.01) where *r* for root length by species was *R. sativus* with *C. racemosa* (*r* = 0.987), *C. amomum* (*r* = 0.894), and *C. florida* (*r* = 0.852), respectively. Comparisons of root lengths showed that *C. florida* correlated more closely with radish than with the other dogwood species. The Pearson correlation coefficient for root length by species for *C. florida* was lower with *C. racemosa* (*r* = 0.803) and *C. amomum* (*r* = 0.758) with a lower level of significance (*P* < 0.05). These data demonstrate that the radish seedling bioassay could be used for detection of phytotoxic compounds in culture filtrates of *D. destructiva*. There was significant inhibition in noninoculated PDB controls when compared to water and MS controls. The inhibition was reduced in cultures inoculated with Type 2 *Discula* isolate 99.

Evaluation of *Discula* isolates. Type 1 *D. destructiva* (isolates 100, 123, and 125) were not significantly different from each other but were significantly different from Type 2 *Discula* sp. (isolate 99; Table 1), indicating a significant difference in the toxicity of the Type 2 isolate and the Type 1 *D. destructiva*. Filtrates from isolate 100 grown in MS inhibited root growth significantly when compared to the MS control for both radish and *Cornus* spp.

The PPCF original culture filtrate, membrane retentates, water, and PDB controls were compared utilizing the radish seedling bioassay. Isolate 100 results (Table 2) show a significant growth inhibition of the PPCF when compared with all other separation fractions and controls. The original culture filtrate inhibited root growth significantly compared to the PPCF, indicating some loss of activity during the molecular sieving. These comparisons indicated that the PPCF contained low-molecular-weight compounds (<3,000 Da) that were inhibitory to root growth. Stimulation of root growth was observed in root growth with the retentate from the 3,000-Da ultrafiltration membrane.

Significant inhibitory activity was detected in two fractions, 0 to 5 and 20 to 25

Table 1. Comparison of mean root growth of radish (*Raphanus sativus*), *Cornus racemosa*, and *C. amomum* seedlings exposed to culture filtrates of *Discula destructiva* (Isolates 100, 123, and 125) and *Discula* sp. (Isolate 99) grown on potato-dextrose broth (PDB) and Murashige-Skoog (MS) media following filtration to remove hyphae^y

Filtrate	Root length by species (mm) ^z			
	<i>R. sativus</i>	<i>C. racemosa</i>	<i>C. amomum</i>	<i>C. florida</i>
Water control	41.8 a	20.6 a	14.5 a	16.8 a
MS control	30.1 b	12.0 bc	12.7 a	8.8 ab
PDB control	14.4 c	3.5 d	2.4 b	12.7 a
Isolate 100-PDB	8.4 c	2.7 d	2.4 b	2.9 b
Isolate 100-MS	7.9 c	2.4 d	1.9 b	2.0 b
Isolate 123-PDB	10.9 c	4.7 cd	2.2 b	2.7 b
Isolate 125-PDB	11.9 c	4.1 cd	2.6 b	2.5 b
Isolate 99-PDB	33.3 ab	15.8 ab	11.7 a	12.8 a

^y Mean root length for seedlings is listed for plant species by fungal isolate. Treatment in each experiment consists of three replicate plates with 10 seeds per plate. Results are averages of two experiments.

^z Means within species followed by the same letter are not significantly different using Tukey's honestly significant difference (*P* < 0.05).

min, isolated from HPLC analysis runs of *Discula* culture filtrates grown in MS or PDB medium (Table 3). The PDB control had inhibitory activity associated with the 0- to 5-min fraction. No inhibition was observed in any fraction from the MS control and this fraction of the culture filtrate was eliminated from the further study.

The 20- to 25-min fraction was further separated into five 1-min intervals for use in the root growth bioassay for the comparison of the different isolates (Table 4). Significant differences in activity were demonstrated between Type 1 (*D. destructiva*, isolates 100, 123, and 125) and the Type 2 *Discula* isolate (isolate 99) in the 23- to 25-min fraction ($P < 0.001$). No differences were observed between the three Type 1 isolates (100, 123, and 125). The 23- to 25-min interval was further separated into four 30-s intervals. Mean root inhibition was greatest for roots exposed to the 23.5- to 24.5-min fractions ($P < 0.001$). No differences in growth inhibition among Type 1 isolates (100, 123, and 125) were detected.

In vivo assay. The HPLC 23.5- to 24.5-min fraction, from isolate 100 grown in MS broth, was tested on 10 *Cornus* spp. (*C. florida*, *C. controversa*, *C. alba*, *C. nuttallii*, *C. kousa*, *C. kousa* var. *chinensis*, *C. canadensis*, *C. amomum*, *C. racemosa*, and *C. angustata*) using a leaf overlay technique. Relative necrotic lesion size formed following application of the active fraction was measured 1, 3, and 7 DAT of leaves. All species developed necrotic lesions in a dose-related response, while no necrosis or wound response was noted in control plants inoculated with the MS control fraction. Typical symptoms of *D. destructiva* on *C. florida* in natural habitats include blighting of young leaves and formation of necrotic lesions forming shot holes bordered with a raised, red-purple margin. Based on the necrotic lesions 1 DAT, the various species were placed in three major groups (Table 5). The most

sensitive group included *C. nuttallii* and *C. controversa*, and intermediate sensitivity included *C. racemosa* and *C. alba*. Six *Cornus* spp. demonstrated less sensitivity to the fraction, including *C. kousa*, *C. amomum*, *C. florida*, *C. canadensis*, *C. kousa* var. *chinensis*, and *C. angustata*.

Translocation of fungal metabolites within leaves was observed in *C. alba* 3 DAT, when secondary lesions were noted along the leaf margin, indicating distal transport of the toxic fraction. These lesions coalesced with the primary lesion by 7 DAT. A red margin developed along the edge of the lesion. This symptom is commonly seen in lesions on naturally infected dogwood. Leaf curling and necrosis along the leaf margin occurred in newly emerging leaves above the secondary leaf pair used for the leaf overlay assay in *C. canadensis*, indicating apical transport of the active fraction from the application site.

DISCUSSION

Seedling bioassays using radish and various dogwood species were useful for in vitro and in vivo detection of toxic fungal metabolites associated with *D. destructiva*. The relative elapsed time for the radish bioassays and their sensitivity was consistent with previous reports (24). Results from the leaf puncture assay were much more variable due to the variability of plants and the confines of the growth system which resulted in less sensitivity in determining differences when compared to the radish or *Cornus* spp. seedling bioassays. The radish bioassay proved to be a sensitive and simple test for the initial detection of toxic metabolites produced by *D. destructiva* without the problems associated with stratification and breaking of dormancy requirements of *Cornus* spp. seeds.

HPLC was efficient for the separation and partial purification of *D. destructiva* phytotoxins. There are probably several compounds in this fraction, and further

research is necessary to separate and identify the specific toxic compound or compounds and compare them with those previously identified in *Discula* sp. which was most likely *D. destructiva* (18). The inhibition associated with the PDB control emphasizes the importance of controls and suggests that PDB may not be the most suitable medium for toxicological studies of plant pathogens. The use of a chemically defined medium would be a much more suitable growth medium for toxicological studies. The HPLC fraction, 0 to 5 min, needs to be further isolated and its involvement in symptom development studied.

The dogwood leaf overlay with the active fraction purified using HPLC indicated that *D. destructiva* produces one or more phytotoxins in culture filtrates that can produce in vivo symptoms similar to those observed in the field, demonstrating the involvement of a phytotoxin or phyto-

Table 4. Comparison of mean root growth of radish seedlings exposed to 1-min fractions of the 23-25 min interval collected from HPLC runs for three *Discula destructiva* Type 1 isolates (100, 123, and 125) and *Discula* sp. Type 2 isolate (99) culture filtrates grown on potato-dextrose broth (PDB) or Murishige-Skoog (MS) media^y

Isolate	1-Min HPLC intervals ^z	
	23 to 24	24 to 25
100-PDB	9.5 ± 0.6 a	8.5 ± 0.5 a
123-PDB	11.6 ± 0.7 a	12.2 ± 0.7 a
125-PDB	10.1 ± 0.6 a	13.5 ± 0.8 a
99-PDB	46.9 ± 2.3 b	42.9 ± 2.2 b
100-MS	14.1 ± 0.8 a	13.7 ± 0.8 a

^y Means are the average of two experiments, each with three replicate plates per fraction with 10 seeds per plate.

^z Mean ± standard error. HPLC retention time fractions with significant growth inhibition ($P < 0.001$). Means within a time interval followed by the same letter are not significantly different using Tukey's honestly significant difference ($P < 0.05$).

Table 2. Comparison of mean root growth in radish seedlings following separation and molecular sieving of culture filtrates of *Discula destructiva* (Isolate 100) grown on potato-dextrose broth (PDB)^y

Fraction	Mean ± SE ^z
PDB control	25.22 ± 1.16 bc
Water control	23.93 ± 1.85 bc
Retentate from YM100 membrane	29.43 ± 1.53 b
Retentate from YM3 membrane	35.28 ± 1.86 a
Partially purified culture filtrate	14.55 ± 1.42 d
Original culture filtrate	8.51 ± 0.43 e

^y Mean root lengths are of three replicate plates with 10 seeds per plate.

^z Length (mm); SE = standard error. Treatment means followed by the same letter are not significantly different using Tukey's honestly significant difference ($P < 0.05$).

Table 3. Comparison of mean root growth of radish seedlings exposed to 5-min fractions collected from high performance liquid chromatography (HPLC) runs separating *Discula destructiva* Isolate 100 potato-dextrose broth (PDB) and Murashige-Skoog (MS) culture filtrates and the corresponding controls^x

Time (min) ^y	100-PDB ^z	PDB Control	100-MS	MS Control
0-5*	5.0 ± 0.5 a	6.25 ± 1.0 c	20.4 ± 1.1 ac	34.2 ± 1.4 a
5-10	35.2 ± 1.9 b	49.8 ± 2.8 a	35.8 ± 1.7 bc	38.0 ± 2.0 a
10-15	39.0 ± 2.2 b	42.5 ± 2.8 ab	43.8 ± 2.0 b	39.8 ± 2.4 a
15-20	34.4 ± 2.4 b	36.7 ± 1.8 b	41.9 ± 2.4 b	41.2 ± 1.9 a
20-25*	8.1 ± 0.5 a	35.3 ± 1.6 b	11.3 ± 0.7 a	38.8 ± 1.7 a
25-30	34.9 ± 1.9 b	42.2 ± 2.4 ab	38.7 ± 1.6 bc	40.8 ± 2.0 a
30-35	39.0 ± 2.1 b	44.8 ± 2.5 a	55.4 ± 9.4 b	43.1 ± 2.0 a

^x Means are the average of two experiments each with three replicate plates per fraction with 10 seeds per plate.

^y HPLC retention time fractions. Fractions demonstrating significant growth inhibition are indicated with an asterisk ($P < 0.001$). Contrasted means for 100-PDB versus PDB control and 100-MS versus MS control were significantly different for the 20- to 25-min intervals using Tukey's honestly significant difference (HSD; $P < 0.05$).

^z Means ± standard error. Means within columns for various culture filtrates followed by the same letter are not significantly different using Tukey's HSD ($p < 0.05$).

Table 5. Comparison of necrotic lesion size (mm²) and sensitivity of various *Cornus* spp. in planta 1 day after application of partially purified culture filtrate (PPCF) separated on high performance liquid chromatography^x

<i>Cornus</i> spp.	Lesion size ^y	Sensitivity to PPCF
<i>C. nuttallii</i> ^z	46.0 ± 19.1 a	High
<i>C. controversa</i>	31.8 ± 3.7 b	High
<i>C. racemosa</i>	19.2 ± 4.2 bc	Intermediate
<i>C. alba</i>	17.7 ± 7.2 bc	Intermediate
<i>C. kousa</i>	14.7 ± 2.6 c	Low
<i>C. amomum</i>	12.7 ± 3.5 c	Low
<i>C. florida</i>	12.0 ± 5.1 c	Low
<i>C. canadensis</i>	11.2 ± 2.0 c	Low
<i>C. kousa</i> var. <i>chinensis</i>	8.5 ± 3.6 c	Low
<i>C. angustata</i>	7.8 ± 2.0 c	Low

^x Mean lesion diameters are the average of six plants from two experiments.

^y Necrotic lesion size (mm²) ± standard error. Means followed by the same letter are not significantly different using Duncan's multiple range test.

^z From one experiment, *n* = 2.

toxins in symptom expression of dogwood anthracnose. These findings support previous histological evidence in leaves with few visible hyphae, indicating the involvement of a toxic metabolite (19). Daley (2) stated that minimum proof for the role of a toxin in disease is the reproduction of that symptom by extracellular products of the pathogen. This has been demonstrated in this study with the results of the leaf overlay assay of purified culture filtrates.

Yoder (24) stated that pathological significance should be assessed by evaluations of the correlation between (i) toxin production and disease production by the pathogen and (ii) sensitivity to the toxin and susceptibility of the plant to disease. Bioassay data indicate that the three *D. destructiva* Type 1 isolates tested produced toxic metabolites. No production of toxic metabolites was detected in the *Discula* Type 2 isolate, lending further evidence that this isolate is not *D. destructiva*. The Type 2 *Discula* sp. isolate was consistently shown to be different, toxicologically, from *D. destructiva*. This supports the designation that *D. destructiva* (Type 1) is taxonomically different from the unidentified *Discula* sp., Type 2 (21). Further studies are needed to compare toxin production and disease development by various isolates. This has been hampered by problems associated with the inoculation of *Cornus* spp. with *D. destructiva* to produce disease.

In *Cornus* spp. seedlings, the *D. destructiva*-active fraction caused rapid necrosis of leaf tissue (less than 24 h), suggesting a possible attack on the plasma membrane. In older tissue, necrosis is prominent in 3 days and in younger tissue, the whole leaf can be blighted. Symptoms indicate that these compounds diffuse apoplastically, causing an increase in leaf necrosis between 1 and 3 DAT. Xylem

translocation of the active agent can cause secondary necrotic lesions to appear in sites distal to the wound site between 3 and 7 DAT. In *C. canadensis*, translocation of the active fraction from the second leaf pair upwards resulted in marginal necrosis in emerging leaves. It appears that a primary response of tissue necrosis results on contact, with subsequent translocation to sensitive meristematic areas along the leaf margin or upwards to younger emerging leaves. These symptoms are consistent with those seen in forested settings and may help to explain the loss of canopy in flowering dogwoods and sensitivity of young seedlings.

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