CHARACTERIZATION OF TOXIC POLYSACCHARIDES PRODUCED IN VITRO BY COLLETOTRICHUM TRIFOLII

by

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INTRODUCTION

Anthracnose of alfalfa, caused by *Colletotrichum trifolii* Bain, is characterized by sunken, oval to diamond-shaped lesions on infected susceptible stems. Lesions enlarge and coalesce, ultimately girdling the stem. Often in the summer and fall light-colored stems, resulting from a crown rot phase of the disease, may be observed scattered throughout the field (14,15). Specifically, leaves become chlorotic followed by necrosis and tissue desiccation. These symptoms begin distally and move inward. After leaf death, stem tips curl and develop a whitish cast. *C. trifolii* was not recoverable from these stems suggesting involvement of a fungal toxin.

Toxins extracted from culture filtrates of various fungi, including *Colletotrichum* species, have been suggested to be involved in numerous diseases. *C. fuscum* produces a glycopeptide *in vitro* which causes wilting and collapse of young *Digitalis* seedlings (13). *C. nicotianae* reportedly produces a number of toxic components *in vitro*, including a high molecular weight compound capable of inducing necrosis (10, 33). *C. gloeosporioides* f.sp. *aeschynomene*, a weed pathogen, produces an acetone precipitable toxin possibly related to aspergillomarasmine A or B (30). This toxin induced a variety of symptoms depending on the host. *C. atramentarium*, *C. capsici*, and *C. musae* also produce phytotoxic compounds *in vitro* (5, 24, 31).

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

MATERIALS AND METHODS

Plant Material

Cloned materials derived from stem cuttings of two alfalfa (*Medicago sativa* L.) cultivars, Arc and Kanza, were used throughout this study. Kanza was selected from a single plant, while Arc was obtained from a plant previously determined to be anthracnose resistant, provided by D. L. Stuteville (Plant Pathology Dept., Kansas State University). Cuttings were rooted in sand and potted in a 1:1 soil-peat mixture in 5.1 cm square pots. Plants were greenhouse grown (20-25 C), watered daily and fertilized bimonthly with Plant Prod 20-20-20 soluble plant food with minor elements.

Hosts for the specificity experiment 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'. Seeds were sown in vermiculite in 10.2 cm diameter pots. Plants were greenhouse grown (23-27 C), watered daily and fertilized weekly with Agrico 20-20-20 soluble plant food.

Hypersensitive reaction (HR) assays involved soybean and red kidney bean (*Phaseolus vulgaris* L.). Seeds were germinated in vermiculite in 24 x 13 x 7 cm pans, at 20-25 C in a greenhouse and watered daily.

Cultures of *C. trifolli*

Three isolates of *C. trifolii* were used in this investigation. KS-1, a Kansas isolate provided by D. L. Stuteville (Kansas State University), was maintained on freshly prepared potato dextrose agar (PDA). Two North Carolina isolates provided by R. E. Welty (USDA-AR-SEA, North Carolina State University) denoted Clayton and FVT-2 were maintained on fresh

lima bean agar (LBA). Cultures were grown at 27 C with a 16 hr photoperiod (1.2 Klux) and were transferred weekly. The KS-1 ioslate was transferred by flooding PDA plates with 1.0 ml of a suspension of ca. 1 x 10^6 conidia/ml sterile distilled water. FVT-2 and Clayton were transferred using pieces of agar containing mycelia.

Toxic compounds were produced in liquid shake culture in 250 ml Erlenmeyer flasks containing 100 ml of modified Richards' medium (12). The medium was inoculated with either mycelial fragments or 1 x 10^4 conidia/100 ml medium, and incubated for 10 days at 27 C in the dark on a gyrotary shaker (200 rpm). The homogenization procedure of Van Etten and Daly was employed to increase the amount of mycelial growth (36).

Bioassay

One ml vials were filled with aqueous test solutions and sealed with Parafilm. Petioles of trifoliolate leaves from alfalfa were inserted into vials through the film. Kanza was used primarily in the bioassay, however Arc was occasionally tested for its reaction. Deionized-distilled water (used thoughout this tudy) was added periodically to maintain the initial solution volume. Bioassays were conducted at 20 C and 7.5 Klux of continuous fluorescent lighting (Westinghouse Cool White). All assays included three leaves and were replicated three times. A dilution series was used to determine the amount of a toxic preparation 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 extracts of uninoculated medium and rarely induced symptoms before 72 hr, after which chlorosis developed.

The toxicity of commercially obtained dextrans was investigated using the leaf bioassay. Solutions (0.5 mg/ml) of various molecular weight dextrans (4×10^4, 8.7×10^4, and 5×10^5 daltons) were assayed on both cultivars.

Excised leaves from wheat, corn, tomato and soybean were used to test for specificity. Leaves from 30-day-old plants were excised under water and immediately transferred to 1.0 ml of a 50% aqueous solution of partially purified toxin from the Clayton isolate or comparably extracted uninoculated medium. This bioassay was conducted as described for the alfalfa leaf assay.

Extracted materials were also tested for HR eliciting ability upon excised soybean cotyledons and bean hypocotyls. Cotyledons and hypocotyls were excised at 7-8 days, sterilized, and assayed by measuring the extent of browning. Partially purified toxin from KS-1 was applied at 100 µl/cotyledon or hypocotyl. The assay employed was identical to that previously described (2).

Toxin Extraction

Ten-day-old cultures were filtered through Whatman #2 filter paper which had been previously washed with acetone and water. The collected mycelia was dried at 90 C for 9-16 hr for dry weight determinations. Culture filtrates 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. This solution could be stored frozen. The solution was then washed with three volumes of water and concentrated in an Amicon ultrafiltration cell (Amicon Corp., Lexington, MA.) using a

PM-10 membrane (1.6 µm pore diameter). The filtrate was discarded and the retained materials frozen (-18 C) for future use. Also, uninoculated medium was similarly extracted, stored and used as a control.

Toxic compounds were partially purified from all three isolates (Table 1). During most of these studies, two separate preparations of the partially purified toxin from KS-1, denoted as KS-1a and KS-1b, were used.

Partially purified toxin from the three isolates was chromatographed on a molecular seive column (66 x 2.6 cm) containing Bio-Gel A.5M, 100-200 mesh (Bio-Rad Laboratories, Richmond, CA.). The column was equilibrated and eluted with 50 mM NaCl at a flow rate of 15 ml/hr. A sample size of 100 mg galactose equivalents (gal eq) was applied to the column. Fractions were exhaustively dialyzed against water prior to bioassaying.

Chemical Assays

The total hexose content of the samples was determined by the anthrone procedure (17) using 1 part sample to 2 parts reagent. Absorbance was read at 620 nm after incubating 15 min at room temperature. A microanthrone assay was also used (26). Galactose was used as a standard and assays were triplicated. The carbazole assay was used to test for uronic acids (7). Amino and N-acetylated amino sugars were assayed using 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. Again assays were triplicated.

Samples were hydrolzed by the procedure of Jones and Albersheim (19). Aqueous samples (2.5 ml) were dried in long-necked 10 ml ampules in a

vacuum oven at 100 C for 48-60 hr. After drying, 2 ml of 2 N trifluoroacetic acid (TFA) was added per ampule which was then sealed under vacuum. The samples were autoclaved for 2 hr at 120 C. Once cool the tubes were opened and the TFA evaporated off in a vacuum oven. Samples were then dried in a vacuum desiccator over Drierite and KOH pellets, resuspended in 2 ml of water, and bioassayed.

Monosaccharide analysis of the hydrolysate was accomplished using the high performance liquid chromatography system (HPLC) of Barr (3), modeled after Mopper (22). The column (25 x 0.3 cm) was an anionic resin DA-X8-11 (Durrum Chemical Corp.) in the borate form and was operated at 78 C and 450-550 psi. The buffer was 0.5 M boric acid, pH 8.63. The flow rate was 19 ml/hr. Detection of aldoses was with Cu^{++}-bicinchonate-aspartic acid color reagent (23). Reagent was injected into the column effulent at 23 ml/hr. Reagent color development was hastened with a 124 C bath with a reaction time of 1.0 min. Absorbance at 560 nm was followed with a P2 differential absorption monitor (Dionex Corp.). Amino, N-acetylated amino and acid sugars are not detected with this system. While pentoses are detectable none would have survived the hydrolysis (P. Nordin, Biochemistry Dept., Kansas State University, private communication).

Paper chromatography was used to confirm the above monosaccharide analysis. Hydrolysates were spotted onto Whatman #1 paper along with standard sugars (1.0 mg/ml): galactose, mannose, glucose, galacturonic acid and glucuronic acid. Two solvent systems were employed: n-butanol: pyridine:water (10:3:3) for neutral sugars, and pyridine:ethyl acetate: water:acetic acid (5:5:3:1) for acid sugars. Chromatograms were produced by descending solvent flow in equilibrated tanks. Carbohydrates were located using Ag^{+}/OH^{-} staining (28).

Isolate Pathogenicity

The pathogenicity of the three isolates studied was determined using the methods of Devine et al (6). Alternate rows of Arc and Kanza were shown in sterile sand in 24 x 13 x 7 cm pans. Each pan had four rows of each cultivar with 10 plants per row. Four replications per isolate were made. Plants were grown at 20 C and 16.1 Klux of fluorescent light (cool white) with a 16 hr photoperiod. Pans were watered daily with 10% Hoagland solution with 100% KNO_3 (29).

Two weeks after seeding, each pan of plants was sprayed with 10 ml of a water suspension of conidia (ca. 2 x 10^5 conidia/ml). Inoculated plants were kept for the first three days in darkened plastic boxes, relative humidity ca. 100%, at 20 C. After this period plants were covered with clear plastic covers that had small air holes to allow a gradual decrease in the humidity. During this period a 16 hr photoperiod, 8.1 Klux, was instituted. Five days after spraying, plants again received 16.1 Klux. Symptoms were rated 10 days post-inoculation.

Inactivation of the Partially Purified Toxin

A variety of methods were employed in efforts to inactivate the partially purified toxin. Each test involved extracts from isolate KS-1, either a or b, or as noted. Tests were replicated three times with triplicate bioassays for each replication. If not specifically noted controls were water and comparably extracted uninoculated medium.

The effect of hydrolysis on toxic activity was tested using the TFA procedure previously outlined. To test the effect of autoclaving aliquots (1.0 ml) of partially purified toxin and the appropriate controls were treated at 120 C for 1.5-2 hr and bioassayed after cooling.

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

The effect of periodate oxidation upon toxic activity was also studied (16, 21). Partially purified toxin (KS-1b), 0.366 mg gal eq/ml, was treated with 50 mM sodium *m*-periodate by incubating a mixture of 1.0 ml of each solution for 48 hr at 5 C in the dark. The oxidation was terminated by the addition of 100 µl of ethylene glycol and reincubating for 6 hr at 5 C in the dark. The treatments were desalted using a PD-10 column (Sephadex G-25, 5 x 1.5 cm, Pharmacia, Sweden). Only material in the first 2 ml after the void volume was collected and used. Since the toxin concentration was below the dilution end point extra replications of each treatment were pooled. After desalting, the replicates were combined, taken to dryness by lyophilization, resuspended in 1.0 ml 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 9 times and the controls (b, c, d) were repeated 3 times each. Periodate modification of the partially purified toxin was confirmed by submitting it to the Smith degradation (11), and demonstrating a molecular weight change with PD-10 column chromatography.

Tests for enzymatic inactivation (21) involved only extracts from KS-1b. Test samples included 1.0 ml of an appropriate enzyme (0.01-0.4 mg protein/ml) plus 1.0 ml of extract (a 50% dilution with buffer). Buffers used: 50 mM pH 4.5 citrate-phosphate and 50 mM pH 7.2 phosphate (for β-galactosidase); all buffers included 25 µg/ml of tetracycline

and streptomycin sulfate. Controls included: autoclaved enzyme plus extract and buffer, enzyme and buffer, extract and buffer, and buffer alone. Treatments were incubated 48 hr at 25 C in the dark. Reactions were terminated by immersing the treatments in boling water for 10 min and then centrifuging out the precipitate. The treatments were then dialyzed exhaustively against water and finally bioassayed.

The enzymes used were tested for activity and specificity under the conditions employed in the inactivation tests. 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 0.02 units/mg gal eq. Proteinase K was obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN; all substrates and the other enzymes were from Sigma Chemical Co., St. Louis, MO. All enzymes demonstrated expected activity and specificity when assayed with the following substrates: 0.5 mg/ml of *p*-nitrophenyl-β-galactoside, *p*-nitrophenyl-α-galactoside, *p*-nitrophenyl-β-galactoside, *p*-nitrophenyl-α-mannoside, 0.65% casein and a chitin suspension. Procedures for the assay of proteinase K and chitinase were obtained from Sigma Chemical Co.; glycoside assays were after English et al (8).

RESULTS

Partial Purification of Toxic Polysaccharides

Culture filtrates of *C. trifolii*, typically 800 ml per extraction, were collected ten days post-inoculation. Filtrates were acetone precipitated, centrifuged and the pellets resuspended in water (Table 1). Materials retained on the ultrafiltration membrane (nominal retention for globular proteins is 1×10^4 daltons for PM-10 membranes) contained the partially purified toxin as determined by the excised leaf bioassay. Controls of comparably treated uninoculated medium demonstrated no toxic activity after ultrafiltration. Crude filtrates of samples and medium controls were toxic due to culture media components, while little phytotoxicity was observed in PM-10 filtrates with either samples or medium controls.

Bioassay activity (Table 1) was reported as the lowest dilution at which 50% of the leaves developed symptoms. All isolates produced essentially the same amount of gal eq material per mg mycelium. While the carbohydrate and protein content generally increased, the protein: carbohydrate ratio changed little through the extraction (ca. 98-99% carbohydrate). In early experiments, Dowex 1 and 50 ion exchange chromatography followed ultrafiltration (18). Essentially all of the carbohydrate and biological activity was not retained by the column. Also, little change in protein content occured (data not shown). Therefore, this step was omitted in latter extractions.

Gel permeation chromatography of the extracts from all three isolates following ultrafiltration produced similar elution profiles (Fig. 1). All produced broad anthrone-positive peaks starting at the void volume (V_o) and extending into the fractionation range of the

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

Extraction Step	Isolate	Carbohydrate[1] (mg/ml)	Protein (mg/ml)	Protein:Carbohydrate Ratio	Activity[2] dilution end point	Activity[2] mg gal eq/ml at end point	mg gal eq/ mg mycelium
Crude	KS-1a[3]	1.5	0.01	0.008	NB[4]	-	-
	KS-1b[3]	2.2	0.05	0.022	NB	-	-
	FVT-2	ND[4]	ND	-	-	-	-
	Clayton	ND	ND	-	-	-	-
Resuspended 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
Filtrate	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

1) Expressed as galactose equivalents (gal eq).
2) Dilutions at which 50% of the leaves showed symptoms, calculated relative to original volume.
3) Two preparations of KS-1 used, denoted a and b.
4) ND - not determined. NB - not bioassayed. Media components were toxic making bioassays of crude and sometimes resuspended precipitates meaningless
5) Mycelial dry weights (mg/ml filtrate): KS-1a 16, KS-1b 17, FVT-2 16, Clayton 16.

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

One hundred mg gal eq of each preparation were applied to a Bio-Gel A.5M column, 5 ml fractions were collected at an elution rate of 15 ml/hr at 1-5 C. The column, 66 x 2.6 cm, was equilibrated and eluted with 50 mM NaCl. Results are expressed as mg gal eq/ml. Void volume (V_o) of the column was 145 ml.

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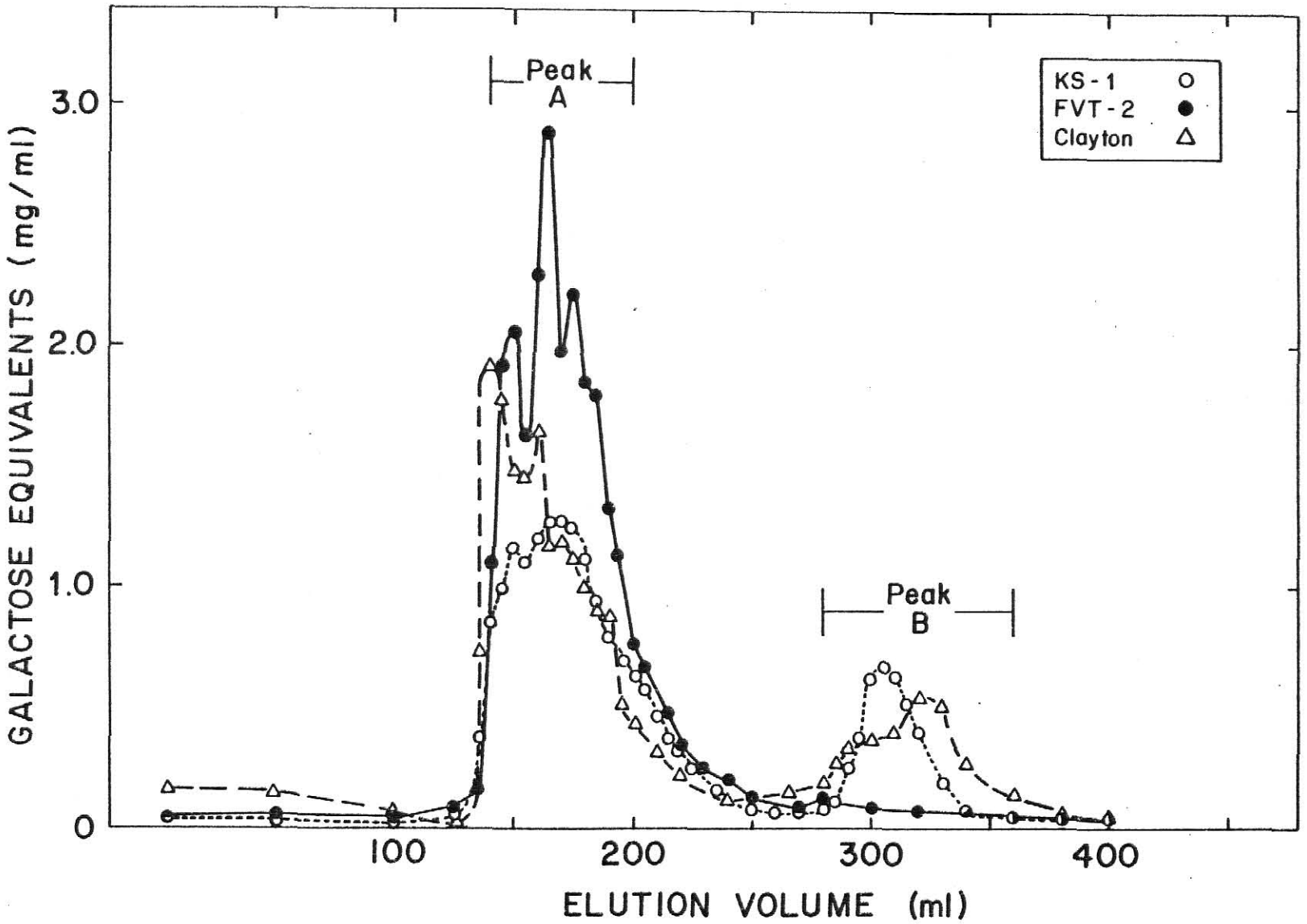
Peak
A
Peak
B
KS-1
FVT-2
Clayton
GALACTOSE EQUIVALENTS (mg/ml)
3.0
2.0
1.0
0
100
200
300
400
ELUTION VOLUME (ml)

column. Clayton and KS-1 also produced small secondary polysaccharide peaks. Phytotoxic activity was primarily located in the pooled peak fractions of the large peaks, while the smaller peaks showed only traces of activity.

Phytotoxicity of Extracted Culture Filtrates

The partially purified extract was phytotoxic to excised leaves, seedlings, and older shoots (18). Symptoms of excised leaves showed the following sequence: leaflet margins became pale green, within 6-12 hr, and the discoloration spread inwardly. With higher dilutions symptom onset was delayed. Desiccation followed also moving from the margins inward. Eventually the tissues became necrotic and brittle. At low dilutions the entire sequence was complete within 48-60 hr. Both Arc and Kanza demonstrated similar symptom development in the bioassay and were equally sensitive in dilution series. All three isolates produced compounds with similar activities (dilution endpoint, Table 1), however, the amounts of mg gal eq/ml at the dilution end points differed. These variations between the isolates may reflect experimental variation, minor differences in the polysaccharides produced, or inactive contaminating oligo-/polysaccharides.

Due to the preponderance of carbohydrate in the extracts it was of interest to study the effects of unrelated dextrans of various molecular weights on alfalfa. These compounds at 500 µg/ml induced similar symptoms to those of the *C. trifolii* toxin.

Isolate Pathogenicity

All isolates were more pathogenic on Kanza ("susceptible") than on Arc ("resistant") (Table 2). The mean scores varied only slightly except for Clayton which was more pathogenic on Kanza than the other isolates.

Table 2. Pathogenicity of *Colletotrichum trifolii* isolates on "resistant" and "susceptible" alfalfa cultivars

Isolate	Cultivar[1]	Percent of plants in each score class[2]					Mean Score	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

1) Arc is anthracnose resistant, Kanza is anthracnose susceptible.

2) Score classes are: 1 - no lesions, 2 - long, narrow lesions, 3 - large lesions nongirdling, 4 - coalescing and girdling lesions, 5 - lethal to seedling.

Host Specificity

Excised leaf assays involving extracts from the Clayton isolate demonstrated limited host specificity. Symptoms developed on tomato, soybean, and corn while wheat remained unaffected. 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 greyish cast by 24 hr. Symptoms developed rapidly on soybean leaves. They became pale and began to wilt within 4 hr and quickly became desiccated. No symptoms were evident on controls of water and uninoculated medium extracts.

Hypersensitivity Induction

Soybean cotyledons and bean hypocotyls treated with culture filtrate extracts demonstrated a HR. Bean hypocotyls became brown in treated areas while soybean cotyledons developed a brown to reddish color within 24 hr. Controls of water and comparably extracted uninoculated medium showed no significant response.

Inactivation of Toxic Activity

Partially purified toxin exposed to autoclaving and pH extremes (<2 and >11) remained active while toxin hydrolyzed with TFA was inactivated. Periodate oxidation induced a 12 hr delay in symptom onset as compared to controls (inactivated periodate plus extracts and extracts alone). Materials treated after the Smith degradation showed a decrease in molecular size as shown by its elution near galactose (Fig. 2).

Of the enzymes tested, only β-galactosidase and α-mannosidase affected activity (Table 3). The effect of the two active enzymes was

Fig. 2. Elution pattern of treated and untreated partially purified toxin as compared to galactose.

Partially purified toxin from KS-1b was treated by either periodate oxidation or the Smith degradation and eluted through a PD-10 column (Sephadex G-25, 5 x 1.5 cm) with deionized-distilled water and fractions of 0.5 ml were collected. Galactose and untreated extracts are for reference only, and were applied at different concentrations than the treated extracts. Results are compared to the elution patterns of galactose and untreated extracts. Results are expressed as absorbance at 620 nm following an anthrone assay.

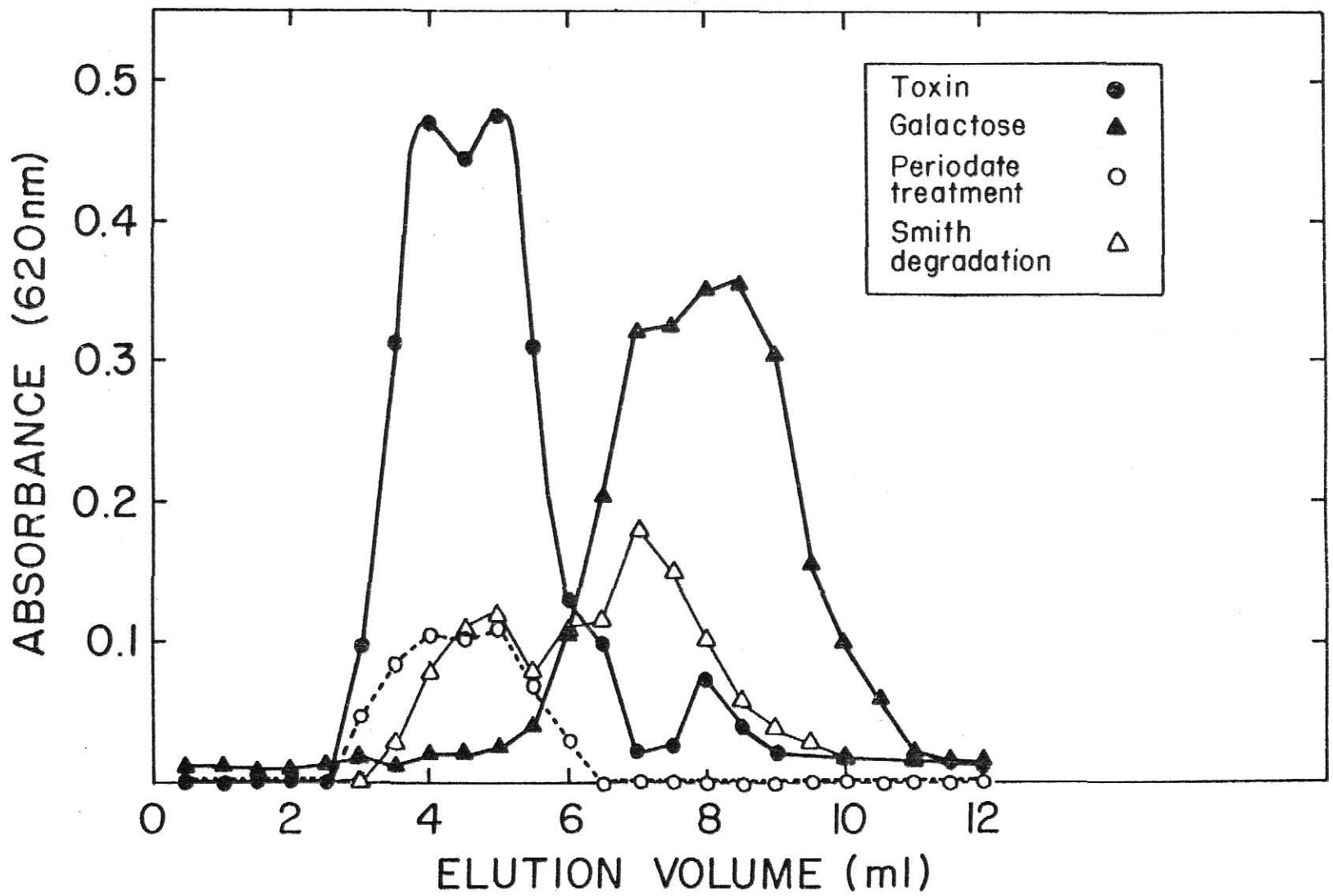
ABSORBANCE (620nm)
0.5
0.4
0.3
0.2
0.1
0
2
4
6
8
10
12
ELUTION VOLUME (ml)
Toxin
Galactose
Periodate treatment
Smith degradation

Table 3. Effect of various enzymes upon the toxic activity of polysaccharides from *Colletotrichum trifolii*

Enzyme[2]	Bioassay time (hr)				
	0	12	24	36	48
β-Glucosidase	0±0	2±0	3±0	4±0	4±0
α-Galactosidase	0±0	2±0	3±0	4±0	4±0
β-Galactosidase	0±0	1±0	1.5±0	2.4±.2	3.4±.2
α-Mannosidase	0±0	0±0	0.6±.5	1.4±.8	2±.8
Chitinase	0±0	2±0	3±0	3.5±0	4±0
Proteinase K	0±0	2±0	3±0	3.5±0	4±0
Controls[3]					
Autoclaved enzyme, buffer, toxin	0±0	1.7±.5	2.4±.8	3±1.1	3.4±.8
Enzyme, buffer	0±0	0±0	0±0	0±0	0±0
Toxin, buffer	0±0	1.6±.5	2.4±.8	3.3±.8	3.5±.7
Buffer	0±0	0±0	0±0	0±0	0±0

1) KS-1 (preparation b) toxin was exposed to anzymes for 48 hr before bioassay. Symptom score classes: 0 - no symptoms, 1 - marginal paling, 2 - general paling (>50% of surface), 3 - marginal desiccation, 4 - total desiccation and death.
2) Mean score of 9 leaves per enzyme (±S.D.).
3) Mean score of 18 leaves (±S.D.).

similar to that of low polysaccharide concentration. Symptom severity was not as pronounced early in the bioassay.

Monosaccharide Analysis

Toxic materials purified by acetone precipitation, ultrafiltration, and column chromatography were hydrolyzed, and the resulting aldoses separated and identified by HPLC (Appendix). Table 4 presents estimations of each sugar (µg/ml). The predominant sugar in the primary peak (peak A) of all three isolates was galactose, followed by mannose with a trace of glucose. The secondary peak (peak B) of the Clayton and KS-1 isolates contained only glucose. These results were confirmed by paper chromatography. Trace amounts of hexuronic acid component were also suggested by co-chromatography with galacturonic and glucuronic acids, and by the carbazole assay. Also, trace amounts of amino and/or N-acetylated amino sugars were suggested by colorimetric assay, however, no absorbance was observed in partially purified materials at 205 nm.

Differences between microanthrone assays of total hexoses before and after hydrolysis may result from sugar degradation. Degradation was confirmed by the presence of a brown color in the hydrolysate and the occurrence of an absorbance peak at 280 nm after hydrolysis. The disparity between the amount of anthrone-positive material after hydrolysis and the monosaccharides recovered following HPLC may be explained as follows. Not all sugars react identically in the anthrone assay. However, only galactose was used as a standard possibly biasing the results. The extracts may also possess materials detectable via the anthrone assay but not by HPLC. Degradation of the sugars during hydrolysis may contribute to data disparity. The single hydrolysis

Table 4. Estimation of monosaccharides present in extracts separated by chromatography as determined by high performance liquid chromatography

Isolate	Elution Peak[1]	Monosaccharide[2] (µg/ml)				Total Hexose[3] (µg/ml)	
		Mannose	Galactose	Glucose	Total	Before Hydrolysis	After Hydrolysis
KS-1	A	21	52	5	78	421	360
	B	0	0	3	3	10	3
FVT-2		16	70	6	92	484	435
Clayton	A	6	21	4	31	380	241
	B	0	0	10	10	130	56

1) As separated on a Bio-Gel A.5M column, 100-200 mesh (Fig. 1). Peak A represents high molecular weight component.
2) Estimation by area triangulation of peaks and comparison to standard curves.
3) Determination by microanthrone procedure.

preceeding HPLC may not be sufficiently effective to reduce the entire polysaccharide to monomers, and breakdown products may not be separated or detected by HPLC. Any or all of the above may lead to the disparity of the results in Table 4.

DISCUSSION

The toxic compounds produced *in vitro* by *C. trifolii* are largely, if not totally, polysaccharide, as judged by the following criteria: they exhibit a positive anthrone reaction, are hydrolyzed to monosaccharides, are stable to autoclaving and pH extremes, and have a high molecular weight as shown by Bio-Gel A.5M column elution and ultrafiltration retention characteristics. 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 are less conclusive, but α-mannosidase and β-galactosidase do partially inactivate the toxic materials as expressed by a decrease in symptom severity in the bioassay. The 1-2% protein found in the partially purified toxin is apparently not requisite for biological activity, because toxicity persists after pronase (9) and proteinase K treatments and ion exchange chromatography.

The role of these polysaccharides *in vivo* during disease development has yet to be established. The symptoms induced in excised leaves, shoots, and seedlings (18) are similar to those observed in crown-infected plants. All three isolates tested produced similar toxic polysaccharides *in vitro*, and they did not vary significantly in pathogenicity. A comparison with recently reported highly virulent *C. trifolii* "races" (25, 32) would be of interest. However, these polysaccharides do not appear to be involved in host specificity, as both Arc and Kanza are equally affected and non-hosts of the pathogen, i.e. tomato, soybean, and corn, were also effected by the toxin.

The lowest concentration of toxic polysaccharides (Table 1) to induce symptoms appears high (ca. 600 µg/ml) as compared to other fungal polysaccharides from culture filtrates. Polysaccharides from *Phytophthora cinnamomi, P. cryptogea,* and *P. nicotianae* were active down to 50 µg/ml (34). 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 the extent of vascular obstruction (35). The size of fungal polysaccharides probably vary with the cultural and extraction conditions. Presently, the reasons for this variation in toxic activity is unclear. Additional fractionation of the heterogenous polysaccharide mixture is needed to establish whether specific components of high toxic activity exist, as are additional studies on the influence of cultural conditions upon production of these polysaccharides.

The mode of action of these materials remains inconclusive. Commercial dextrans of $>2.5 \times 10^5$ daltons can cause vascular obstruction in alfalfa leaves at picomole levels (35). Yet wilt-inducing β-1, 3-glucans isolated from several *Phytophthora* species *in vitro* are extensively metabolized during wilt induction, suggesting toxin:cell interactions (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 to cause these responses than toxin:cell interactions because toxic activity was retained by periodate-treated polysaccharides, which, while oxidized, showed little change in molecular size as shown by column

chromatography. Presently, generalizations are difficult especially with a heterogeneous population of molecules, because the possibility of some cell-specific molecules cannot be eliminated.

Anderson (1) has reported a culture filtrate polysaccharide from *C. trifolii*, of somewhat different monosaccharide composition, that elicits HR in red kidney bean (*Phaseolus vulgaris* L.) cotyledons. The *C. trifolii* polysaccharides reported here are similarly active on red kidney bean and soybean in HR production. Thus, these preparations are capable of both HR induction and toxic activity, depending upon the plant species and bioassay. Presently, it is not possible to distinguish between induction of both responses by all components of the polysaccharide mixture, and specific induction of each response by singular components of the mixture. Until attempts are made to separate the HR elicitor and the toxin, the suggestion that elicitor vs. toxin functions are dependent only upon the choice of assay (21) cannot be evaluated.

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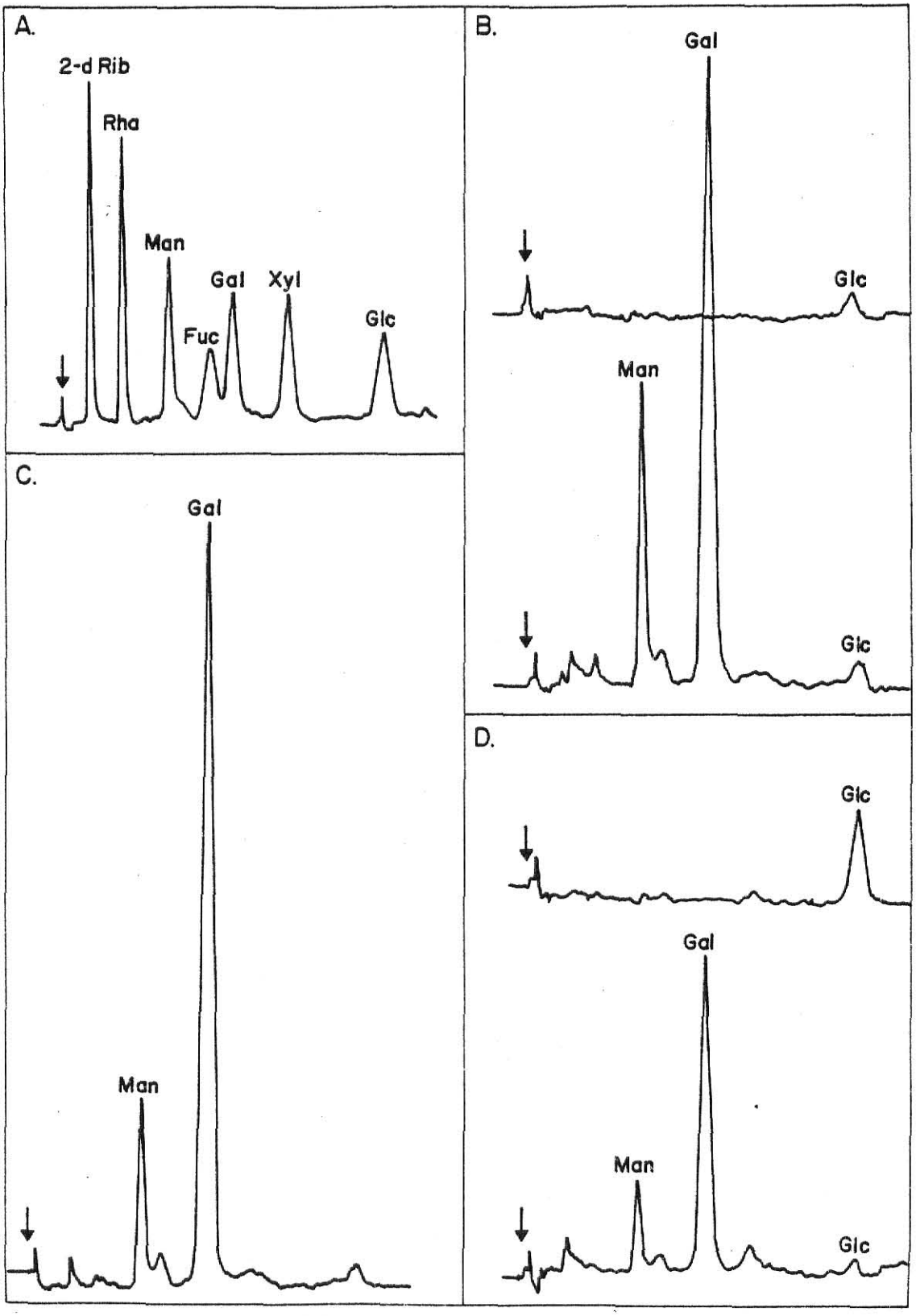
APPENDIX

The following graphs are the HPLC recorder outputs generated by injections of: standard aldoses, toxin from KS-1 peak A, FVT-2, Clayton peak A, Clayton peak B and KS-1 peak B, respectively.


A.
2-d Rib
Rha
Man
Fuc
Gal
Xyl
Glc
B.
Gal
Glc
Man
Glc
C.
Gal
Man
D.
Glc
Gal
Man
Glc

CHARACTERIZATION OF TOXIC POLYSACCHARIDES PRODUCED IN VITRO BY COLLETOTRICHUM TRIFOLII

by

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ABSTRACT

Polysaccharides produced *in vitro* by *Colletotrichum trifolii* were found to cause 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 isolate of *C. trifolii* tested produced similar phytotoxic polysaccharides. Symptoms were induced in cuttings from susceptible (Kanza) and resistant (Arc) alfalfa, tomato, corn, and soybean, and a hypersensitive 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 what is probably a uronic acid were found in hydrolysates. Toxic activity was reduced by periodate oxidation and treatment with α-mannosidase and β-galactosidase, and totally removed by hydrolysis. Treatment with proteinase K, autoclaving, and a pH of less than 2 or greater than 11 did not alter activity. Excised alfalfa leaves fed commercial dextrans (40 to 500 x 10^3) daltons) developed symptoms similar to those induced by the *C. trifolii* polysaccharides.