Production and Nature of a Host-Specific Toxin from Alternaria alternata f. sp. lycopersici

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ABSTRACT

Cell-free culture filtrates from isolates of *Alternaria alternata* f. sp. *lycopersici* pathogenic to certain cultivars of tomato contained a toxin which elicited foliar symptoms characteristic of the stem canker disease on naturally infected tomatoes. Tomato cultivars, resistant to the pathogen were at least 1,000-fold less sensitive to the toxin than susceptible cultivars. Nonpathogenic *A. alternata* isolates did not produce bioassayable toxin when grown in standing liquid culture. Of representatives from nine families tested, only tomato was susceptible to the pathogen and the toxin. Resistance to the pathogen in tomato is inherited as a single gene expressing complete dominance, while sensitivity to the toxin is controlled by a single locus with two alleles expressing incomplete dominance when heterozygous. The toxin is a highly stable, low-molecular-weight, cationic molecule, which based upon ion exchange, gel filtration, and paper chromatographic behavior, is produced in culture as a single molecular species. Furthermore, based upon its host-specific nature and chemical properties, the toxin has not been reported heretofore as a phytotoxin produced by *Alternaria* spp.

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Additional key words: Lycopersicon esculentum, tomato stem canker.

Only certain cultivars of tomato (*Lycopersicon esculentum* Mill.) are susceptible to a stem canker disease caused by *Alternaria alternata* (Fr.) Keissler f. sp. *lycopersici* (3). The disease is characterized by the formation of dark-brown to black cankers on the stem near the soil line, or on the stems aboveground in naturally infected plants. Associated with the cankers are foliar symptoms comprised by epinasty of the petiole with inward rolling and angular interveinal necrotic areas on one or both sides of the midrib (3). Culture filtrates of the pathogen, when absorbed by excised aerial portions of susceptible tomato cultivars, elicited foliar symptoms indistinguishable from symptoms expressed on naturally infected plants (2). Absence of fungus in leaves with foliar symptoms induced either by the pathogen or the cell-free culture filtrate, indicated that these symptoms are caused by a translocatable toxin.

Phytotoxins of fungal origin have been demonstrated to cause symptoms in a number of plant diseases (12). Phytotoxins which exhibited the same host specificity as the pathogen which produced them are classified as host-specific, and are generally assumed to play an important role in host specificity of the pathogen, and subsequent disease development (10). Toxins classified as host-specific, also possess the common feature of capability for eliciting all or most of the symptoms of the disease. Thus, the ultimate disease reaction in these cases seems to be based upon the relative sensitivity of the host to specific secondary metabolite(s) produced by the pathogen, while pathogenicity is conditioned (at least in part) by toxin-producing ability. Consequently, to establish such a role information is required on the presence, biological activity, chemistry, and mode of action of host-specific toxins, coupled with inheritance studies of any differential host response. Practical application of the information regarding the presence and role of phytotoxins in host-parasite interactions has resulted in development of breeding programs to select for disease resistance based upon reaction of the host to the toxin (1, 5, 10, 16).

The objectives of the present study were: (i) to verify the presence of a phytotoxin in culture filtrates of the pathogenic *A. alternata;* (ii) to compare the host ranges of the pathogen and the toxin; (iii) to compare pathogenic and nonpathogenic *A. alternata* for toxin-producing capability; (iv) to explore the feasibility of using the toxin to screen tomatoes for stem canker resistance; and (v) to evaluate some fundamental chemical characteristics pursuant to purifying and characterizing the toxin(s).

MATERIALS AND METHODS.—Cultures of *A. alternata* f. sp. *lycopersici* were obtained from cankers of naturally infected tomato plants with typical stem-canker symptoms as previously described (3). Twelve single-spore isolates from active cankers were tested for pathogenicity under greenhouse and field conditions, and all produced consistent infection on field-susceptible cultivars, but not resistant ones. Pathogenicity tests on Earlypak 7 and other cultivars were made by spraying uninjured 10- to 15-cm or larger plants to runoff with spore suspensions containing approximately 250,000 spores/ml followed by 48 hours of incubation in a moist chamber, after which the plants were returned to the greenhouse bench. Greenhouse temperatures ranged from 23-29 C. Infection, generally evident in 7-8 days, was first observed as necrotic spotting on inoculated leaves, petioles, and stems. Within 10-15 days, the stem spots enlarged, forming cankers which eventually girdled the stem and resulted in the death of susceptible plants.

The single-spore-derived isolate 1012-5SS-1 which was used for toxin production in this study, was maintained on potato-dextrose agar (PDA) or cornmeal agar (CMA) at 22 C under continuous fluorescent illumination. Subculture of the fungus for 10 months on PDA or CMA did not alter colony appearance, capacity for sporulation, pathogenicity on susceptible tomato cultivars, or efficacy for toxin production in liquid culture. Noncanker-inducing isolates of *A. alternata* derived from ripe rotted tomato fruit, apricot, prune, or barley were obtained from K. A. Kimble, Department of Plant Pathology, University of California, Davis.

Seed of tomato cultivars ACE, Earlypak 7, and F_1 and F_2 generations from the cross ACE × Earlypak 7 was the generous gift of Paul G. Smith, Department of Vegetable Crops, University of California, Davis. Tomato plants used for routine bioassay were grown from seed in standard UCD soil mixture in a heated greenhouse to a height of 15 cm before individual leaves were removed. Plants utilized from the segregating F_2 generation were maintained in controlled growth chambers under an alternating light-temperature regime of 12 hours light (27 C) and 12 hours darkness (16 C) and were allowed to reach 15 cm (1 month) before being tested for sensitivity to the pathogen and the toxin.

For toxin production, *A. alternata* was grown at 21-23 C under artificial light in 500-ml flasks containing 100 ml potato-dextrose broth (PDB) or modified Fries basal medium (MF) (6) supplemented with 0.1% (w/v) yeast extract (Difco). The sterile media were inoculated with 1 ml of a spore suspension containing approximately 250,000 spores/ml, shaken gently, and incubated as a standing culture. The culture filtrates were collected by successive passage through glass wool, Whatman No. 1 filter paper, and a Millipore filter (0.45 μm). The culture filtrate which has been passed through the Millipore filter and unfiltered filtrate gave similar results in toxin bioassays. Sterile uninoculated media and media from nonpathogenic *A. alternata* cultures were used as controls. The sterile filtrates were stored at 4 C or −10 C pending assay or fractionation. Toxin preparations stored for 6 months showed no apparent loss in toxicity based upon dilution end-point bioassay.

Toxin activity in culture filtrates, as expressed by stem and foliar necrosis, was evident when intact seedlings, excised shoots, excised stem sections or leaves were allowed to absorb the culture filtrate through their vascular system. Symptoms were not induced by direct application of the culture filtrate to leaves (either attached to the plant or detached). For routine determination of toxin activity, a detached-leaf bioassay was developed. This procedure involved excising a fully expanded leaf with a razor blade by cutting across the petiole base at an oblique angle so that the cut surface of the petiole was in the same plane as the underside of the leaf thus allowing maximum contact of the cut surface with surface upon which the leaf was placed. The toxin preparation was diluted with H_2O to the desired concentration and 2.0 ml of the diluted preparation was applied to a 7.0-cm filter paper disk (Whatman No. 1) on the bottom of a petri dish. The excised leaf was placed cut-surface down on the filter paper after which the petri dish was covered and placed under continuous illumination (Gro-Lux lamps at 60 cm) at 21-23 C. Dilution end points of serial 2-fold series of

toxin preparations were determined by scoring leaves with 25% or more of the leaf surface showing interveinal necrosis after 48 hours of incubation. The rate and degree of symptom development was concentration (dilution) dependent up to 48 hours after which no further symptom change was generally observed. There was some variation in the sensitivity of leaves of different ages; thus three or more replications were used for bioassay of each extract or dilution therefrom. The pH of nonbuffered toxin preparation, in the range of pH 4 to pH 8, had no significant effect on symptom expression. The interveinal necrosis of sensitive tomato cultivars was induced only by *A. alternata* f. sp. *lycopersici* culture filtrates or toxin preparations obtained from these culture filtrates.

All chemicals were purchased from commercial sources in the highest grades obtainable and were used without further purification. Chromatographic materials, BioGel P-2, Dowex 1-X^4, and Dowex 50W -X^8 (BioRad Laboratories) were prepared according to the manufacturer's specifications.

RESULTS.—*Foliar symptoms.*—Stem inoculations through leaf scars of the susceptible tomato variety Earlypak 7 with spore suspensions of isolate 1012-5SS-1 produced necrotic sunken lesions at the site of inoculation within 5-7 days. From the lesion, discontinuous streaks of necrotic tissue developed on stem and uppermost leaves of the plant became necrotic. As reported by Grogan et al. (3) the foliar symptoms were comprised of epinasty of the petiole with inward rolling and angular interveinal necrotic areas that developed on one or both sides of the midvein. Although the fungus was readily isolated from inoculated portions of stems, leaves, and petioles, it was not present in uninoculated necrotic tissues. Cell-free 10-day-old culture filtrates of the pathogenic isolates elicited typical necrotic stem streaks and foliar symptoms on susceptible tomato seedlings when 10 cm seedlings, excised at the crown, were immersed in the culture filtrates. Culture filtrate absorption by excised leaves and stem sections also induced necrosis (Fig. 1). No symptoms were produced when tissues similarly were exposed to sterile culture media or culture filtrates from nonpathogenic *A. alternata* isolates.

Host-specific toxicity.—Host-specific toxicity of the culture filtrate of the pathogen was demonstrated qualitatively with either excised shoots or leaves of the *A. alternata* resistant cultivar ACE by the absence of foliar symptoms following exposure to the filtrate. However, some concentrated culture filtrates did produce foliar symptoms on ACE similar to those on the susceptible variety, but dilution end-point bioassays with excised leaflets indicated that the pathogen-resistant leaflets of ACE were at least 1,000-fold less sensitive to the toxin than the susceptible leaflets of Earlypak 7 (Table 1). The most active culture filtrates had a dilution end point of 1:2048 for the susceptible and 1:2 for the resistant cultivar. The rate of development and severity of symptoms on detached leaves appeared to be a function of the relative toxin concentration. Bioassays were terminated after 48 hours to avoid differences in degree of expression as a function of time.

Host range of the pathogen and the toxin.—Preliminary results with toxin assays showed positive correlation between sensitivity to the toxin and susceptibility to infection (2). In a subsequent test, 38 lines
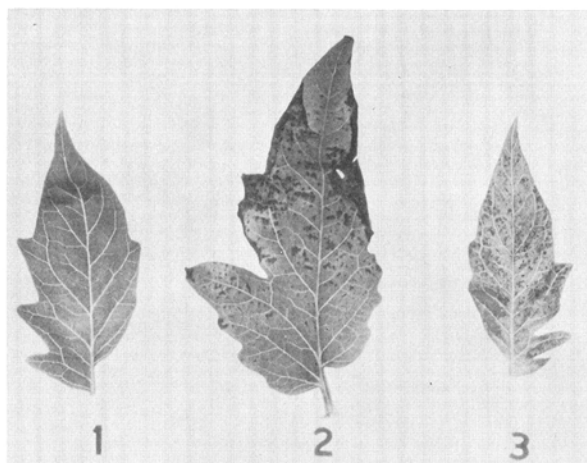
Fig. 1-(A to C). Foliar symptoms induced by *Alternaria alternata* f. sp. *lycopersici* toxin. A) Leaf from a healthy tomato plant; B) leaf from a tomato plant exhibiting typical stem canker symptoms following inoculation with the pathogen; C) leaf from a healthy susceptible tomato plant which was excised and treated with a cell-free culture filtrate of the pathogen. Note the interveinal necrosis in the leaf from the diseased plant and toxin-treated leaf. All photographs were taken with backlighting.

or cultivars of tomato were assayed by leaf bioassay for sensitivity to the toxin and also inoculated with a spore suspension of the pathogen. Of the 38 lines, representing both susceptible and resistant types, 13 were susceptible to the pathogen and 25 were resistant when bioassayed at a culture filtrate dilution sufficient to cause symptoms on the susceptible cultivar Earlypak 7 but not on ACE; the 38 cultivars fell into two discrete classes of resistant and susceptible to the the toxin. Significantly, in all cases

there was complete agreement of the host-fungal reaction and the host-toxin reaction.

In addition to the tomato cultivars, eight solanaceous species and at least one representative of eight other families were tested with three replications for reaction to the pathogen and the toxin as follows: (i) solanaceous species; *Capsicum frutescens, Solanum esculentum, S. tuberosum, Nicandra physalodes, Nicotiana tabacum, Datura stramonium, Physalis floridana,* and *P. exocarpa;* (ii) other families; Chenopodiaceae, Cucurbitaceae, Compositae, Convolvulaceae, Cruciferae, Gramineae, Leguminosae, and Umbelliferae. The toxin was adjusted to a concentration sufficient to cause symptoms on the resistant variety ACE to insure detection of toxin sensitivity equal to or greater than that of the most tolerant cultivar. When challenged by the pathogen or the toxin all plants were resistant to fungal infection and at least as tolerant to the toxin as resistant ACE.

Production and accumulation of the toxin in standing cultures of various isolates of A. alternata.—Potato-dextrose broth supported the growth of all *A. alternata* isolates listed in Table 1. The *A. alternata* f. sp. *lycopersici* isolates were all derived from single spores obtained from active cankers on naturally infected tomatoes. Isolates UCP-1 and 1551 were obtained from ripe-rotted tomato fruit with typical black mold symptoms. Isolates PP1-D, fA[2] and 1038-1 were isolated respectively from prune, apricot, and barley leaves and were identified as *A. alternata* on basis of spore morphology (3). Isolate QM 736 was obtained from the type culture collection of the Army Quartermaster Corps as *A. alternata*. Filtrates of the respective isolates incubated at room temperature, were collected after 10 and 20 days. All four stem-canker isolates were pathogenic to susceptible cultivars and produced bioassayable host-specific toxin for Earlypak 7 at the 10-day sampling. By the 20-day sampling the

TABLE 1. Toxin production in culture and pathogenicity of various isolates of *Alternaria alternata*

Isolate	Source[a]	Pathogenicity[b]		Toxin production[c]			
				10 days		20 days	
		ACE	EP-7	ACE	EP-7	ACE	EP-7
1024-5	Tomato	NP	P	NS	512	2	2048
1057-1	Tomato	NP	P	NS	512	2	2048
1011-1SS1	Tomato	NP	P	NS	512	2	2048
1012-5SS1	Tomato	NP	P	NS	512	2	2048
UCP-1	Tomato	NP	P	NS	512	2	2048
PP1-2	Prune	NP	NP	NS	NS	NS	NS
5-A2	Apricot	NP	NP	NS	NS	NS	NS
1038-1	Barley	NP	NP	NS	NS	NS	NS
1552	Tomato	NP	NP	NS	NS	NS	NS
1557	Type culture	NP	NP	NS	NS	NS	NS

[a]Isolates 1024-S, 1057-1, 1011-1SS1, 1012-5SS1, UCP-1 were isolated from plants showing typical stem canker symptoms. All other isolates were from senescent decayed tissue, with the exception of isolate 1557, which was obtained from the Army Quartermaster Corps as *Alternaria alternata* type culture QM 736.

[b]Disease ratings were made 14 days after inoculation with spore suspensions of the respective isolates and were rated as pathogenic (P) or nonpathogenic (NP) based on presence of canker symptoms on susceptible cultivar Earlypak 7.

[c]Toxin production was determined by a dilution point bioassay. Culture filtrates of the respective isolates were bioassayed at 10 days and 20 days after inoculation of the culture media. Relative toxin concentration was estimated following 48-hour exposure of leaves of the cultivars ACE and Earlypak 7. The data are maximum 2-fold dilution expressing symptoms on the differential varieties. NS indicates complete absence of symptoms.

concentration was sufficient to cause symptoms on the pathogen-resistant cultivar ACE (Table 1). The dilution end points of 20-day-old culture filtrates of the pathogenic isolates revealed a 1,000-fold difference in toxin concentration required to elicit symptoms on pathogen-resistant vs. susceptible cultivars. All other isolates tested including the *A. alternata* type culture were nonpathogenic on susceptible tomato seedlings and did not produce bioassayable toxin at either the 10- or 20-day sampling periods.

The time course of toxin accumulation was followed by sampling from standing cultures of the pathogen grown in 25-ml aliquots of modified Fries basal medium in 125-ml Erlenmeyer flasks with glutamine as the sole nitrogen source in the medium. Each flask was seeded with 1ml of a spore suspension containing approximately 25,000 spores/ml from a culture grown on CMA. Cultures were incubated at 23 C under artificial light. Culture filtrates from duplicate flasks were harvested on alternate days and assayed for toxin by the excised leaf bioassay. Mycelial dry weight and pH measurements were taken at the time of harvest. Sufficient toxin to produce typical foliar symptoms had accumulated by day 4 (Fig. 2). In this experiment toxin accumulation reached a maximum within 8 to 10 days. Mycelial dry weight accumulation paralleled toxin accumulation throughout the experiment. In potato dextrose broth toxin accumulation did not plateau until approximately day 15 with a slightly higher apparent dilution end point (1:2048) but the kinetics of mycelial growth and toxin production were similar.

The toxin also was produced on V-8 juice and unsupplemented tomato infusion media, the latter prepared from leaves of either ACE or Earlypak 7 cultivars.

Genetic control of the host reaction to the pathogen and the toxin.—The F_1 hybrid of a cross between resistant ACE × susceptible Earlypak 7, was resistant to *A. alternata* f. sp. *lycopersici* in both field and greenhouse inoculation tests (3). Dilution end-point bioassay of F_1 plants challenged by either crude culture filtrates or highly purified toxin preparations, however, indicated a toxin sensitivity intermediate to the parents with the hybrid 50 times more sensitive than ACE as compared to Earlypak 7 which exhibited 1,000-fold greater sensitivity than ACE. The genetic control of the host reaction to both the pathogen and the toxin was examined with F_2
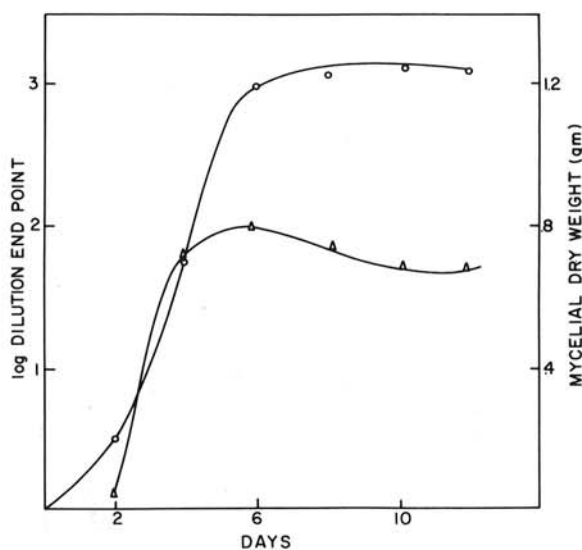

Fig. 2. Toxin production in standing liquid culture by *Alternaria alternata* f. sp. *lycopersici* as a function of mycelial dry weight. Toxin production (o———o), determined by the detached leaf bioassay procedure, and mycelial dry weight measurements (△———△) were conducted on two replicate cultures (25 ml) at each sampling period. The respective data points represent the average of the replicates.

plants of the cross ACE × Earlypak 7. The plants used in this study were grown in a controlled growth chamber (daily cycle of 12 hours light, 27 C and 12 hours dark, 15.5 C) to a height of 15 cm (20-days-old) before sampling. The plants were tagged individually for identification, sampled for toxin bioassay by removing three leaflets, and then sprayed with a spore suspension of the pathogen. The toxin preparation was a 14-day-old cell-free culture filtrate produced on PDB, pH 6.0, with a dilution end point of 1:1000 for production of symptoms on Earlypak 7 and 1:50 dilution for symptom expression on the hybrid (Table 2).

The 100 ACE plants tested were uniformly resistant to the pathogen and developed necrotic spotting only after exposure of leaflets to undiluted culture filtrate. All Earlypak 7 plants developed toxin symptoms at all three dilutions and were killed by inoculation with the pathogen. The F_1 hybrid was completely resistant to

TABLE 2. Disease reaction of resistant ACE, susceptible Earlypak 7, and the F_1 and F_2 generations of the cross between these cultivars compared with the sensitivity to the toxin produced by *Alternaria alternata* f. sp. *lycopersici*

Cultivar[a]	Disease reaction[b]			No. plants showing toxin sensitivity[c]		
	Resistant	Susceptible	tested	1:1	1:50	1:1000
ACE	100	0	100	100	0	0
Earlypak 7	0	100	100	100	100	100
F_1	100	0	100	100	96	0
F_2	36	15	51	51	21	15

[a]Plants of ACE and Earlypak 7 are homozygous resistant and susceptible, respectively. The F_1 plants were derived from the cross ACE × Earlypak 7. The F_2 plants were the selfed progeny of F_1.

[b]The plants were sprayed with a spore suspension of *A. alternata* f. sp. *lycopersici* isolate 1012-5SS1, incubated for 48 hours in a mist chamber (29 C), followed by 10 days of incubation in a greenhouse at 23-29 C prior to being rated for disease reaction.

[c]Detached leaves were exposed to toxin dilutions of 1:1, 1:50, 1:1,000. Leaves were rated for toxin sensitivity following 48 hours of incubation with the toxin.
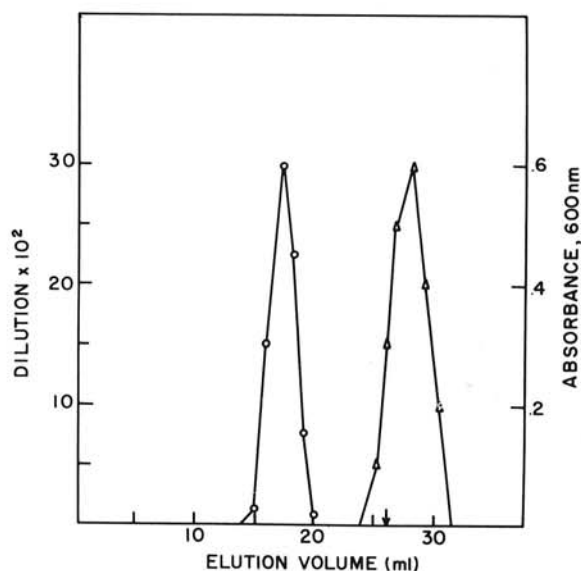
Fig. 3. Polyacrylamide gel filtration of the host-specific toxin produced in standing liquid culture by *Alternaria alternata* f. sp. *lycopersici*. The preparation containing toxin activity was obtained by continuous extraction of a cell-free culture filtrate with ethyl acetate followed by aqueous recovery of the toxin after ethyl acetate removal *in vacuo*. The electron patterns were obtained from a Bio Gel P-2 column (1.5 × 26 cm) in the presence of 0.01 M potassium phosphate buffer, pH 6.8. The toxin elution profile ($\triangle$——$\triangle$) revealed a single component with a V_3/V_o ratio of 1.67 which was indistinguishable from a separate elution of the crude cell-free culture filtrate. The elution of the void volume marker (o———o) was monitored by absorbance determinations at 600 nM. A V_e/V_o ratio of 1.53 was calculated for the elution of sucrose from the same column (arrow indicates V_o for sucrose).

inoculation with the pathogen and the toxin at the dilution of 1:1000, but was sensitive to the toxin at the 1:50 dilution; necrosis developed on at least 25% of the leaf surface after 48 hours of incubation. The undiluted culture filtrate caused complete blackening of the hybrid leaflets in 48 hours just as the undiluted and 1:50 diluted preparations had done on the Earlypak 7 leaflets. Only two classes were distinguishable by inoculation with the pathogen; of 51 plants inoculated 36 were completely resistant and 15 susceptible. A $\chi^2 = 0.32$ was calculated for the hypothesis of a 3:1 segregation expressing a single gene dominant resistant reaction to the pathogen. On the basis of the toxin bioassay these same 51 plants were classified into three discrete classes with 15 plants being sensitive only to the undiluted culture filtrate, 21 plants expressing intermediate sensitivity at the 1:50 dilution, and 15 plants were sensitive to all three dilutions thus expressing the Earlypak 7 level of sensitivity to the toxin. A $\chi^2 = 1.11$ was calculated for the hypothesis of a 1:2:1 ratio for a single locus with two alleles expressing incomplete dominance. In addition, the 15 plants susceptible to the pathogen were the same 15 plants showing the highest level of sensitivity to the toxin preparation. No evidence was obtained for independent assortment of the phenotypes exhibiting susceptibility to the pathogen and sensitivity to the toxin.

Properties of the Alternaria alternata toxin.—Culture filtrates, pH 7.0, stored at −10 C for 6 months showed no loss in toxin activity when tested by the dilution end-point bioassay. Experiments were carried out to determine the effect of pH and temperature on the stability of the toxin in culture filtrates. The pH of the culture filtrates was adjusted to pH 3, 7, and 10 with 1N HCl or 1N NaOH; aliquots were boiled for 0, 10, and 20 minutes or autoclaved for 9 and 10 minutes in stoppered tubes. After cooling, all tubes were adjusted to pH 7.0 and 2-fold serial dilution with distilled water were bioassayed. Boiling or autoclaving at all time periods at pH 3 and 7 did not reduce the toxicity of the preparations. In contrast, both boiling and autoclaving treatments eliminated the biological activity of the preparation at pH 10, but toxicity was not reduced in the unheated control at pH 10.

The biological activity of the toxin preparation was dialyzable and could be recovered from the water against which the preparation was dialyzed. Elution patterns of the toxin from gel filtration on a poly-acrylamide P-2 column (199-200 mesh) revealed a single active component with a molecular weight of less than 400 (Fig. 3). The V_e/V_o ratio for the toxin was 1.67 while that for sucrose was 1.53. The toxin activity of a culture filtrate was not retained by an anion exchange resin (Dowex 1-X^4 − Cl^- form) at pH 6.0 or pH 8.0. However, toxin activity was retained by a cation exchange resin (Dowex 50W − Na^+ form) at pH 3.5 and was desorbed as a single component by elution with 0.10 M sodium phosphate, pH 7.1.

The solubility of the toxin in various water-immiscible organic solvents was tested at pH 3.0, 6.0 and 8.0 at room temperature (20 C). The culture filtrate was brought to the desired pH with 1 N HCl or 1N NaOH, aliquots (10 ml) were transferred to a 50-ml separatory funnel, shaken with three volumes of the particular solvent, and the two phases separated. The organic phase was taken to dryness under a stream of N_2 and the aqueous phase was taken to dryness in vacuo. Both phases then were resuspended in 5.0 ml distilled water and bioassayed. The preparations were extracted with *n*-hexane, petroleum ether, ethyl acetate, diethyl ether, and 1-butanol. At pH 3.0 and 6.0 the toxin activity remained in the aqueous phase with all solvents tested. At pH 8.0, the toxin activity was sparingly partitioned into the organic phases of ethyl acetate and 1-butanol but remained with the aqueous phase in the presence of *n*-hexane, petroleum ether, and diethyl ether. The efficiency of the extraction by ethyl acetate and 1-butanol was improved by the addition of crystalline KCl to a final concentration of 1.0 M. Continuous extraction for 24 hours in a Kutscher-Steudel extractor with ethyl acetate of the effluent from a Dowex 50W column amended to 1.0 M with KCl removed 80% of the toxin activity from the aqueous solution to the organic solvent.

The toxin did not precipitate when acetone was added to a 10-fold concentrated culture filtrate to give a 60% acetone solution.

The cell-free culture filtrate recovered from modified Fries medium, a toxin preparation eluted from the cation exchange resin, and a preparation obtained by 24 hours of continuous extraction with ethyl acetate were examined by paper chromatography. A portion (0.20 ml) of each preparation, concentrated to obtain a final dilution end

point of 1:1000 in the toxin bioassay, was streaked across a 10×50 cm strip of Whatman No. 1 chromatography paper, equilibrated in a sealed developing tank with the solvent-saturated atmosphere for 30 minutes prior to initiation of the descending separation. Separation was continued until the solvent front had migrated 40-42 cm; the paper was then removed from the tank and air-dried for 1 hour in a forced air hood. A longitudinal strip, 3 cm wide, cut from the center of the chromatogram was further divided into 2×3 cm sections which were bioassayed directly for toxin activity. The direct bioassay of the chromatogram sections was done by placing petioles of excised leaflets of the differential varieties ACE and Earlypak 7 in contact with the sections previously placed on 7-cm filter paper dishes in the standard petri dish bioassay. Each petri dish received 1.5 ml H_2O to initiate the bioassay. A single area of toxin activity, R_f 0.73 − 0.75, was detected when the chromatograms of all three toxin preparations were developed with 1-propanol: $NH_4OH:H_2O$ (6:3:1, v/v). The R_f range represents the extremes recorded in six individual separations. Development of the chromatograms with benzene:glacial acetic acid:H_2O (80:17:3, v/v) revealed toxin activity confined to 1.5 cm band at $R_f = 0.20$, with no discernible difference apparent in the three toxin preparations.

The toxin activity was not associated with fluorescent or UV absorbing bands when chromatograms were viewed under UV light although a number of fluorescent areas were revealed. The area of the chromatogram corresponding to the toxin activity did not react with ninhydrin, $FeCl_3$ in 0.1N HCl in 95% ethanol, or diazotized p-nitroaniline indicating the apparent absence of free primary or aromatic amines and aromatic acids at the sensitivity limit of the respective diagnostic tests. Further characterization of the toxin will be conducted when sufficient quantities of a homogeneous preparation is available.

DISCUSSION.—The experiments reported here confirm an earlier observation that A. alternata f. sp. lycopersici produces in still culture a toxin capable of eliciting foliar symptoms on tomato which are indistinguishable from those that develop on naturally infected plants (2). The sunken necrotic cankers associated with the site of fungal infection was not produced by contact of sensitive tissue with the toxin and thus appeared to require fungal activity for formation. All tested isolates of the stem-canker pathogen were shown capable of producing the toxin. In addition, the toxin did not appear to be a staling product of fungal growth, but was produced throughout the entire growth period of the fungal cultures. The fungus also produced the toxin on all media tested, including modified Fries, PDB, V-8 juice, and an unsupplemented tomato infusion media, but nonpathogenic isolates of A. alternata, which were morphologically indistinguishable from the pathogen, failed to produce any bioassayable toxin during the same time of incubation. The sensitivity of the bioassay should have enabled detection of toxin in the nonpathogenic isolates at a concentration 1/2000th that present in undiluted culture filtrates of the pathogen. Additional evidence that this toxin conforms to the criteria of a host-specific toxin include: (i) Sixteen plant genera representing nine families apparently were not affected by

the pathogen or the toxin; only certain cultivars of tomato were susceptible to the pathogen and sensitive to the toxin, (ii) tomato cultivars susceptible to the pathogen were most sensitive to the toxin. Cultivars resistant to the pathogen were relatively insensitive to the toxin.

Phytotoxins have been isolated from culture filtrates of other species of the genus Alternaria (13). Pound and Stahman isolated and characterized alternaric acid from culture filtrates of A. solani, the causal organism of early blight of tomato and potato (9). Tentoxin, purified from culture filtrates of A. alternata = A. tenuis was capable of causing chlorosis of most dicotyledonous species tested with the exception of tomato and members of the Cruciferae (7, 14). Starratt reported the production of a penta substituted benzene by A. zinniae which closely resembled quadrilineatin, a fungal inhibitor produced by Aspergillus guadrilineatus (11). The toxin from A. zinniae, given the trivial name of zinniol, induced a complex of symptoms including chlorosis, stem withering, and curling of leaf tips on zinnia as well as a number of plants not susceptible to the pathogen.

The first reported example of an Alternaria-produced phytotoxin exhibiting the same host specificity as the pathogen was from the blackspot disease of Japanese pear caused by A. kikuchiana (4). The fungus is pathogenic only on a few varieties of pear related to the variety Nijisseki. Three compounds isolated from culture filtrates and mycelial mats of A. kikuchiana were shown to cause the blackspot symptom when introduced into susceptible pear leaves. The compounds have been tentatively identified as low-molecular weight proteins and one of them, phytoalternarin A, had the same host specificity as the fungus on 17 pear cultivars. Alternaria mali, the causal organism of blotch of apple, also has been reported to produce a host-specific toxin (15). The trivial name of Alternariolide was suggested for the toxin which has been characterized as a depsipeptide (8).

From our limited studies of the genetic control of host resistance to the stem canker pathogen, it appears that compatibility of the host-parasite interaction was controlled by a single gene with two alleles, resistance being expressed as a complete dominant. Susceptible plants were also more sensitive to the toxin as indicated by dilution end-point bioassays. However, the heterozygote F_1 resulting from a cross between the homozygous resistant cultivar ACE and the homozygous susceptible cultivar Earlypak 7, expressed a toxin sensitivity intermediate between the two parents. The F_2 progeny segregated into three discrete classes with a good fit to a 1:2:1 ratio for a single locus with two alleles expressing incomplete dominance. Evaluation of individual plants in the F_2 generation did not reveal any differential segregation for the host-fungal reaction and the host-toxin reaction; all susceptible plants were most sensitive to the toxin. These observations taken with the apparent host-specific nature of the toxin support the thesis of a significant role for the toxin in the etiology of the stem canker disease. However, exact definition of the role of the toxin in the disease must await the complete purification of the toxin, isolation of the toxin from diseased tissue, and a study of the biological relationship between toxin production and pathogenicity. It appears that the toxin may be an effective tool for selecting homozygous disease resistant plants through a simple

nondestructive bioassay of potential breeding material.

The results of gel filtration, cation exchange and paper chromatographic separation do not indicate the presence of more than a single chemical entity. The identity of the toxin is unknown. However, based upon its host specificity and general chemical behavior it does not appear to have been detected heretofore (13).

Current efforts are concentrated on purification and characterization of the toxin as a prelude toward examination of the role of the toxin in pathogenesis or expression of virulence.

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