Partial Characterization of Phytotoxic Polysaccharides Produced in vitro by *Colletotrichum trifolii*

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ABSTRACT

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Polysaccharides produced in vitro by *Colletotrichum trifolii* caused paling, desiccation, wilting, and death of excised leaves, shoots, and seedlings of alfalfa (*Medicago sativa*). They were partially purified by acetone precipitation, ultrafiltration, and column chromatography. All three *C. trifolii* isolates tested produced similar phytotoxic polysaccharides. Symptoms were induced in cuttings of susceptible (Kanza) and resistant (Arc) alfalfa, tomato, corn, and soybean, and a hypersensitivelike response was elicited on soybean cotyledons and bean hypocotyls. Partially purified materials were 98–99% carbohydrate and 1–2% protein. Galactose, mannose, glucose, and a trace of a probable uronic acid were found in hydrolysates. Toxic activity was reduced by periodate oxidation or treating with α-mannosidase or β-galactosidase, and was totally removed by hydrolysis. Proteinase K, autoclaving, or pH of 2 or 11 did not alter activity. Excised alfalfa leaves fed commercial dextrans (40 to 500×10^3 daltons) developed symptoms similar to those induced by the *C. trifolii* polysaccarides.

Additional key word: anthracnose.

Anthracnose of alfalfa, caused by *Colletotrichum trifolii* Bain & Essary, is characterized by sunken lesions on infected susceptible stems. Crowns may become infected later in the season. In advanced stages of symptom development, leaves and stems become chlorotic and then tissues become desiccated and necrotic (14,15). *C. trifolii* cannot be recovered from these stems, suggesting the involvement of a fungal toxin.

Toxins extracted from culture filtrates of various fungi, including *Colletotrichum* species, may be representative of toxins that have a role in disease development. *C. fuscum* produces a glycopeptide in vitro that causes wilting and collapse of young *Digitalis* seedlings (13). *C. nicotianae* reportedly produces several toxic components in culture, including a high molecular weight compound capable of inducing necrosis (10,35). *C. gloeosporioides* f. sp. *aeschynomene*, a weed pathogen, produces an acetone-precipitable toxin that may be related to aspergillomarasmine A or B (33). *C. atramentarium* (32), *C. capsici* (24), and *C. musarum* (5) also produce phytotoxic compounds in vitro.

This report describes the partial purification and characterization of phytotoxic polysaccharides from *C. trifolii* culture filtrates. Preliminary work has been reported (9,18).

MATERIALS AND METHODS

Plant material. Cloned materials derived from single plants from alfalfa (*Medicago sativa* L. 'Arc' and 'Kanza') were used. The Arc plant was previously determined to be highly resistant to *C. trifolii*; the Kanza plant was susceptible. Stem cuttings were rooted in sand, potted in a soil-peat mixture (1:1, v/v), grown in the greenhouse (20–25 C), and fertilized every 2 wk with Plant Prod 20-20-20 soluble plant food with minor elements (Patterson Chemical Co., Kansas City, MO 64101).

Other plant species bioassayed against the *C. trifolii* polysaccharide were: tomato (*Lycopersicon esculentum* Mill. 'Walter'), wheat (*Triticum aestivum* L. 'Parker'), soybean (*Glycine max* L. Marr. 'Harosoy 63'), and corn (*Zea mays* L. 'Pioneer Valley 82-S'). Plants were grown in vermiculite in the greenhouse (23–27 C), and fertilized weekly with Agrico 20-20-20 soluble plant food (Continental Oil Co., Memphis, TN 38101).

Assays for the hypersensitive-type response to the *C. trifolii* polysaccharide were done with soybean and red kidney bean (*Phaseolus vulgaris* L.) grown at 20–25 C in a greenhouse.

Cultures of *C. trifolii*. Three isolates of *C. trifolii* were used. An isolate from Kansas (KS-1) was maintained on potato-dextrose agar (PDA) prepared from fresh potatoes. Two isolates from North Carolina designated Clayton and FVT-2 (provided by R. E. Welty) were maintained on lima bean agar (LBA). Cultures were grown at 27 C with a 16-hr photoperiod (1,200 lux) and were transferred weekly. The KS-1 isolate was transferred by flooding PDA plates with 1.0 ml of a suspension of $\sim 10^6$ conidia per milliliter of sterile distilled water. FVT-2 and Clayton were transferred with pieces of agar containing mycelia.

Phytotoxic polysaccharides were harvested from shake cultures in 250-ml Erlenmeyer flasks containing 100 ml of modified Richards' medium (12). The medium was inoculated with either mycelial fragments or 1×10^4 conidia per 100 ml of medium, and the cultures were incubated for 10 days at 27 C in the dark on a gyrotary shaker (200 rpm). Cultures were homogenized to increase mycelial growth (31).

Bioassay. One-milliliter vials were filled with aqueous test solutions and sealed with Parafilm (American Can Co., Greenwich, CT 06830). Trifoliolate leaves from alfalfa were excised under water and their petioles were then inserted into vials through the film. Kanza was used unless otherwise designated. Deionized distilled water (used throughout this study) was added periodically to maintain the initial solution volume. Bioassays were conducted at 20 C and 7,500 lux of continuous fluorescent lighting (Westinghouse cool-white). Each assay included three leaves and was replicated three times. A dilution series was used to determine the amount of polysaccharide required to induce symptoms in 50% of the treated leaves. Symptoms were rated: 0 = no symptoms; 1 = marginal paling; 2 = general paling; 3 = marginal desiccation; and 4 = total desiccation and death. Controls consisted of water and appropriately diluted extracts of uninoculated medium.

Phytotoxicity of commercially obtained dextrans was tested with the leaf bioassay. Solutions (0.5 mg/ml) of various molecular

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weight dextrans (4×10^4, 8.7×10^4, and 5×10^5 daltons) were assayed with both cultivars.

Excised leaves from wheat, corn, tomato, and soybean also were bioassayed. Thirty days after seed was planted, leaves were excised under water and their petioles or cut ends were immediately submerged in 1.0 ml of either a 50% aqueous solution of partially purified polysaccharides from the Clayton isolate or a comparable extract of uninoculated medium. This bioassay was conducted as described for alfalfa leaves.

Extracted materials also were tested by published procedures (2) for their ability to elicit a hypersensitive-type response on excised soybean cotyledons and red kidney bean hypocotyls. Cotyledons and hypocotyls were excised at 7–8 days and surface sterilized in 1% sodium hypochlorite for 1 min. Partially purified polysaccharides from KS-1 (~600 μg/ml) were applied in 100-μl drops to cut surfaces.

Extraction of polysaccharides from *C. trifolii*. Ten-day-old shake cultures were filtered through Whatman No. 2 filter paper that had been washed with acetone and water. The collected mycelia were dried at 90 C for 9–16 hr for dry-weight determinations. Culture filtrates, typically 800 ml, were mixed with three volumes of acetone (−18 C) and immediately centrifuged at 12,000 *g* for 10 min. The supernatant was discarded and the air-dried pellet was resuspended in water. The resulting suspension was homogenized with a Potter-Elvejhem homogenizer and filtered through glass wool. The solution was then washed with three volumes of water and concentrated in an Amicon ultrafiltration cell (Amicon Corp., Lexington, MA 02173) using a PM-10 membrane (1.6-μm pore diameter). The retained material contained the phytotoxic polysaccharide and was frozen (−18 C) for future use in bioassays and analyses. Uninoculated medium was similarly extracted and stored for use as controls.

Phytotoxic polysaccharides were partially purified from culture filtrates of all three isolates (Table 1). During most of these studies, two preparations from KS-1, KS-1a, and KS-1b were used.

Partially purified preparations from the three isolates were chromatographed on a molecular sieve column (66 × 2.6 cm) containing Bio-Gel A-.5 m, ~80–150 μm (100–200-mesh) Bio-Rad Laboratories, Richmond, CA 94804). The column was equilibrated and eluted with 50 mM NaCl at a flow rate of 15 ml/hr. Column void volume was 145 ml. A sample of 100 mg galactose-equivalents (gal eq), as determined by the anthrone procedure, was applied to the column. Fractions were exhaustively dialyzed against water before they were used in the bioassay.

Chemical assays. The total hexose content of the samples was determined by the anthrone procedure (17,26) for 15 min at room temperature and with galactose as the standard. One part of the sample was incubated with two parts of reagent. Absorbance was read at 620 nm. The carbazole assay was used to test for uronic acids (7). Amino and *N*-acetylated amino sugars were determined with 3-methyl-2-benzothiazolone hydrazone hydrochloride (27). Protein content was estimated with the Coomassie brilliant blue G-250 dye assay (4) with bovine serum albumin as a standard. Triplicate samples were analyzed in all assays.

For monosaccharide analyses, samples were hydrolyzed by the procedure of Jones and Albersheim (19). Aqueous samples (2.5 ml) were dried in 10-ml ampules and hydrolyzed with 2 N trifluoroacetic acid (TFA) for 2 hr at 120 C. After evaporation of TFA under vacuum, dried samples were resuspended in 2 ml of water and bioassayed. Analysis of neutral sugars in the hydrolysate was accomplished with the high-performance liquid chromatography (HPLC) system of Barr and Nordin (3), modeled after Mopper (22). The column (25 × 0.3 cm) was an anionic resin DA-X8-11 (Durrum Chemical Corp., Palo Alto, CA 94303) in the borate form and was operated at 78 C and 31.6–38.7 kg/cm^2 (450–550 psi). The buffer was 0.5 M boric acid, pH 8.6. The flow rate was 19 ml/hr. The color reagent to detect aldoses was Cu^{++}-bicinchoninate-aspartic acid (23). Reagent was injected into the column effluent at 23 ml/hr. Reaction time was 1 min at 124 C. Absorbance at 560 nm was followed with a P2 differential absorption monitor (Dionex Corp., Sunnyvale, CA 94086).

Paper chromatography was used to confirm the monosaccharide analysis obtained by HPLC. Hydrolysates were spotted on Whatman No. 1 paper along with standard sugars (1.0 mg/ml): galactose, mannose, glucose, galacturonic acid, and glucuronic acid. Two solvent systems were used: *n*-butanol:pyridine:water (10:3:3, v/v) for neutral sugars, and pyridine:ethyl acetate:water:acetic acid (5:5:3:1, v/v) for sugar acids. Chromatography was by descending solvent flow in equilibrated tanks. Carbohydrates were located with Ag^+/OH^- staining (28).

Isolate pathogenicity. The pathogenicity of the three isolates was determined by the methods of Devine et al (6). Alternate rows of Arc and Kanza were seeded in sterile sand in 24 × 13 × 7-cm pans. Each pan had four rows of each cultivar thinned to 10 plants per

TABLE 1. Extraction of toxic polysaccharides from culture filtrates of *Colletotrichum trifolii* in modified Richards' medium

Extraction step	Isolate	Carbohydrate[a] (mg/ml)	Protein (mg/ml)	Protein:carbohydrate ratio	Activity[b]		mg gal eq/mg mycelium[e]
					Dilution end point	mg gal eq/ml at end point	
Crude filtrate	KS-1a[c]	1.5	0.01	0.008	NB[d]	-	-
	KS-1b[c]	2.2	0.05	0.022	NB	-	-
	FVT-2	ND[d]	ND	-	-	-	-
	Clayton	ND	ND	-	-	-	-
Resuspended acetone precipitate	KS-1a	6.5	0.05	0.007	NB	-	-
	KS-1b	17.3	0.25	0.015	NB	-	-
	FVT-2	11.0	0.20	0.018	NB	-	-
	Clayton	22.8	0.24	0.011	NB	-	-
Ultrafiltration-retained materials	KS-1a	18.5	0.06	0.003	1/20	0.925	1.2
	KS-1b	18.3	0.26	0.014	1/30	0.621	1.1
	FVT-2	25.6	0.38	0.015	1/20	1.28	1.6
	Clayton	14.7	0.19	0.013	1/20	0.736	0.92
Ultrafiltrate	KS-1a	0.0	0.0	0.0	0	-	0.0
	KS-1b	0.7	0.05	0.07	0	-	0.04
	FVT-2	0.2	0.0	0.0	0	-	0.012
	Clayton	0.4	0.2	0.05	0	-	0.025

[a] Expressed as galactose equivalents (gal eq).
[b] Dilutions at which 50% of the leaves showed symptoms, calculated relative to original volume.
[c] Two preparations of KS-1 used, denoted a and b.
[d] ND = not determined. NB = not bioassayed. Medium components were toxic, thus bioassays of crude filtrates and some resuspended acetone precipitates were meaningless.
[e] Mycelial dry weights (mg/ml filtrate): KS-1a 16, KS-1b 17, FVT-2 16, Clayton 16.

row. Four replicate pans were used per isolate. Plants were grown at 20 C and 16,100 lux of fluorescent light (cool-white) with a 16-hr photoperiod, and irrigated daily with Hoagland's solution (29) with all salts except KNO_3 diluted one-tenth.

Two weeks after seeding, plants in each pan were sprayed with 10 ml of water containing ca 2×10^5 conidia per milliliter. Inoculated plants were kept for three days in darkened plastic boxes (relative humidity ~100%, 20 C). Plants were then placed in clear plastic boxes that had small air holes to maintain a somewhat lower humidity. During this period a 16-hr photoperiod of 8,100 lux was used. Five days after inoculation, plants again received 16,100 lux. Symptoms were rated 10 days after inoculation.

Inactivation of the partially purified polysaccharide. Various procedures were used in attempts to inactivate the partially purified polysaccharide. Each test involved extracts from isolate KS-1, denoted as either KS-1a or KS-1b, or other extracts as noted. Experiments were replicated three times with three bioassays for each replication. Unless otherwise noted, controls were water and comparably extracted uninoculated medium.

The effect of hydrolysis on phytotoxicity was tested by the TFA procedure previously outlined. To test the effect of heat, we autoclaved both the partially purified polysaccharide and the appropriate controls at 120 C for 1.5–2.0 hr and bioassayed them after they had cooled.

To investigate the effect of pH extremes, we adjusted aliquots from KS-1a and Clayton extracts to pH ~2.0 and 11.0 with 1 N HCl and 1 N NaOH, respectively. Treated extracts were incubated at 3 C for 48 hr, returned to pH 7.0, and bioassayed.

The effect of periodate oxidation on phytotoxicity was also studied (16,21). One milliliter of a partially purified preparation (KS-1b), 0.366 mg gal eq/ml, was treated with an equal volume of 50 mM sodium *m*-periodate and incubated for 48 hr at 5 C in the dark. Oxidation was terminated by adding 0.1 ml of ethylene glycol, and the sample was incubated for 6 hr at 5 C in the dark. Treatments were desalted with a PD-10 column (Sephadex G-25, 5 × 1.5 cm, Pharmacia, Sweden). Only material in the first 2 ml after the void volume was collected for bioassay. Since the toxin concentration was below the dilution end-point, several replications of each treatment were pooled, lyophilized, resuspended in 1.0 ml of water, and bioassayed. Treatments included: a) extracts and periodate; b) extracts and previously inactivated periodate (with ethylene glycol); c) extracts and water; and d) water and periodate. Treatment 'a' was repeated nine times and the controls (b, c, and d) were repeated three times each. Modification of the partially purified toxin by periodate was confirmed by submitting it to Smith degradation (11) and demonstrating a molecular weight change with PD-10 column chromatography.

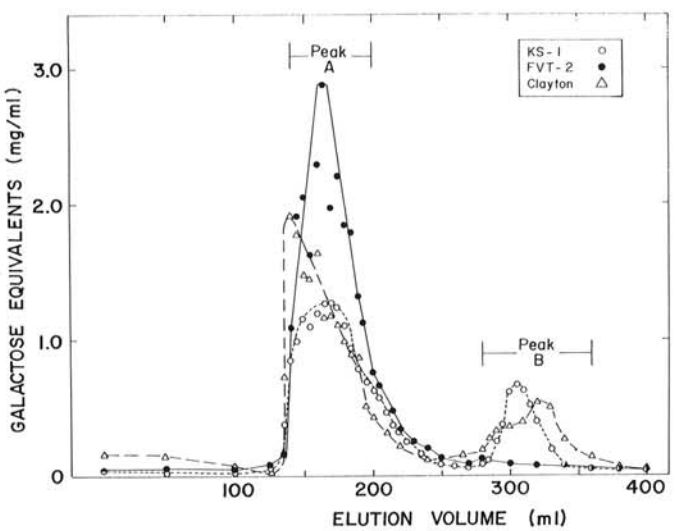

Fig. 1. Fractionation of partially purified polysaccharides from *Colletotrichum trifolii* isolates KS-1, FVT-2, and Clayton.

Tests for enzymatic inactivation (21) were done on partially purified polysaccharide from KS-1b. Reaction mixtures included 1.0 ml of an appropriate enzyme (0.01–0.4 mg protein/ml) and 1.0 ml of polysaccharide solution (1.1 mg gal eq/ml in buffer). All assays were buffered with 50 mM citrate-phosphate, pH 4.5, except β-galactosidase, which contained 50 mM phosphate, pH 7.2. All buffers contained 25 μg/ml each of tetracycline and streptomycin sulfate. Controls included autoclaved enzyme plus extract and buffer, enzyme and buffer, extract and buffer, and buffer alone. Reactions were carried out for 48 hr at 25 C in the dark and were terminated by immersing the tubes in boiling water for 10 min. Precipitates were removed by centrifugation. Treated samples were then dialyzed exhaustively against water and bioassayed. The enzymes used were: proteinase K (E.C. 3.4.21.14) at 0.9 units/mg gal eq, chitinase (E.C. 3.2.1.23) at 0.08 units/mg gal eq, β-glucosidase (E.C.3.2.1.21) at 0.04 units/mg gal eq, α-galactosidase (E.C. 3.2.1.22) at 0.02 units/mg gal eq, β-galactosidase (E.C. 3.2.1.23) at 5 units/mg gal eq, and α-mannosidase (E.C. 3.2.1.24) at 0.10 units/mg gal eq. Proteinase K was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN 46250; all substrates and the other enzymes were from Sigma Chemical Co., St. Louis, MO 63178. Each of the enzymes had the expected activity and specificity when assayed individually under the above conditions with each of the following substrates: 0.65% (w/v) casein, a chitin suspension, 0.5 mg/ml of *p*-nitrophenyl-β-glucoside, *p*-nitrophenyl-α-galactoside, *p*-nitrophenyl-β-galactoside, or *p*-nitrophenyl-α-mannoside. Procedures for the assay of proteinase K and chitinase were obtained from Sigma Chemical Co.; glycosidase assays were after English et al (8).

RESULTS

Partial purification of phytotoxic polysaccharides. Crude culture filtrates of *C. trifolii* were acetone precipitated, centrifuged, and the pellets resuspended. Materials retained on the ultrafiltration membrane (nominal retention for globular proteins is 10^4 daltons for PM-10 membranes) contained the active components as determined by the excised leaf bioassay (Table 1). Controls of comparably treated uninoculated medium contained no activity after ultrafiltration. Crude filtrates of samples and medium controls were toxic due to components of the culture medium, whereas little phytotoxicity was observed in the diluted PM-10 culture filtrates of either samples or medium controls.

All isolates produced essentially the same amount of material (gal eq) per mg mycelium (Table 1). Although the carbohydrate and protein content generally increased during purification, the protein:carbohydrate ratio changed little (~98–99% carbohydrate). In early experiments, ion exchange chromatography on Dowex-1 and Dowex-50 followed ultrafiltration (18). Essentially none of the carbohydrate or biological activity was removed by these columns, and little change in protein content occurred (*unpublished*). Therefore, this step was omitted in later extractions.

Following ultrafiltration, gel permeation chromatography of the polysaccharides from all three isolates produced similar elution profiles (Fig. 1). All produced broad anthrone-positive peaks starting at the void volume and extending into the fractionation range of the column. Clayton and KS-1 produced a smaller peak in addition to the larger one. Phytotoxicity was primarily in the pooled fractions of the large "A" peaks, while the small or "B" peaks showed only traces of activity.

Phytotoxicity of extracted culture filtrates. The partially purified extract was phytotoxic to alfalfa seedlings and older shoots (18). Leaves of treated seedlings became pale and wilted, and eventually the emerging unifoliolate leaves became necrotic. Leaflet margins of excised leaves became pale green within 6–12 hr. This lighter color spread inwardly and was followed by desiccation. Higher dilutions (~600 μg gal eq/ml) delayed the onset of symptoms. Eventually the tissues became necrotic and brittle. At low dilutions (~3 mg gal eq/ml) the entire sequence was complete within 48–60 hr. Excised shoots exhibited wilting, occasional chlorosis, and eventual necrosis beginning at the tip. Both Arc and Kanza demonstrated similar symptom development in the excised leaf

bioassay and were equally sensitive in dilution series. All three *C. trifolii* isolates produced compounds with similar activities (dilution end-point, Table 1). However, the dilution end-points differed in milligrams of galactose equivalents per milliliter. The variations between isolates may reflect experimental variation or minor differences in the polysaccharides produced and their activity. Controls receiving water or appropriately diluted extracts of uninoculated medium rarely showed symptoms before 72 hr, when chlorosis first appeared.

Because the active preparations were largely carbohydrate, we studied the effects on alfalfa of commercially available dextrans of various molecular weights. These dextrans (4×10^4, 8.7×10^4, and 5×10^5 daltons) at 500 μg/ml induced symptoms similar to those caused by the *C. trifolii* phytotoxin.

Isolate pathogenicity. All isolates were more pathogenic on Kanza than on Arc (Table 2). Mean scores varied only slightly, but the Clayton isolate was most pathogenic.

Response of other plant species to polysaccharides of *C. trifolii*. Excised leaf assays with partially purified polysaccharides from the Clayton isolate were used. Tomato leaves became severely desiccated and were brittle after 36 hr. The symptoms on corn were less pronounced, but the leaf margins curled inward and developed a grayish cast by 24 hr. Soybean leaves became pale, began to wilt within 4 hr, and quickly became desiccated. No symptoms were evident on wheat or on controls receiving water or extracts of uninoculated medium.

Induction of a hypersensitive-type response by polysaccharides of *C. trifolii*. Soybean cotyledons and bean hypocotyls treated with partially purified polysaccharides (~600 μg gal eq/ml) developed a hypersensitivelike response. Bean hypocotyls became brown in treated areas while soybean cotyledons developed a brown to reddish color within 24 hr. Water or comparably extracted uninoculated medium caused no significant response.

Inactivation of phytotoxic activity. Partially purified polysaccharide exposed to autoclaving and pH extremes (pHs 2 and 11) remained active, while activity was lost after hydrolysis with TFA. Periodate oxidation induced a 12-hr delay in the onset of symptoms compared with untreated polysaccharides or polysaccharides treated with inactivated periodate. Molecular size of the polysaccharide toxin decreased after the Smith degradation as shown by its elution near galactose on a PD-10 column (Fig. 2).

Of the six enzymes tested for their ability to alter symptom induction by the *C. trifolii* polysaccharides in the alfalfa leaf bioassay, four had no significant effect: β-glucosidase, α-galactosidase, chitinase, and proteinase K (Table 3). In contrast, a 48-hr incubation in either β-galactosidase or α-mannosidase reduced the ability of the polysaccharide to induce symptoms, relative to both untreated polysaccharide and polysaccharide treated with boiled enzymes. Pretreatment of the polysaccharide with either enzyme only slowed but did not destroy the symptom-inducing ability, and symptom development resembled that obtained with reduced polysaccharide concentrations.

Monosaccharide analysis. *C. trifolii* polysaccharides purified by acetone precipitation, ultrafiltration, and gel permeation chromatography were hydrolyzed, and the resulting aldoses separated and identified by HPLC (Table 4). The predominant sugar in the primary peak (peak A) of all three isolates was galactose, followed by mannose, then glucose. The secondary peak (peak B) of the Clayton and KS-1 isolates contained only glucose. The same sugars were also observed in paper chromatographs. Trace amounts of one or more hexuronic acid components also were indicated both by paper chromatography, in which a faint spot cochromatographed with both galacturonic and glucuronic acid standards, and by the carbazole assay. Also, trace amounts of amino and/or *N*-acetylated amino sugars were indicated by colorimetric assay (27), although no absorbance was observed in partially purified materials at 205 nm.

Differences between anthrone assays of total hexoses before and after hydrolysis probably resulted from sugar degradation, as indicated by both the light brown color of the hydrolysate and an absorbance peak at 280 nm after hydrolysis. The disparity between the amount of anthrone-positive material after hydrolysis and the monosaccharides recovered after HPLC (Table 4) may result from

TABLE 2. Pathogenicity of *Colletotrichum trifolii* isolates on the alfalfa cultivars Arc and Kanza

Isolate	Cultivar	Plants (%) in class[a]					Mean class	Number of plants tested
		1	2	3	4	5		
KS-1	Arc	79.8	2.5	1.9	5.0	10.7	1.6	159
	Kanza	13.5	16.8	8.4	25.8	35.5	3.5	155
Clayton	Arc	77.5	0.64	0.64	9.6	11.5	1.8	156
	Kanza	7.5	6.25	0.62	15.6	70.0	4.3	160
FVT-2	Arc	85.9	1.3	0.0	8.9	3.8	1.4	156
	Kanza	21.6	3.2	1.9	26.8	46.5	3.7	157

[a] Classes are: 1 = no lesions; 2 = long, narrow lesions; 3 = large lesions, nongirdling; 4 = coalescing and girdling lesions; and 5 = plant dead.

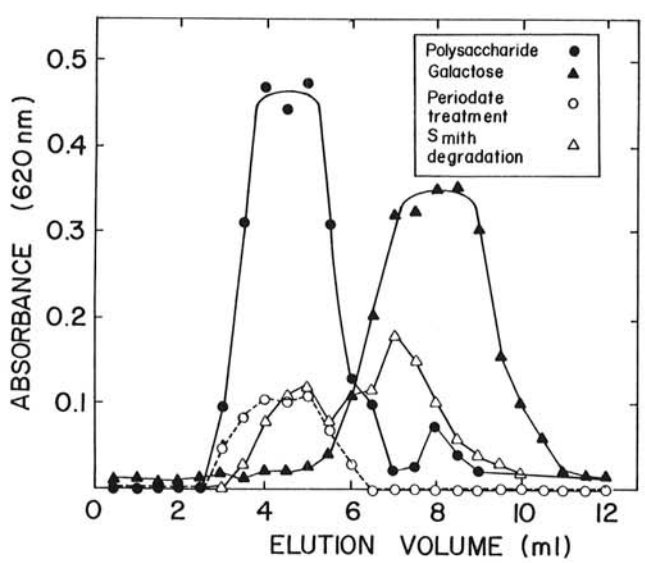

Fig. 2. Elution pattern of treated and untreated partially purified *Colletotrichum trifolii* polysaccharide compared with galactose.

TABLE 3. Effect of various enzymes on the phytotoxicity of *Colletotrichum trifolii* polysaccharides to alfalfa leaves[a]

Enzyme[b]	Average symptom ratings at intervals during bioassay[c]		
	12 hr	24 hr	48 hr
Chitinase	2±0	3±0	4±0
α-Galactosidase	2±0	3±0	4±0
β-Galactosidase	1±0	1.5±0	3.4±0.2
β-Glucosidase	2±0	3±0	4±0
α-Mannosidase	0±0	0.6±0.5	2±0.8
Proteinase K	2±0	3±0	4±0
Controls			
Autoclaved enzyme, buffer, polysaccharide[d]	1.7±0.5	2.4±0.8	3.4±0.8
Enzyme, buffer[d]	0±0	0±0	0±0
Polysaccharide, buffer[e]	1.6±0.5	2.4±0.8	3.5±0.7
Buffer[e]	0±0	0±0	0±0

[a] KS-1 (preparation b) toxin was exposed to enzymes for 48 hr before bioassay.
[b] Mean score of nine leaves per enzyme (±S.D.).
[c] Symptom classes: 0 = no symptoms; 1 = marginal paling; 2 = general paling (>50% of surface; 3 = marginal desiccation; and 4 = total desiccation and death.
[d] Mean score of nine leaves, three leaves per enzyme for each enzyme. Scores were pooled because results were indistinguishable.
[e] Mean score of 18 leaves per control (±S.D.).

several factors. First, not all sugars react identically in the anthrone assay. The extracts may possess materials detectable via the anthrone assay but not by HPLC, including sugar degradation products from hydrolysis. In addition, hydrolysis may have failed to totally reduce the polysaccharide to monomers. Any or all of the above may have contributed to the apparent reduced recovery after HPLC.

DISCUSSION

The phytotoxic substances produced in vitro by *C. trifolii* are largely, if not totally, polysaccharide, as they exhibit a positive anthrone reaction, are hydrolyzed to monosaccharides, are stable to autoclaving and pH extremes, and have high molecular weights. Activity is somewhat reduced by periodate oxidation and, as expected of polysaccharides, Smith degradation causes a decrease in molecular weight. Activity is totally lost by TFA hydrolysis. Enzyme treatments, because of the probable presence of unknown enzyme contaminants, are inherently less conclusive, but both α-mannosidase and β-galactosidase reduced biological activity of the toxic material in the alfalfa leaf bioassay. The 1–2% protein found in the partially purified toxin, based on both Lowry (18) and Coomassie brilliant blue assays, apparently is not necessary for biological activity, because biological activity persists after pronase (9) and proteinase K treatments and ion-exchange chromatography.

The role of these polysaccharides during disease development was not established. The symptoms induced in excised leaves, shoots, and seedlings (18) are similar to those observed in crown-infected plants. All three *C. trifolii* isolates produced similar toxic polysaccharides in vitro, and they did not vary significantly in pathogenicity. A comparison of polysaccharide production and pathogenicity of recently reported, highly virulent *C. trifolii* "races" (25,34) would be of interest. However, our results suggest that *C. trifolii* polysaccharides are not involved in host specificity, because Arc and Kanza were equally sensitive, as were several nonhosts of the pathogen, including tomato, soybean, and corn.

The lowest concentration of toxic polysaccharides (Table 1) to induce symptoms (~600 μg/ml) appears high compared with polysaccharides from culture filtrates of other fungal pathogens. Polysaccharides from *Phytophthora cinnamomi*, *P. cryptogea*, and *P. nicotianae* were active at concentrations as low as 50 μg/ml (36). Mycolaminarins (β-1,3-glucans) from *P. cinnamomi*, *P. palmivora*, and *P. megasperma* var. *sojae* induced symptoms at concentrations from 10 to 250 μg/ml, depending on the plant species assayed (20). Early in this study (data not shown), some extracts from *C. trifolii* were active below 100 μg/ml (18). Both the plant material bioassayed and the size of the polysaccharide can influence phytotoxicity if vascular obstruction is involved (30). The size of fungal polysaccharides may vary with cultural and extraction conditions. Additional fractionation of the heterogeneous polysaccharides of *C. trifolii* is needed to establish whether specific components of high toxic activity exist and if cultural conditions influence their production.

The mode of action of these materials is unknown. Commercial dextrans of $>2.5 \times 10^5$ daltons can cause vascular obstruction in alfalfa leaves at picomole concentrations (30). Yet wilt-inducing β-1,3-glucans isolated from the mycelium of several *Phytophthora* spp. in vitro are extensively metabolized during wilt induction, indicating possible interactions at the cellular level (20). Alfalfa leaflets respond to the vascular uptake of *C. trifolii* polysaccharides by a fading of the normal green color followed by tissue desiccation and necrosis. Vascular obstruction appears more likely than polysaccharide-cell interactions to cause these responses, because toxic activity was retained by periodate-treated polysaccharides, which, while oxidized, were not reduced in molecular size (Fig. 2). However, generalizations are difficult with a heterogeneous population of molecules, because the possibility cannot be eliminated that some portion of the molecules is directly toxic to leaf cells.

Anderson (1) reported a polysaccharide in culture filtrates from *C. trifolii* that was of somewhat different monosaccharide composition and that elicited both browning and phytoalexin accumulation by red kidney bean (*Phaseolus vulgaris* L.) cotyledons. The *C. trifolii* polysaccharides reported here were similarly active on both red kidney bean and soybean. Thus, these preparations can induce both the hypersensitive-type response in bean and soybean and phytotoxicity in the alfalfa leaf bioassay. It is not yet possible to distinguish between specific induction of each response by different components of the mixture and induction of both responses by all or the same components of the polysaccharide mixture. Until attempts are made to separate these activities, the suggestion that elicitor or toxic functions depend only upon the choice of assay (21) cannot be evaluated.

TABLE 4. Monosaccharides in hydrolyzed *Colletotrichum trifolii* polysaccharides as determined by high-performance liquid chromatography (HPLC)

Isolate	Elution peak[a]	Monosaccharides (% of HPLC total) Mannose	Galactose	Glucose	HPLC total[b] (μg/ml)	Total hexose[c] (μg/ml) Hydrolysis (−)	(+)
KS-1	A	27	67	6	78	421	360
	B	0	0	100	3	10	3
FVT-2		17	76	7	92	484	435
Clayton	A	19	68	13	31	380	241
	B	0	0	100	10	130	56

[a] As separated on a Bio-Gel A.5 m column ~80–150 μm (100- to 200-mesh) (Fig. 1). Peak A represents the high molecular weight component.
[b] Estimation of peaks by triangulation and comparison with standard curves.
[c] Determined by microanthrone procedure, before (−) and after (+) hydrolysis.

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