

Biological Activity and Specificity of a Toxin Produced by *Cladosporium fulvum*

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ABSTRACT

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The biological activity of a glycoprotein toxin produced in vitro by *Cladosporium fulvum* was studied by necrosis, callose deposition, and ion leakage assays. Concurrent appearance of callose and necrosis was not altered by the toxin's degree of purification or concentration. Increased leakage of electrolytes from toxin-treated tomato leaf tissue was concentration-dependent, and this assay proved to be the most sensitive. As judged by necrosis and callose deposition, *C. fulvum* toxin from races 1, 10,

and 12 had similar effects on all tomato cultivars regardless of gene for resistance. Similarly, quantitative comparisons of ion leakage from different tomato cultivars treated with purified race 1 toxin failed to support an earlier report that the toxin was cultivar-specific. Nonhost plant species were unaffected by the toxin. The toxin stimulated the respiratory rates of tomato and of nonhost species tobacco and pepper.

Additional key words: tomato leaf mold.

Van Dijkman and Kaars Sijpesteijn (27-29) suggested that the gene-for-gene relationship between tomato and *Cladosporium fulvum* Cooke is based on production by the pathogen of specific toxins that interact with genetically controlled receptor sites on the host cell membrane. They proposed that interaction of the toxin with the receptor sites in a resistant cultivar results in expression of resistance because membrane damage causes necrosis and prevents further growth of the fungus. More recently, histological studies (9,20,21) showed that in addition to necrosis, callose deposition on host cell walls is one of the dominant features seen in tomato cultivars infected by an incompatible race of *C. fulvum*. In preliminary studies (19), callose deposition and host cell necrosis occurred in tomato leaves injected with high molecular weight components isolated from culture filtrates or spore germination fluids of *C. fulvum*. This reproduction of some of the observed symptoms supported involvement of the toxic material in leaf mold disease.

Our study details the effect of the toxin on callose formation, necrosis, and ion leakage in tomato leaf tissue and examines the specificity of the toxin on tomato cultivars and nonhost species. Concurrently, the toxin was characterized as a polydisperse glycoprotein (18).

We have retained Bailey's (6) terminology for the differential responses of tomato to *C. fulvum*. Histological examinations (20,21) have confirmed three distinct reactions under optimal environmental conditions. In the susceptible response, symptoms are absent until sporulation is complete. In a resistant plant, fungal growth is extensive but the emergence of aerial mycelium is accompanied by considerable necrosis. The highly resistant (immune) reaction is a typical hypersensitive necrotic response that occurs soon after penetration. Under suboptimal conditions, infected susceptible tissues also become necrotic, a reaction termed "susceptible killing" by Bailey (6). We have continued to use the term "immune," although "highly resistant" is preferable, to conform to the tables (23) outlining differential responses of cultivars to *C. fulvum*.

MATERIALS AND METHODS

Cultivation of tomato plants. Seeds were obtained from the Vineland Horticultural Experimental Station. Host lines were checked initially for uniformity of behavior under the planned experimental conditions, and the same seed lot was used throughout the study. Plants were grown from seed (1-4 plants per 4-in. pot) in a standard soil mix and were maintained in a controlled-environment chamber calibrated to the specifications outlined by Patrick et al (23), including a day length of 12 hr at about 13,000 lux and day and night temperatures of 24 and 18 C, respectively. The humidistat was kept at the highest setting, so that relative humidity was at or close to 100% when the chamber was filled with plants.

Culture of *C. fulvum* and toxin production. *C. fulvum* (Cf) races 1, 10, and 12 were obtained from the collection of Z. A. Patrick and were the isolates used in the differential race studies of Patrick et al (23). Single-spore cultures were established and maintained on V8 juice agar or stored in sterilized soil culture (23). Modified Fries' medium was used for liquid culture of *C. fulvum* and contained K₂HPO₄ (1 g), (NH₄)₂NO₃ (1 g), MgSO₄·7H₂O (0.5 g) or MgSO₄ (0.244 g), CaCl₂ (0.13 g) or CaCl₂·2H₂O (0.172 g), NaCl (0.10 g), ammonium tartrate (5 g), sucrose (30 g), and yeast extract (1 g) per liter of distilled water. The yeast extract was prepared by dissolving 50 g of yeast extract (Difco) in 100 ml of water and adding 900 ml of 100% methanol. The mixture was stirred, and insoluble material was removed by filtration. The soluble fraction was concentrated at 40 C under reduced pressure, and the final volume was adjusted to a concentration of 0.5 g of yeast extract per milliliter after correction for loss of the methanol-insoluble material.

Ten agar disks (7 mm diam) from V8 agar plates bearing an even layer of sporulating *C. fulvum* were transferred to 100 ml of sterile medium in 500-ml or 1-L Erlenmeyer flasks, which were gently swirled to dislodge spores from floating colonies. The cultures were incubated for 3 wk at 24 C under an illumination of 4,000 lux for 15 hr a day. The liquid medium then was filtered through a glass-wool plug and readjusted to original volume with the distilled water that had been used to rinse the mycelial mat. The crude filtrate, now considered to be at the original concentration, was filtered through Whatman No. 1 filter paper and stored at -20 C.

Filtrates were purified by a combination of methanol precipitation or dialysis, DEAE-cellulose chromatography, affinity chromatography on concanavalin A-Sepharose 4B, and gel chromatography on Sephadex G-100 and Sepharose 4B-CI. These procedures are described in detail elsewhere (18). Toxin described as partially purified had undergone methanol precipitation and chromatography on DEAE-cellulose and Sephadex G-100, unless otherwise noted. Toxin described as purified had been through all steps. Data for the purified Cf race 1 toxin used in ion leakage assays is given in Lazarovits et al (18).

Bioassay technique. The toxin's ability to cause host cell necrosis and callose production was tested by injecting it into the intercellular areas of tomato leaves, at the junction of the midrib and lateral vein. Generally, 5–15 μ l of liquid was injected through a fine glass needle at one site, an area of about 1 cm². At least one leaflet on each of four plants was injected. The plants were maintained 15–24 hr at about 95% relative humidity in the growth chamber and were examined after exposure to a less humid environment for at least 30 min.

A scale of 0–4 based on the visible reaction of injected areas was used to assess toxin activity: 0 = no symptoms by 48 hr; 1 = slight darkening of tissue viewed through background light and pinpoint necrotic spots; 2 = pitting where groups of cells had collapsed and discoloration evident without background light; 3 = extensive but not uniform necrosis; 4 = completely collapsed and uniformly necrotic tissue.

Callose deposition was determined in treated tissue by decolorizing leaf pieces in boiling methanol and staining them in 0.01% aniline blue in 0.1 M K₂HPO₄ at pH 9.0 for at least 24 hr. The tissue was mounted on quartz slides and examined on a Zeiss or Reichert microscope using an Osram Mercury Super Pressure Lamp 200W/4 and an exciter filter with transmission range of 280–400 nm and barrier filter 41 (transmission only above 400 nm). Callose deposits were identified by bright yellow fluorescence in the presence of aniline blue (7, 14). Fluorescence in the tissues was rated on a scale of 0–4 or as Q (quenching): 0 = no fluorescence at injected site; 1 = flecking reaction, walls with small areas of fluorescence, or fluorescence in individual cells; 2 = groups of cells with strong fluorescence in walls but most with only flecks or no fluorescence, bright fluorescence often in parenchymal cells associated with veins; 3 = uniform fluorescence in walls of many cells so that entire cells distinguishable, large groups of cells with very brilliant fluorescence; 4 = some degree of fluorescence in entire area, large patches of cells with extremely brilliant fluorescence, remaining cells fluorescent along part of a wall; Q = severe necrosis, weak fluorescence where browning extensive, brilliant fluorescence where browning absent.

Data for necrosis and callose deposition are given as either individual or average ratings. Average ratings should be compared on a semiquantitative basis only because the response of different plants to a particular sample varied.

Ion leakage assay. Entire leaflets of tomato plants at the 6–7 leaf stage were infiltrated with test solutions (about 0.5 ml/leaflet) by injection into the interveinal areas. Solutions to be compared for activity generally were injected into opposite or adjoining leaflets of the same leaf. Leaves 3, 4, and 5 usually were selected. Injected plants were covered with a polyethylene bag to maintain high relative humidity and incubated 14–16 hr (overnight) in a growth chamber at 68 C. Injected leaflets then were excised, and 9-mm leaf disks were removed with a cork borer and placed immediately in 50 ml of distilled water. After two rinses with 50 ml of water, the disks were drained for 1–2 min on filter paper and transferred as eight disks per replicate to 25-ml flasks containing 5 ml of distilled water. Normally, two leaflets per treatment yielded enough disks for four replicates. As soon as all treatments were prepared, conductivity of the 5 ml of bathing solution was measured with a Radiometer Type CDM 2 conductivity meter with a CDC 104 cell. Disks were incubated on a wrist-action shaker at laboratory temperatures of 25–27 C, and conductivity measurements were made at 1- or 1.5-hr intervals for at least 6 hr. Increase in conductivity with time (net conductivity) was obtained by subtracting the initial background reading from subsequent measurements.

Measurement of respiratory rate. Leaf disks (9 mm) were prepared from leaflets injected with test solutions as described for ion leakage experiments. Six disks were floated on 3 ml of distilled water in the outer area of respirometer flasks. The center well contained 0.5 ml of Pardee buffer (25) at equilibrium with 1% CO₂. Oxygen uptake at 26 C was measured on a Gilson Differential Respirometer in the dark. The respiratory rate as microliters of O₂/hr/six leaf disks was established from the slope of the graph after five or six readings at 1-hr intervals using three or four replicates per treatment.

RESULTS

General aspects of callose deposition and necrosis. The studies using callose deposition and host cell necrosis as bioassays were designed to determine (i) cultivar specificity of the toxin, (ii) time required for a detectable reaction, (iii) distribution of necrotic cells and callose in treated tissue, (iv) minimum effective dose, and (v) whether necrotic response and callose deposition were separable. Initially, the toxin's specificity for cultivars with different genes for resistance to *C. fulvum* (Cf genes) was tested by injecting serial dilutions of crude culture filtrates from Cf race 1 into the following tomato cultivars: Potentate (Cf0), Stirling Castle (Cf1), Vetomold (Cf2), V121 (Cf3), Purdue 135 (Cf4), and Vinequeen (Cf2, Cf4). *Lycopersicon pimpinellifolium*, the source of genes Cf3 and Cf4, also was tested. All toxin-treated plants showed callose formation and host cell necrosis, despite inclusion of cultivars susceptible (Cf0), resistant (Cf1, Cf3), and immune (Cf2, Cf4) to race 1. In most subsequent tests, Potentate, V121, and/or Vinequeen were used, and their reactions to a given race of *C. fulvum* were designated (S), (R), or (I), for susceptible, resistant, and immune, respectively.

In preliminary experiments, relative humidity strongly influenced the early (< 24 hr) development of symptoms. For example, a leaflet injected with a relatively high toxin concentration (> 100 μ g glucose equivalents/ml) might be given a rating of 1 when first removed from the humid growth chamber 15 hr after injection. This rating might change to 4 after about 30 min as the injected site collapsed and became desiccated in the drier laboratory environment. The progression to a rating of 4 might take up to 30 hr if the plant remained in the growth chamber. Consequently, in experiments in which tissue collapse was undesirable (eg, callose development and ion leakage assays), a high relative humidity was maintained until leaf samples were removed.

Partially purified Cf race 10 toxin at 50 μ g glucose equivalents/ml was used to study the time required by tomato leaf cells to become necrotic and develop callose after toxin treatment. Potentate (S),

TABLE 1. Necrosis and callose formation in tomato in response to injection of partially purified toxin from *Cladosporium fulvum* race 10^a

Time after injection (hr)	Potentate (susceptible)		V121 (resistant)		Vinequeen (immune)	
	Necrosis ^b	Callose ^c	Necrosis	Callose	Necrosis	Callose
3,6,9	0	0	0	0	0	0
12	1.0	2.3	1.0	2.0	0.3	1.3
15	1.0	2.3	1.0	2.3	1.0	1.7
18	2.0	2.3	2.3	2.3, Q	1.7	2.0, Q
21	3.3	3.0, Q	3.3	Q	2.7	2.7
24	4.0	3.0, Q	3.3	3.0, Q	3.0	2.5, Q

^a Toxin was purified by dialysis and by chromatography on DEAE-cellulose and Sephadex G-100 (20). Toxin concentration injected into leaflets was 50 μ g glucose equivalents/ml.

^b Necrosis was rated macroscopically on a 0–4 scale in which 0 = no necrosis and 4 = total collapse of injected site. Values are average ratings for three leaflets.

^c Callose deposition was estimated microscopically by fluorescence of aniline blue-stained tissue and rated on a scale of 0–4 in which 0 = no fluorescence and 4 = maximum fluorescence; Q = quenching of fluorescence by browning products in at least one sample. Values are average rating for three leaflets.

V121 (R), and Vinequeen (I) were injected at 3-hr intervals over a period of 24 hr without removing the plants from the growth chamber. Leaves injected with distilled water served as controls. At the end of 24 hr, the injected sites were excised, rated for necrosis, and prepared for callose determinations.

Cell necrosis and callose deposition were first detected in the three cultivars 12 hr after injection (Table 1), indicating that detectable levels developed sometime between 9 and 12 hr. The amount of necrosis and callose increased up to about 21 hr (Table 1). No distinct or consistent differences among the three cultivars were noted.

Fluorescence usually was first detectable in the walls of cells bordering the vascular bundles. Palisade and spongy mesophyll cells generally reacted either as individual cells with some fluorescence along one wall or as clumps of cells with more uniform fluorescence along the entire wall. Later, increased numbers of mesophyll cells plus some epidermal cells had entirely fluorescent walls. Only cells in the injected area were fluorescent, the veins apparently acting as boundaries between injected and noninjected sites. Generally, the injected sites were not uniformly fluorescent but were characterized by large clumps of cells with brilliant uniform fluorescence separated by zones where only a few cells were fluorescent. Spongy mesophyll cells showed more extensive callose development than palisade cells.

Correlation of necrosis with fluorescence depended on whether tissue was examined microscopically for necrosis. Tissue without macroscopic necrosis often had clumps of necrotic cells or individual necrotic cells dispersed throughout the injected site. Necrotic and neighboring cells sometimes were associated with fluorescent material, and apparently healthy cells frequently showed extensive fluorescence. Thus, callose production was a more sensitive test for toxin activity than macroscopic estimation of necrosis, but microscopic observation for necrosis was comparable to the callose test.

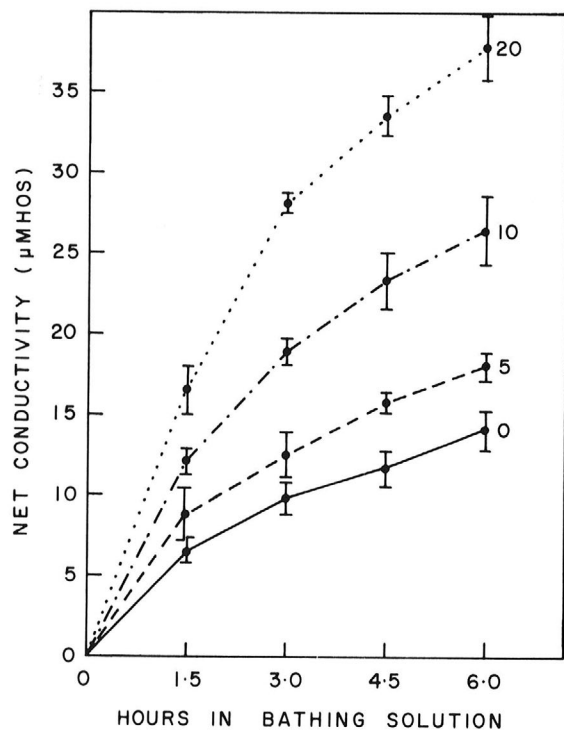


Fig. 1. Effect of the concentration of purified toxin from *Cladosporium fulvum* race 1 on electrolyte loss from tomato (Potentate) leaflets. Injected leaflets were incubated for 15 hr before preparation of 9-mm disks (eight per replicate); disks were placed in 5 ml of distilled water on which conductivity was measured. Net conductivity is the reading at each interval minus the background reading at 0 hr. Values are the means of three or four replicates \pm the standard deviation of the mean. Toxin concentration is expressed as μ g glucose equivalents/ml.

Partially purified Cf race 10 toxin at concentrations of 10, 20, 30, 40, and 50 μ g glucose equivalents/ml was injected into Potentate (S) and V121 (R). The minimum effective dose for necrosis or for callose deposition was defined as that concentration at which all injected sites gave a rating of at least 1. The minimum effective dose for both callose deposition and necrosis was 20 μ g glucose equivalents/ml, but three of four injected leaflets showed callose at 10 μ g glucose equivalents/ml (Table 2). Necrosis was rated macroscopically, which accounts for the greater sensitivity of the callose assay. In similar tests performed with race 10 toxin at other stages of purification or with race 1 or 12 toxin, the minimum effective dose ranged from 10 to 20 μ g for callose and from 20 to 30 μ g for necrosis. No consistent differences between the cultivars were observed in dosage response tests.

Ion leakage of toxin treated tissue. In preliminary experiments, electrolyte leakage from leaflets injected with toxin preparations was significantly greater than that from leaflets injected with distilled water (or with Fries' medium if a crude toxin preparation was used). Increased ion leakage could be detected in disks cut as early as 3 hr after injection of the leaflet. The time (3-4 hr) required to inject several test solutions, however, made a short incubation time technically impractical. Incubation overnight gave comparable results if tissue collapse was prevented by maintaining a high humidity.

Ion leakage from treated leaf tissue increased with increasing concentration of toxin within the range of 5-20 μ g glucose equivalents/ml. Data for Potentate (S) injected with purified toxin from Cf race 1 are typical (Fig. 1). At toxin concentrations above 20 μ g glucose equivalents/ml, the magnitude of ion leakage varied considerably among experiments. Routine assays were done at 10 μ g glucose equivalents/ml, a concentration at which the variability was reduced but not eliminated. Although the rate of electrolyte loss to the bathing solution declined with time, toxin-stimulated loss was still evident after 6 hr (Fig. 1). Conductivity as a percentage of the control treatment remained relatively constant with time. Consequently, some comparisons are summarized by presenting net conductivity at 4.5 hr and/or conductivity as a percentage of the control at 4.5 hr.

At the completion of some ion leakage assays, chloroform (to 2%) was added to the flasks and conductivity of the bathing solutions was measured after several hours of shaking. This measurement was termed "total leakable ions" and was in the range of 450-500 μ MHOS. Toxin-induced ion leakage by 6 hr of incubation rarely exceeded 40 μ MHOS, or less than 10% of total leakable ions.

Respiration of toxin-treated tissue. Toxin-stimulated oxygen uptake was consistently observed for tomato leaflets injected with toxin and incubated overnight before removal of disks for respirometry. Typical results for Potentate (S) are given in Table 3. In all experiments, a constant rate of oxygen uptake was established within the first hour and was maintained to the

TABLE 2. Effect of different concentrations of partially purified toxin from *Cladosporium fulvum* race 10 on callose deposition and necrosis in tomato cultivars Potentate (susceptible) and V121 (resistant)

Toxin concentration ^a (μ g glucose equiv./ml)	Potentate (S) ^b		V121 (R) ^b	
	Necrosis ^c	Callose ^d	Necrosis	Callose
10	0,0,0,0	0,1,1,2	0,0,0,2	0,1,1,1
20	1,2,2,2	1,1,3,3	1,1,2,2	2,2,2,3
30	1,1,2,3	2,3,3,4	1,1,1,2	1,2,3,3
40	1,2,2,3	1,2,3,3	1,1,1,2	3,3,4,Q
50	2,2,2,4	2,2,3,Q	2,2,2,4	1,2,3,Q

^aToxin was purified by dialysis and by chromatography on DEAE-cellulose and Sephadex G-100 (20).

^bOne leaflet on each of four plants was injected and each leaflet rated.

^cNecrosis was rated macroscopically on a 0-4 scale in which 0 = no necrosis and 4 = total collapse of injected site.

^dCallose deposition was estimated microscopically by fluorescence of aniline blue-stained tissue and rated on a scale of 0-4 in which 0 = no fluorescence and 4 = maximum fluorescence; Q = quenching of fluorescence by browning products.

conclusion 4–9 hr later.

Effect of *C. fulvum* toxin on nonhosts of *C. fulvum*. Dilutions of partially purified Cf race 1 toxin ranging from 50 to 2,000 μg glucose equivalents/ml were injected into one leaf of each of four plants of the following species: potato, tobacco, pepper, Jerusalem cherry, cowpea (primary leaves only), sunflower, cucumber, squash, cabbage, and tomato. Potato and Jerusalem cherry were grown in the greenhouse and the other species were grown in growth chambers as described for tomato. Callose formation and necrosis were not detected in any of the nonhost species, whereas necrosis developed in tomato at 50 μg glucose equivalents/ml. This experiment was repeated using toxin isolated from *C. fulvum* cell wall preparations (18), with similar results.

Leaves of tobacco (Havana) and pepper plants (4 wk old) injected with 100 μg glucose equivalents/ml of purified Cf race 1 toxin or with distilled water were compared by ion leakage and respiration assays. The toxin did not cause increased ion leakage in either pepper or tobacco but did stimulate oxygen uptake of both species (Table 3). Comparative data for tomato (Potentate) showed that ion leakage was stimulated to 260% of the control (Table 3).

Effect of high molecular weight polysaccharides on tomato. The possibility that callose production, necrosis, and ion leakage are general responses of tomato leaf tissue to high molecular weight compounds was examined by injecting starch and laminarin (K & K Labs, Division of ICN Pharmaceuticals, 121 Express St., Plainview, NY 11803), a β -1,3-glucan, into Potentate and Vinequeen. Laminarin and starch solutions containing 2 mg

glucose equivalents/ml failed to cause callose production or necrosis. Laminarin at 100 $\mu\text{g}/\text{ml}$ had no significant effect on ion leakage of Vinequeen (Table 3).

Cultivar specificity compared by ion leakage assays. Ion leakage and respiration of Potentate (S) and Vinequeen (I) were consistently stimulated by crude Cf race 1 toxin (methanol-precipitated only). With purified Cf race 1 toxin, results of simultaneous comparisons of ion leakage from Potentate and Vinequeen were inconsistent, Potentate and Vinequeen each

TABLE 3. Effect of purified toxin from *Cladosporium fulvum* race 1 (Cf R1) and of laminarin on electrolyte leakage and respiration of tobacco, pepper, and tomato

Plant	Treatment	Net conductivity ^a (μMHOS)	Rate of O ₂ uptake ^b ($\mu\text{l/hr/6 disks}$)
Tobacco (Havana)	Control ^c	12.7 \pm 2.5	6.2
	Cf R1 toxin ^d	14.3 \pm 2.1	8.2
Pepper	Control	10.7 \pm 0.7	6.5
	Cf R1 toxin	11.1 \pm 0.4	10.0
Tomato (Potentate)	Control	15.6 \pm 3.3	21.3
	Cf R1 toxin	40.5 \pm 2.2	44.3
Tomato (Vinequeen)	Control	15.4 \pm 3.3	...
	Laminarin ^e	14.0 \pm 0.4	...

^a Conductivity reading at 4.5 hr of incubation (of bathing solutions containing eight disks from treated leaves) minus conductivity reading at 0 hr. Values are average of three or four replicates \pm standard deviation of mean.

^b Established by readings over 6-hr incubation period on six disks cut from treated leaflets.

^c Leaflets injected with distilled water.

^d Concentration was 100 μg glucose equivalents/ml.

^e Dissolved in distilled water to give 100 $\mu\text{g}/\text{ml}$.

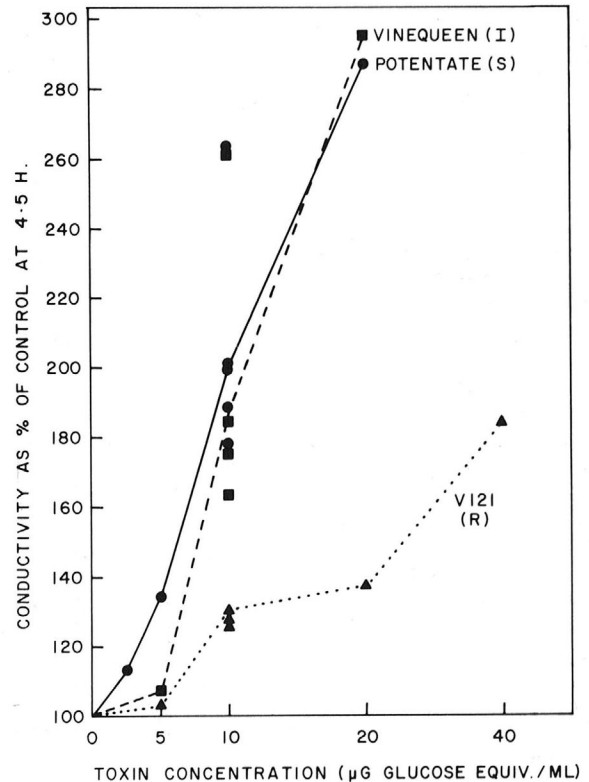


Fig. 2. Comparison of the effect of the concentration of purified toxin from *Cladosporium fulvum* race 1 on electrolyte loss from the tomato cultivars Potentate (susceptible), V121 (resistant), and Vinequeen (immune). Injected leaflets were incubated for 15 hr before preparation of 9-mm disks (eight per replicate); disks were placed in 5 ml of distilled water on which conductivity was measured at 1.5-hr intervals. Only the results for 4.5 hr are presented. The control treatment contained leaflets injected with distilled water. Unjoined points (● Potentate, ■ Vinequeen, ▲ V121) represent data from Table 4 and are included to illustrate the variation between experiments at toxin concentrations of 10 μg glucose equivalents/ml.

TABLE 4. Comparison of electrolyte loss from tomato cultivars Potentate, Vinequeen, and V121 after treatment with 10 μg glucose equivalents/ml of purified toxin from *Cladosporium fulvum* race 1

Cultivar	Treatment	Net conductivity ($\mu\text{MHOS} \pm \text{SD}$) at 4.5 hr ^a				
		Experiment ^b				
		1	2	3	4	5
Vinequeen (immune)	Control ^c	8.8 \pm 0.6	9.9 \pm 1.1	8.4 \pm 0.2
	Toxin	23.1 \pm 1.9 (263%) ^d	17.2 \pm 1.5 (174%)	13.7 \pm 2.2 (163%)
Potentate (susceptible)	Control	8.3 \pm 1.0	10.1 \pm 0.7	10.6 \pm 1.6	11.2 \pm 0.9	17.4 \pm 1.3
	Toxin	15.5 \pm 2.2 (187%)	20.2 \pm 0.9 (200%)	27.9 \pm 1.3 (263%)	19.8 \pm 0.6 (177%)	31.1 \pm 5.0 (179%)
V121 (resistant)	Control	11.2 \pm 0.4	10.3 \pm 0.6
	Toxin	14.3 \pm 1.3 (128%)	11.7 \pm 1.2 (114%)

^a Injected leaflets were incubated for 15 hr before preparation of 9-mm disks (eight per replicate). Disks were placed in 5 ml of distilled water, and conductivity was measured on the solution at 1.5-hr intervals. Net conductivity is reading at 4.5 hr minus reading at 0 hr and is average of three or four replicates.

^b Each experiment represents treatments compared simultaneously.

^c Leaflets injected with distilled water.

^d Conductivity of toxin treatment listed as percentage of control treatment.

appearing most sensitive to the toxin in at least one of three experiments (Table 4). When conductivity was expressed as a percentage of the control at 4.5 hr, the range for both cultivars was similar (Table 4). V121 was consistently less sensitive to the toxin than Potentate. Dosage response experiments done at separate times for each cultivar also indicated that Potentate and Vinequeen were similar in responses to race 1 toxin and that V121 was less sensitive than the other two cultivars when assayed by ion leakage (Fig. 2). The data suggest that Vinequeen may be less sensitive than Potentate at very low toxin levels, but more assays at concentrations $< 10 \mu\text{g}$ glucose equivalents/ml are required to confirm this possibility. The data from Table 4 superimposed as points on Fig. 2 show the degree of variability for experiments done at $10 \mu\text{g}$ glucose equivalents/ml and further illustrate the similarity of Potentate and Vinequeen in the leakage assays.

DISCUSSION

Reports by van Dijkman and Kaars Sijpesteijn (27-29) that *C. fulvum* produces a high molecular weight toxin that causes host cell membrane damage were confirmed in this study. The toxin also was shown to cause host cell necrosis and production of callose wall appositions, two symptoms known to occur in infected plants (20,21). Callose deposition and cell necrosis seemed to appear simultaneously in toxin-injected sites but were not necessarily present in the same cells. The coappearance of callose and necrosis was not altered by changes in toxin concentration and purity or by the source of the toxin. None of the observations make it possible to predict whether the toxin induces both callose and necrosis directly or whether products leaking from affected or dying cells stimulate callose deposition in adjacent cells.

The *C. fulvum* toxin also stimulated the respiratory rate of tomato leaf tissue, but this effect may not be linked to callose deposition, necrosis, or increases in electrolyte leakage, as respiratory rates also increased in nonhost species tobacco and pepper, in which the other effects were not detected. For this reason, respirometry was not used as a routine assay for toxin activity.

The *C. fulvum* toxin, a polydisperse glycoprotein (18), can be readily isolated from culture filtrates, from material enriched by the mycelial wall (18), and from fungal spores (17). Origins, level of activity, and composition suggest this toxin is comparable to the phytoalexin elicitors (26). Although some elicitors appear to contain only glucans (1,2,4,5), others also have protein components (8,11,15,24). The effect of elicitors on host cells has received scant attention, but those from *Phytophthora megasperma* var. *sojae* (Pms) (26), *P. infestans* (22), *Colletotrichum* spp. (1), and *Rhizopus stolonifer* (24) are reported to cause browning of host tissue. The minimum effective dose of the *C. fulvum* toxin for necrosis was calculated to be about 5×10^{-12} mol based on an average molecular weight of 60,000 (18), which is in the range of the 10^{-12} mol calculated for the Pms elicitor (3). There is a paucity of information on phytoalexins produced by tomato foliage, but recently de Wit and Flach (10) reported that unidentified phytoalexins in tomato leaves accumulated more rapidly in an incompatible *C. fulvum*-tomato combination than in a compatible combination.

Cultivar specificity of the *C. fulvum* toxin was reported by van Dijkman and Kaars Sijpesteijn (27-29), who found that leaf tissue treated with toxin from an incompatible race had greater leakage of a ^{32}P label than tissue treated with toxin from a compatible race or with buffer control solutions. Although Dow and Callow (12) initially reported similar correlations using isolated mesophyll cells in electrolyte leakage assays, they found more recently (13) that the observed specificity was not reproducible.

We found no indication that toxin damage was greater in incompatible combinations. Toxin from any race caused necrosis and callose deposition in any tomato cultivar tested. This response, however, could be compared only on a qualitative or, at most, semiquantitative basis. Comparisons of cultivar specificity by the more quantitative electrolyte leakage assay were restricted to the use of purified *C. fulvum* race 1 toxin on cultivars giving different responses to that race. Potentate (S) and Vinequeen (I) responded

similarly at toxin concentrations of 10 and 20 μg glucose equivalents/ml, but V121 (R) was less affected, as judged by ion leakage. This difference with V121 and the apparent differences among cultivars at lower toxin concentrations require verification by comparisons using other incubation times, other environmental conditions, other races, and, if possible, other techniques. The disparities between our results and those of van Dijkman and Kaars Sijpesteijn (27-29) are not easily resolved because of the differences in toxin purification and application methods and in the concentrations used.

In situ production of the toxin has not been shown, but the low level required to produce necrosis and callose deposition, the seeming specificity for tomato, and the ability to reproduce some of the symptoms all implicate the toxin in leaf mold disease. If the toxin is a cell wall component, as indicated by its isolation from material enriched by the cell wall (18), its presence in the infected plant is to be expected. Consequently, evidence that the toxin is released from the fungal cell wall into the intercellular area of the leaf may be needed to support activity in situ.

In the hypothesis proposed by van Dijkman and Kaars Sijpesteijn (27-29), the fungal product was responsible for inducing the hypersensitive response in incompatible interactions. Our data do not exclude involvement of the toxin in any cultivar-race combination, including the compatible interaction. In compatible tissues, necrosis normally does not occur until after sporulation unless humidity is reduced. Under such conditions, infected sites rapidly become necrotic and collapse, a response that has been termed "susceptible killing" (6). Comparable symptoms were observed in toxin-injected leaves. Although the necrotic reactions in these infected and toxin-injected sites appear to be identical, the mechanism producing the necrosis may be quite different. Nevertheless, the phenomenon of susceptible killing suggests that the *C. fulvum* toxin may not be released in lethal amounts from the cell wall of the invading hyphae except under conditions of stress. In susceptible killing, the stress may be decreased humidity. In immune and resistant responses, the toxin release may be caused by stress or injury resulting from a host resistant response; thus, necrosis may be a consequence of the resistance response as proposed by Kiraly et al (16).

In the susceptible interaction, *C. fulvum* has an extremely compatible relationship with its host, deriving all its nutrients from the intercellular space surrounding apparently healthy mesophyll cells (20,21). Sublethal levels of the toxin could facilitate nutrition of the fungus in this interaction. The observation that electrolyte leakage was stimulated by toxin concentrations that were too low (eg, 2.5 or 5 μg glucose equivalents/ml) to cause callose deposition or necrosis supports this idea. Thus, the hypothesis that the toxin normally occurs at sublethal levels in the infected plant but increases to lethal concentrations as a consequence of a host resistance response is currently under investigation.

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