Toxin Production by *Alternaria solani* and its Related Phytotoxicity to Tomato Breeding Lines

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ABSTRACT

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The fungus *Alternaria solani* causes early blight and collar rot diseases on tomatoes. *A. solani* has been reported to synthesize phytotoxic metabolites, especially alternaric acid and zinniol, in culture. Culture filtrates of several *A. solani* isolates were tested for their phytotoxicity to tomato genotypes previously evaluated for resistance to early blight and collar rot. Tomato seedlings exposed to culture filtrates for 20 h exhibited marginal and interveinal leaf necrosis and wilting. At a 1:2 dilution, the filtrate was severely phytotoxic to all genotypes tested, but at greater dilutions differences in susceptibility were observed. The collar rot and early blight resistant genotypes (C1943 and NC EBR-2) were tolerant of the filtrate, whereas genotypes that were resistant to early blight but not collar rot (71B2, 87B187, NC EBR-1) had phytotoxic symptoms. The phytotoxic metabolites were not extractable with chloroform, ethyl acetate, or isopropyl alcohol, but were present in the culture filtrate aqueous fraction. Alternaric acid and zinniol could not be detected in any significant quantity.

Alternaria solani (Ellis & Martin) Jones & Grout causes two types of disease on tomato (*Lycopersicon esculentum* Mill.) plants: early blight and collar rot. Early blight, which defoliates mature plants and contributes to major economic losses for growers, is considered a more serious disease than collar rot. Collar rot is primarily a seedbed disease carried to the field on tomato transplants. Collar rot symptoms are dark, sunken stem lesions near the soil line (16). Some genotypes exhibit resistance to one or both phases of *A. solani* infection (1,4,10).

Investigations of the early blight disease are complicated by the synthesis of phytotoxins by the causal organism. Early blight lesions on tomato leaves are typically surrounded by chlorotic regions that are less extensive in nonvascular tissue than in vascular tissue, indicating that *A. solani* toxins are translocated systemically and may be responsible for the lesions observed (17). The role of the phytotoxins in disease development has not been established, and the use of culture filtrates to screen tomatoes for resistance has not been investigated.

Two principal components, alternaric acid and zinniol, have been recovered from *A. solani* culture filtrates (5,8,17). Alternaric acid produces symptoms characteristic of early blight on tomatoes, with leaf lesions, chlorosis, and necrosis as visible symptoms. Zinniol causes stem wilting and leaf necrosis on zinnias, marigolds, and carrots, but its effects on tomato plants are unknown (2,9,22). Zinniol is nonhost specific (2,9,22), whereas alternaric acid exhibits a concentration-dependent host-specificity (11). Both compounds are considered secondary components in the disease development process.

Whereas previous *A. solani* toxin research evaluated the phytotoxic effects on host and nonhost plant species, differences in phytotoxic response among genotypes within the host species, tomato, have not been established for either culture filtrates or isolated toxic metabolites. To our knowledge, early blight-resistant and susceptible tomato genotypes have not been evaluated for their sensitivity to *A. solani* toxins.

The objectives of this study were to 1) determine if tomato responses to *A. solani* culture filtrates could be used as a reliable screening technique for early blight resistance; 2) test the phytotoxicity of *A. solani* culture filtrates on tomato breeding lines previously screened for resistance to early blight and collar rot (12–14); and 3) analyze *A. solani* culture filtrates for the production of alternaric acid and zinniol.

MATERIALS AND METHODS

Fungal culture and filtrate preparation. *A. solani* isolates ATCC 11078, ATCC 44204, A159, A131, A88-6, and A88-9 were maintained in petri dishes containing lima bean agar according to Barksdale (3). Isolates ATCC 11078 and ATCC 44204 were obtained from the American Type Culture Collection, Rockville, MD, and previously were reported as producers of toxic metabolites in culture (19,20). Isolates A159 and A131 were obtained from Dr. Thomas Barksdale, USDA, Beltsville, MD; A88-6 and A88-9 were isolated from a tomato field at Salisbury, MD, in 1987. Isolates A159, A131, A88-6, and A88-9 were used extensively in field and greenhouse disease-screening experiments and were prolific sporulators in culture (13,14). Small disks (2 mm) from 1-wk-old cultures were removed and placed in 25 ml of sterile liquid medium containing 1.0 g of KH_2PO_4, 0.5 g of $MgSO_4$, 6.0 g of casein hydrolysate, 100 g of sucrose, 1 mg of $FeSO_4$, 0.15 mg of $CuSO_4$, 0.10 mg of $ZnSO_4$, and 0.10 mg of Na_2MoO_4 per liter of distilled water (6). The medium was adjusted to pH 4.9 with 0.1 M HCl and autoclaved before inoculation. The pH remained at 4.9 after autoclaving. The cultures were incubated for 6 wk at 24 C in the dark under stationary conditions.

Phytotoxicity tests of culture filtrates on tomato seedlings. Culture filtrates of *A. solani* were tested for phytotoxicity on seedlings of tomato genotypes previously evaluated for resistance to early blight and collar rot (12–14). Mycelial mats were removed by filtration from culture filtrates of isolates A159, A131, A88-6, and A88-9. The filtrates were bulked to represent the combination of isolates used for spore suspension preparations in the previous field and greenhouse disease evaluations (12–14). Aqueous dilutions of 1:2, 1:5, 1:10, 1:50, and 1:100 were prepared and autoclaved for 15 min. In earlier studies of *Alternaria* toxic metabolites, autoclaving did not affect phytotoxicity of the culture filtrates (17). Control treatments included dilutions of uninoculated medium with distilled water. Culture filtrates were stored at 4 C in the dark until used.

Four-week-old seedlings of the genotypes C1943, 71B2, 87B187, NC EBR-1, NC EBR-2, and Castlejay were exposed to the various culture filtrates. Breeding lines C1943 and NC EBR-2 are resistant to early blight and collar rot, whereas the lines 71B2, 87B187, and NC EBR-1 are resistant to early blight but susceptible to collar rot (4,10,12–14). Castlejay is susceptible to both diseases (12–14). The seedlings were excised at the soil line and placed in vials containing 2 ml of filtrate solution. Four replications were used for each genotype and treatment combination in a completely randomized design. After 20 h at laboratory conditions of ambient temperature and natural plus fluorescent light, the seedlings were rated for the degree of phytotoxicity on a scale of zero to three in which 0 = no symptoms, 1 = slight necrosis, 2 = moderate necrosis and wilting, and 3 = severe necrosis and wilting. The experiment was repeated using a second collection of culture filtrate. Assumptions for homogeneity of variance were met, and a combined analysis of variance was performed using data from both experiments. Mean separations were according to Fisher's least significant difference test. All subsequent phytotoxicity tests were analyzed with the same statistical methods.

In an additional experiment, intact seedlings of C1943, NC EBR-1, NC EBR-2, and Castlejay were used. Roots were washed in distilled water and then immersed in a 1:2 dilution of culture filtrate for 2, 4, 6, 8, 10, 15, 30, or 60 min to determine the rate of uptake of phytotoxic compounds. A 1:2 dilution of uninoculated medium and distilled water were used as controls. The roots then were rinsed in distilled water and the seedlings were placed in distilled water overnight. After 20 h, seedlings were rated for phytotoxicity using the 0–3 rating scale. There were four replications in a completely randomized design, and the experiment was repeated once.

In order to determine whether differences in phytotoxic symptoms among genotypes could be associated with differences in transpiration rates, total water loss of 4-wk-old tomato seedlings was measured. Four seedlings each of C1943, NC EBR-1, NC EBR-2, and Castlejay were excised at the soil line and placed in vials containing 10 ml of distilled water. Vial openings were covered with Parafilm, and weights of each seedling plus vial were recorded. After 20 h under laboratory conditions, weights were determined. Differences between initial and final weights were attributed to water uptake and evaporative loss through the seedling leaves.

Extraction of zinniol and alternaric acid from culture filtrates. Analyses were done to determine if zinniol and alternaric acid were present in culture filtrates of *A. solani*. The extraction techniques were modified from Cotty and Misaghi (8) for zinniol and Stoessl and Stothers (20) for alternaric acid. Culture filtrates from isolates ATCC 11078, ATCC 44204, A159, A131, A88-6, and A88-9 were analyzed for zinniol and alternaric acid after 21 days of culture in the light or dark. Mycelial mats were removed by filtration, and the filtrates from four flasks per isolate were combined and divided into two 100-ml aliquots. The pH was adjusted to 8.5 for zinniol extraction and 3.5 for alternaric acid extraction. Filtrates were extracted three times with equal volumes of chloroform. The aqueous fraction was retained and the chloroform fraction was washed twice with 50 ml of 0.1 M NaOH (pH 13), three times with 50 ml of 0.1 M KH_2PO_4 (pH 4.5), and then evaporated to dryness by flash evaporation. The residue was redissolved in 2 ml of chloroform and stored at 4 C. Residual chloroform was removed from the aqueous fraction by flash evaporation, the pH was adjusted to 5.5, and the fraction was autoclaved and stored at 4 C.

Zinniol standards were obtained from Dr. J. A. Martin, Roche Products, Welwyn Garden City, England, and alternaric acid standards from Dr. A. N. Starratt and Dr. A. Stoessl, Research Centre, London, Ontario. The chloroform extracts were cochromatographed with zinniol or alternaric acid standards on 0.25-mm-thick, silica-coated, glass thin-layer chromatography (TLC) plates (Silica Gel 60F-254, E. Merck, Darmstadt, Germany). Developing solvents were acetone/hexane/chloroform (1:1:1, v/v/v) for zinniol and chloroform/ethanol (95:5, v/v) for alternaric acid. Compounds were visualized by spraying developed TLC plates with vanillin-sulfuric acid (3 g of vanillin dissolved in 100 ml of ethanol with 3 ml of concentrated sulfuric acid dropped slowly into the ethanolic solution) and heating the plates at 120 C for 10 min or until violet-blue spots appeared.

Phytotoxicity of culture filtrate extracts. The aqueous and chloroform extracts were compared with nonextracted culture filtrate for phytotoxicity to tomatoes. Culture filtrates of isolates A159, A131, A88-6, and A88-9 were pooled and separated into aqueous and chloroform fractions, and a portion of the nonextracted culture filtrate retained. The residue from the chloroform fraction was dissolved in 5 ml of ethanol and brought to the original filtrate volume (270 ml) with distilled water. Dilutions of 1:5, 1:10, 1:25, and 1:50 of the culture filtrate and the aqueous fraction were prepared and autoclaved. The chloroform fraction was used at its original concentration (113 mg of residue/270 ml of filtrate). Controls included a 1:2 dilution of uninoculated medium plus distilled water. Four-week-old seedlings of C1943, 87B187, NC EBR-1, NC EBR-2, and Castlejay were excised at the soil line and placed in vials containing 2-ml aliquots of the various dilutions. A completely randomized design was used with four replications of the nine treatments and two controls. After 20 h, necrosis and wilting symptoms were recorded using a 0–3 scale; the experiment was repeated once.

Polarity of toxic fraction. Solvents of varying polarity were tested for their ability to extract phytotoxic compounds from the culture filtrates. Culture filtrates were divided into three 35-ml aliquots, lyophilized, and then extracted with 10 ml of chloroform, ethyl acetate, or isopropyl alcohol. Extracts were transferred to clean flasks, the solvents were removed by flash evaporation, and 35 ml of distilled water was added to each extract. The residue of each lyophilized sample after extraction was retained as well, and 35 ml of distilled water was added to those flasks. Phytotoxicity tests were conducted for C1943 and Castlejay seedlings using the three solvent samples, the three residue samples, a 1:2 dilution of the original culture filtrate, plus controls of distilled water and a 1:2 dilution of uninoculated medium. Seedlings of C1943 and Castlejay were excised, exposed to 2 ml of each treatment solution for 20 h, and rated on a 0–3 scale. Four replications were used in a completely randomized design, and the experiment was repeated once.

RESULTS

Phytotoxicity of culture filtrates. Culture filtrates of *A. solani* caused wilting and necrosis of tomato seedlings (Table 1). Increasing dilutions of the culture filtrates resulted in decreasing phytotoxicity. Symptom development began with the apparent collapse of epidermal cells, followed by the appearance of diffuse, dark gray spots usually at the edges and interveinal regions of the leaves. Eventually the entire plant wilted; no phytotoxicity was observed for the distilled water and uninoculated medium treatments.

Genotypic differences in response to the culture filtrates after 20 h are summarized in Table 1. At a 1:2 dilution, culture filtrates

TABLE 1. Phytotoxicity of tomato seedlings exposed to *Alternaria solani* culture filtrates for 20 h

Genotype	Dilution[a]					
	1:2	1:5	1:10	1:50	1:100	Controls[b]
Castlejay	3.0[c]	3.0	3.0	3.0	2.8	0.0
71B2	3.0	3.0	3.0	2.6	2.5	0.0
87B187	3.0	3.0	2.8	2.4	2.3	0.0
NC EBR-1	3.0	2.8	2.6	1.9	1.6	0.0
NC EBR-2	3.0	1.9	1.5	0.8	0.4	0.0
C1943	3.0	1.5	0.9	0.3	0.0	0.0

$LSD_{(0.05)} = 0.4$ (within rows and columns)

[a] Combined culture filtrates of isolates A159, A131, A88-6, and A88-9.
[b] Control treatments included sterile medium and distilled water.
[c] Phytotoxicity ratings: 0 = no symptoms, 1 = slight necrosis, 2 = moderate necrosis and wilting, 3 = severe necrosis and wilting.

were severely phytotoxic to all six genotypes, but when further diluted differences in genotype susceptibility were evident. The collar rot and early blight resistant genotypes, C1943 and NC EBR-2, showed less phytotoxicity to culture filtrates at a 1:5 dilution. At a 1:100 dilution Castlejay (susceptible) and both 71B2 and 87B187 (early blight resistant) showed moderate to severe damage, whereas C1943 and NC EBR-2 showed little or no phytotoxic symptoms. The resistance of C1943 and NC EBR-2 decreased with time; phytotoxicity symptoms were observed if the exposure time exceeded 24 h, especially at the 1:5 and 1:10 dilutions.

The experiment to determine the minimum exposure time needed to produce toxicity symptoms on tomato seedlings is summarized in Table 2. Genotypic differences did occur: Castlejay showed slight symptoms after a 4-min exposure to the filtrate, whereas a 15-min exposure was needed for slight necrosis of C1943 and NC EBR-2 to occur. The response of NC EBR-1 was intermediate to that of Castlejay and C1943. After 30-min and 1-h exposure times, the degree of necrosis and wilting of C1943 and NC EBR-2 was about half that of Castlejay.

The possibility that slower transpiration rates are involved in tolerance of seedlings to the filtrate was investigated. NC EBR-2 lost the highest amount of water (3.59 g $\pm$ 0.64), followed by Castlejay (2.99 g $\pm$ 0.36), C1943 (2.54 g $\pm$ 0.31), and finally, NC EBR-1 (1.65 g $\pm$ 0.36). These results do not agree with the ranking of genotypes according to culture filtrate susceptibility. For example, Castlejay was the most susceptible, followed by NC EBR-1, NC EBR-2, and C1943. If tolerance to the culture filtrate was due simply to slower transpiration rates, then the most resistant genotypes would have the lowest water loss, which was not the case in this experiment.

Zinniol and alternaric acid production by *Alternaria solani*. Six isolates of *A. solani* were tested for zinniol and alternaric acid, which previously have been reported as responsible for phytotoxicity in infected plant material. Only trace amounts of zinniol were detected in filtrates of A159, A88-6, and A88-9, while alternaric acid could not be detected for any isolate. Isolate A88-9 produced zinniol in both light and dark, whereas isolates A159 and A88-6 produced zinniol in the dark only. Efforts to isolate zinniol and alternaric acid in quantities large enough for resistance screening tests were unsuccessful. Visual spots on TLC plates were always very faint, indicating very low levels of zinniol.

Phytotoxicity of culture filtrate extracts. Nonextracted and chloroform-extracted culture filtrates were compared as to their phytotoxicity to tomato seedlings. The chloroform fraction was originally hypothesized as containing the phytotoxicity factor; however, the absence of zinniol and alternaric acid in this fraction led to an investigation of the aqueous fraction for the presence of phytotoxic compounds.

The chloroform fraction was not toxic (Table 3), whereas the aqueous fraction was as phytotoxic as the nonextracted culture filtrates. The response of genotypes to the aqueous phase and the nonextracted culture filtrate was the same at all dilutions, indicating that the major phytotoxic metabolites of *A. solani* were present in the aqueous fraction. As in our previous experiments, C1943 and NC EBR-2 were tolerant to the culture filtrate and aqueous fraction, while Castlejay, 87B187, and NC EBR-1 were susceptible.

Polarity of toxic fraction. The presence of the toxic substances in the aqueous phase led to an investigation of solvents having higher polarities than chloroform to remove the toxic fraction from the aqueous phase. Phytotoxicity tests of the three solvent extracts and their respective residues are shown in Table 4. The toxic metabolites remained in the residues and were not extracted by the three solvents. Seedlings exposed to the chloroform, ethyl acetate, or isopropyl alcohol extracts remained normal, while those exposed to the residue solutions or culture filtrate showed severe necrosis, wilting, and desiccation.

TABLE 2. Phytotoxicity of tomato seedlings exposed to *Alternaria solani* culture filtrates (1:2 dilution) for varying periods of time

Exposure time (min)	Genotype			
	Castlejay	NC EBR-1	NC EBR-2	C1943
2	0.1[a]	0.0	0.0	0.0
4	0.6	0.0	0.0	0.0
6	1.5	0.4	0.0	0.0
8	1.8	0.5	0.0	0.1
10	1.9	0.8	0.0	0.1
15	2.5	1.4	0.5	0.6
30	2.9	1.6	1.5	1.2
60	2.9	2.3	1.6	1.5
Controls[b]	0.0	0.0	0.0	0.0

[a] Phytotoxicity ratings: 0 = no symptoms, 1 = slight necrosis, 2 = moderate necrosis and wilting, 3 = severe necrosis and wilting.
[b] Control treatments: 1:2 dilution of sterile medium or distilled water.

TABLE 3. Response of tomato seedlings to *Alternaria solani* aqueous and chloroform culture filtrate extracts

Dilution	Treatment	Genotype				
		Castlejay	87B187	NC EBR-1	NC EBR-2	C1943
1:5	CF[a]	3.0[b]	3.0	3.0	2.1	1.9
	AQ	3.0	3.0	3.0	1.9	2.2
1:10	CF	3.0	2.9	3.0	1.6	1.6
	AQ	3.0	3.0	3.0	1.5	1.3
1:25	CF	3.0	2.8	2.4	1.1	0.9
	AQ	3.0	2.5	2.4	0.9	0.9
1:50	CF	2.9	2.5	2.1	0.6	0.4
	AQ	2.8	2.4	1.9	0.6	0.3
Chloroform extract		0.0	0.0	0.0	0.0	0.0
Controls[c]		0.0	0.0	0.0	0.0	0.0

$LSD_{(0.05)} = 0.6$ (within rows)
$LSD_{(0.05)} = 0.5$ (within columns)

[a] CF = culture filtrate, and AQ = aqueous phase. Culture filtrates of isolates A159, A131, A88-6, and A88-9 were pooled before extraction.
[b] Phytotoxicity ratings: 0 = no symptoms, 1 = slight necrosis, 2 = moderate necrosis and wilting, 3 = severe necrosis and wilting.
[c] Control treatments: 1:2 dilution of sterile medium or distilled water.

DISCUSSION

Isolates of *A. solani* produced metabolites in culture that were phytotoxic to tomato seedlings at dilutions as high as 1:100. Phytotoxic symptoms included marginal and interveinal leaf necrosis, followed in some cases with severe wilting after 20 h. Pound and Stahmann (17) also tested culture filtrates from four isolates of *A. solani* for their toxicity to cut tomato shoots. All four isolates produced toxic metabolites in culture, and the symptoms that resulted were similar to early blight symptoms occurring on tomatoes after inoculation with *A. solani*.

TABLE 4. Phytotoxicity of tomato seedlings to solvent extracts of *Alternaria solani* culture filtrates

Extraction treatment	Genotype	
	C1943	Castlejay
Chloroform		
Solvent extract	0[a]	0
Residue	3	3
Ethyl acetate		
Solvent extract	0	0
Residue	3	3
Isopropyl alcohol		
Solvent extract	0	0
Residue	3	3
Culture filtrate[b]	3	3
Controls[c]	0	0

[a] Phytotoxicity ratings: 0 = no symptoms and 3 = severe necrosis and wilting.
[b] Combined, nonextracted culture filtrates of isolates A159, A131, A88-6, and A88-9.
[c] Control treatments: 1:2 dilution of sterile medium and distilled water.

Differential genotype responses to the culture filtrates occurred; the collar rot and early blight resistant genotypes C1943 and NC EBR-2 were most tolerant and exhibited a delayed phytotoxic reaction to culture filtrates. Genotypes that were resistant to early blight but not collar rot (71B2, 87B187, NC EBR-1) were susceptible to the filtrates. A short exposure time (8–10 min) was sufficient to induce symptom expression on susceptible genotypes, but 30-min or 1-h exposures were needed for symptoms to appear on the resistant genotypes C1943 and NC EBR-2. Differential responses of genotypes to toxins could not be explained by different transpiration rates. These results provide further evidence that collar rot and early blight resistances can be separate, although it does not preclude the possibility that collar rot resistance may be involved in early blight resistance, especially for C1943 and NC EBR-2. The objective to develop an early blight resistance screen using *A. solani* toxins does not appear possible for the 71B2, 87B187, and NC EBR-1 sources of resistance. However, if the C1943 and NC EBR-2 sources of resistance are used, selection with culture filtrates may be feasible, although the existing greenhouse test for detecting resistance is also efficient (10).

The toxins zinniol and alternaric acid, previously reported as responsible for the phytotoxicity of culture filtrates, could not be detected in the filtrates from three isolates of *A. solani.* Zinniol has been detected in filtrates of *A. solani* and related *Alternaria* species, but the quantity varied among species and isolates (8). Similarly, Stoessl et al (21) reported that toxin production varied among isolates of *A. solani* and between experiments with the same isolate. Brian et al (6,7) tested 12 *A. solani* isolates for alternaric acid production, but only two produced the toxin. To say definitely that zinniol and alternaric acid were not present in the culture filtrates of *A. solani* may require more sensitive analytical techniques.

Phytotoxins produced by *A. solani* were in the aqueous phase of the culture filtrate and could not be extracted with chloroform, ethyl acetate, or isopropyl alcohol. The toxic fractions were heat stable and could be stored for several months without losing their phytotoxic activity. Few researchers have investigated the aqueous phase of *A. solani* filtrates for its toxicity, and no researchers have tested the chloroform extracts for phytotoxicity at their original culture filtrate concentrations. Cotty et al (9) extracted zinniol with chloroform from culture filtrates of another "large-spored" *Alternaria* species, *A. tagetica,* and reported that the aqueous fraction contained undefined toxic compounds as well. Matern et al (15) reported that two lipidlike compounds were present in cultures of *A. solani* and both were required for phytotoxicity on potato leaves. Shepard et al (18) failed to purify these toxic compounds with chloroform extraction but found instead that the toxic fraction was water soluble. Recently, Langsdorf et al (11) detected an unidentified susceptibility-inducing factor in the aqueous fraction of *A. solani* spore germination fluid.

Apparently, *A. solani* synthesizes a number of toxins in culture, and their isolation and identification is more difficult than previously thought. The isolates used, pathogen culture conditions, bioassays employed, sensitivity of analytical techniques, and the fact that multiple toxins are being synthesized should be considered in future investigations on pathogen metabolites and their involvement in pathogenicity.

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