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Zinniol Production by *Alternaria* Species

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ABSTRACT

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Thirty-one isolates of 10 pathogenic *Alternaria* spp. were tested for in vitro production of zinniol, a nonselective phytotoxin, on a casamino acids-enriched medium. Analyses were performed by gas-liquid and thin-layer chromatography with synthetic zinniol as a standard. Of the seven pathogenic large-spored, long-beaked *Alternaria* spp. tested, *A. carthami, A. macrospora, A. porri, A. solani, A. tagetica,* and an unnamed isolate from pods of *Phaseolus vulgaris* produced zinniol but *A. brassicae* and a nonpathogenic isolate of *A. zinniae* did not. Zinniol was not detected in filtrates of three pathogenic species lacking large spores and long beaks (*A. alternata, A. citri,* and *A. raphani*). The quantity of zinniol produced varied greatly among species, among different isolates of a single species, and between trials of the same isolate. All hosts of the *Alternaria* spp. tested were sensitive to zinniol at 50–200 μg/ml. The evolutionary conservation of zinniol production in pathogenic large-spored *Alternaria* spp. may be indicative of its importance in pathogenesis.

Zinniol, 3-methoxy-4-methyl-5(3-methyl-2-butenyl-oxy)-1,2-benzenedimethanol, first described as a major phytotoxic metabolite of *Alternaria zinniae* in 1968 (14), only very recently was found to be produced by four other *Alternaria* spp. (1,2,15–17). That zinniol may be important in the pathogenicity of *A. zinniae* (14,18), *A. dauci* (1), and *A. tagetica* (2) has been suggested. In those studies, however, often only single isolates were tested for toxin production (2,14–16,18), or if more than one isolate was studied, zinniol production was measured for only one isolate (1) or was not reported at all (2,11,15).

The production of zinniol by five *Alternaria* spp. and the low metabolic cost of its biosynthesis (16) compared to other phytotoxins of *Alternaria* led us to the supposition that zinniol production may have been a highly conserved characteristic of some plant pathogenic *Alternaria* spp. during coevolution with their hosts.

Our interest in the possible conservation of zinniol production and the general paucity of information on the distribution of the secondary metabolites of *Alternaria* spp. led us to investigate zinniol production within our collection of plant pathogenic *Alternaria* spp.

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MATERIALS AND METHODS

Cultures. Fungi were isolated from various plants in northern Mexico and southern Arizona except for *A. zinniae, A. alternata* f. sp. *lycopersici*, and the rough lemon isolate of *A. citri* (Table 1). Fungal isolates were maintained on a modified V-8 medium containing 5% V-8 vegetable juice (v/v) and 2% agar (w/v) at 27 C under 5,200 lux fluorescent light on a 12-hr diurnal cycle. Most test fungi sporulated adequately under these conditions but *A. solani* and *A. zinniae* did not; their sporulation was induced with Kilpatrick's filter paper technique (6).

Pathogenicity tests. Isolates were tested for pathogenicity on intact plants and/or excised leaves or fruits. Fully developed leaves were excised with a sharp razor blade and placed, abaxial surface down, on 1.5 × 3.1-mm (8 × 16 per inch)-mesh fiberglass screen on moistened filter paper in 9-cm-diameter plastic petri dishes. Ten microliters of 0.005% Triton X-100 solution containing 1–50 spores was placed on the leaf surface. Controls were treated with 10 μl of 0.005% Triton X-100. Bean-pod isolates (Table 1) were tested on detached young bean pods (2–6 cm long) according to the above procedure. Intact plants (25 to 45 days old) were sprayed to runoff with 0.005% Triton X-100 solution containing one to 10 spores per microliter and incubated in a dark humidity chamber (100% relative humidity) at 27 C for 24 or 48 hr. Plants were then held at 28–30 C under 12 hr of 5,800 lux fluorescent light daily until symptoms developed, but not longer than 21 days. Isolates of *A.*

citri that caused black fruit rot (Table 1) were tested by injecting 200 μl of the above spore suspension into the stem end of juice-orange fruits with a hypodermic needle. Inoculated and control fruits (injected with 0.005% Triton X-100) were bisected and evaluated after 30–40 days of incubation in the dark at 27 C.

Pathogenicity tests involved the following pathogen-host combinations: *A. alternata* (Fr.) Keissler f. sp. *lycopersici* on tomato (*Lycopersicon esculentum* Mill. 'Early Pak 7'), *A. brassicae* (Berk.) Sacc. on broccoli (*Brassica oleracea* L. var. *botrytis*, 'Italian Green Sprouting') and on turnip (*Brassica rapa* L., 'Burpee 6121'), *A. raphani* Groves and Skolko on broccoli and turnip, *A. carthami* Chowdhury on safflower (*Carthamus tinctorius* L.), *A. citri* Ell. and Pierce on orange (*Citrus nobilis* Lour.), *A. macrospora* Zimm. on cotton (*Gossypium barbadense* L., 'Pima S5'), *A. porri* (Ellis) Cif. on onion (*Allium fistulosum* L., 'Yellow Sweet Spanish'), *A. tagetica* Shome and Mustafee on marigold (*Tagetes erecta* L., 'Hawaii,' 'Orangeade,' 'Golden Rooster,' and 'Crackerjack,' and *T. patula* L. 'Sparky'), *A. zinniae* Pape on zinnia (*Zinnia elegans* Jacq., 'Giant Cactus Flowered,' 'Giant Double,' and 'Cherrytime'), *Alternaria* sp. isolated from pods of pinto bean (*Phaseolus vulgaris* L., 'Oliathe,' 'Pinamerpa,' and 'Canary 101'). Pathogenicity tests all had a minimum of five replications; they were repeated at least twice, except for the isolates of *A. citri* which were tested once.

Zinniol production. Hyphal tip- or single-spore-derived isolates were used for toxin production. A 2-cm-diameter disk from a 2-wk-old V-8 agar culture was seeded into a 125-ml flask containing 25 ml of casamino acids-enriched medium (18). Cultures were incubated without shaking at 24 C in the dark for 35 days. The incubation time was based on the curve for production of zinniol by *A. dauci* (1).

Zinniol was extracted by a modification of the procedure reported by White and Starratt (18). The culture filtrates from five flasks were pooled, passed through Whatman No. 1 filter paper, adjusted to pH 10 with 5.0 N and 1.0 N NaOH, and extracted three times with 50 ml of chloroform. The alkaline pH permitted partitioning of all the pigments into the aqueous phase. The chloroform fraction was washed twice with 50 ml of 0.1 N NaOH (pH 13) and three times with 50 ml of 0.1 M KH_2PO_4 (pH 4.5) and evaporated under reduced pressure. The residue was dissolved in 4.6 ml of chloroform and stored at 4 C. Isolates were tested one to six times for zinniol production (Table 1).

Identification and quantification of zinniol. Zinniol was initially identified by gas-liquid chromatography (GLC). Analyses were performed with a Varian series 1700 dual flame ionization gas chromatograph (Varian Associates, Inc., Palo Alto, CA 94306) with a 100-cm long, 2.1-mm i.d., stainless steel column of 3.0%

TABLE 1. Zinniol production[a] by *Alternaria* spp. isolated from various hosts

Species	Isolate	Disease	Host	Origin	Trials[b] (no.)	Zinniol[c]
A. alternata f. sp. *lycopersici*	TA-1[d]	Stem canker	Tomato	Davis, CA	2	–
A. brassicae[e]	W1	Leaf spot	Wild Brassica	Litchfield, AZ	2	–
	W7				2	–
	W14				2	–
A. carthami[e]	S1	Blight	Safflower	Guasave, Mexico	4	+
	S2				2	+
	S3				2	+
A. citri	OR1	Black rot	Orange	Tucson, AZ	2	–
	OR3				3	–
	OR4				2	–
A. citri	RL1[f]	Blight	Rough lemon	Florida	2	–
A. macrospora[e]	C3	Leaf spot	Cotton	Cochise County, AZ	5	+
	C8				1	+
	C9				2	+
	C10				4	+
A. porri[e]	07	Purple blotch	Onion	Stuart, AZ	2	+
	010				2	+
	015				2	+
A. raphani	Tu1	Black spot	Turnip	Litchfield, AZ	4	–
	Tu2				3	–
	Tu3				2	–
A. solani[e]	T4	Early blight	Tomato	San Quintin, Mexico	5	+
	T9				2	+
	T15				2	+
A. tagetica[e]	16	Blight	Marigold	Los Mochis, Mexico	3	+
	17				5	+
	23				3	+
A. zinniae[e]	Z18[g]	Blight	Zinnia	Berkeley, CA	6	–
?[e]	B2	Pod canker	Bean	Kansas Settlement, AZ	4	+
	B3				3	+
	B5				3	+

[a] Fungi were incubated in a casamino acid-enriched medium at 27 C for 35 days prior to filtrate analysis.
[b] Number of experiments in which zinniol production was tested.
[c] + = zinniol production detected in at least one experiment.
[d] Received from Ken Kimble, Moran Seed Co., Davis, CA 95616.
[e] Long-beaked, large-spored *Alternaria* sp. ? = Species name not assigned.
[f] Received from J. M. Gardner, AREC, University of Florida, Lake Alfred 33850.
[g] Received from R. B. Hine, Dept. of Plant Pathology, Univ. of Arizona, Tucson 85721.

SE-52 on Gas Chrom Q (2). The column, detector, and injector were kept at 190, 220, and 250 C, respectively, and the carrier gas (N_2) flow rate was 30 ml/min. The performance of this column was superior to that of the 2% SE-30 column used by Barash et al (1).

Initial estimates of zinniol quantities were made on the washed chloroform fractions. Precise identifications and quantifications were performed on fractions containing detectable quantities of zinniol following silylation with Tri-Sil Z (Pierce Chemical Co., Rockford, IL 61105) according to the manufacturer's instructions. A standard curve (micrograms of zinniol per centimeter of peak height) was constructed with silylated authentic zinniol (supplied by J. A. Martin, Roche Products Ltd., Welwyn Garden City, England).

Identification of zinniol was confirmed with thin-layer chromatography (TLC). Ten- to 100-μl aliquots of each of the fractions were cochromatographed with authentic synthetic zinniol on prechanneled, 200-μm-thick, silica-coated glass TLC plates with preadsorption zones (Analtech, Newark, DE 19711) by using acetone-hexane-chloroform (1:1:1, v/v) as a solvent. Zinniol was visualized with vanillin-sulfuric acid (1,2). All washed chloroform fractions were subjected to TLC analysis regardless of the GLC results. To verify the dependability of GLC and TLC for zinniol identification, zinniol fractions from *A. tagetica* and *A. porri* were subjected to mass spectrometry following GLC according to the procedure reported earlier (2).

Percent recovery of zinniol. To determine the percent recovery of zinniol from culture filtrates, a known quantity of zinniol was added to 80 ml of uninoculated medium; then zinniol was extracted, silylated, and quantified by GLC as described above. The test was repeated three times.

Bioassay of zinniol. Zinniol was bioassayed on detached leaves of host plants including tomato, turnip, broccoli, safflower, cotton, onion, marigold, zinnia, and pinto bean. Excised leaves were placed, abaxial side down, on moistened filter papers in 9-cm-diameter plastic petri dishes. Twenty-microliter aliquots of solutions of synthetic zinniol (50, 100, and 200 μg/ml) in 0.02 M phosphate buffer, pH 5.5, containing 10% ethanol were applied to the leaf surfaces. Ethanolic buffer solutions serving as controls were applied to different spots on the same leaves treated with zinniol solutions. Leaves were pricked once through each droplet with a hypodermic needle shortly after application of the test solutions, kept at 25–27 C under 12 hr of fluorescent light (4,500 lux) daily, and after 3 days were rated for sensitivity to the test materials under a dissecting microscope (×30) by using transmitted light. Leaves that exhibited darkening around the wounds were rated positive for toxic reaction. Each solution was bioassayed on a total of 20 leaves.

RESULTS

All *Alternaria* spp. tested were pathogenic on their respective hosts, except the isolate of *A. zinniae*, which was nonpathogenic in each of six trials.

Mass spectra of silylated zinniol produced in cultures of *A. porri* and *A. tagetica* agreed exactly with those previously reported (2) and verified the dependability of the GLC and TLC techniques employed.

Zinniol was produced by all long-beaked, large-spored *Alternaria* spp. tested except *A. zinniae* and *A. brassicae*. None of the other *Alternaria* spp. produced zinniol (Table 1).

The quantity of zinniol produced varied greatly among various species, among different isolates of a single species and in different trials with the same isolate (Table 2). Zinniol was detected by TLC in all filtrates containing >2 μg of zinniol per milliliter. Zinniol spots on TLC plates stained violet-purple with vanillin-sulfuric acid and faded to red-brown within 1 mo. In no case was a zinniol-like substance detected in filtrates of a nonproducer of zinniol by TLC. The recovery of zinniol ranged from 59 to 68% and averaged 62%.

Bioassay of zinniol. All plant species tested were sensitive to zinniol at concentrations ranging from 50 to 200 μg/ml (Table 3). Onion appeared to be slightly less sensitive to zinniol than other plants, probably because of the difficulties involved in treating onion leaves with toxin solutions.

DISCUSSION

Results of experiments such as these must be cautiously interpreted as they largely reflect the limits of the experiment. Detection of in vitro toxin production by test organisms depends upon chemical, nutritional, and environmental factors that may influence toxin production as well as the sensitivity of the analytical techniques employed (10). Moreover, toxin production in vitro is not always correlated with that in vivo (10). Consequently, the distribution of zinniol production under conditions not tested here, particularly in vivo, may or may not be the same as that reported here.

Zinniol production seems to be a common characteristic of large-spored, long-beaked *Alternaria* spp. Of the eight large-spored, long-beaked species tested, only *A. brassicae* and *A. zinniae* did not produce detectable amounts of zinniol in vitro. A pathogenic isolate of *A. zinniae* has been reported to produce zinniol in the culture medium (14,18). Therefore, the inability of one nonpathogenic isolate of *A. zinniae* to produce detectable

TABLE 2. Quantities of zinniol detected per test[a] in filtrates of zinniol-producing isolates of *Alternaria*

Species	Isolate	Zinniol[c] (μg)		
		Highest	Lowest	Average[b]
A. carthami	S2	36,671	32,263	34,468
	S3	17,210	14,905	16,058
	S1	30,656	18,316	23,629
?	B2	7,113	4,087	5,989
	B3	79	6	34
	B5	1,524	89	882
A. macrospora	C8	40[d]	40[d]	40[d]
	C9	26	0	13
	C10	240	0	77
	C3	1,340	0	889
A. porri	07	9,306	6,316	7,811
	010	8,947	8,582	8,765
	015	43,739	9,868	26,803
A. tagetica	16	1,615	416	1,068
	17	6,687	771	3,579
	23	785	547	700
A. solani	T9	60	58	59
	T4	3,760	19	1,044
	T15	50	24	37

[a] In each test fungi were grown in 125 ml of casamino acid-enriched medium for 35 days at 25 C; the resulting filtrate was analyzed for zinniol.
[b] Refer to Table 1 for number of trials.
[c] Quantities have been divided by 0.62 to compensate for the average loss during recovery.
[d] Results of a single trial.

TABLE 3. Percent of excised leaves[a] of various host plants exhibiting toxic reactions to zinniol solutions after 72 hr

Plant	Leaves (%) with toxic reaction to zinniol at:		
	50 μg/ml	100 μg/ml	200 μg/ml
Pinto Bean	15	75	95
Broccoli	20	50	65
Cotton	20	75	100
Marigold	10	75	95
Onion	5	30	50
Safflower	25	85	95
Turnip	20	30	90
Zinnia	10	65	90

[a] Twenty leaves of each host were treated with each zinniol concentration.

levels of zinniol in vitro in this study might have been due to the loss of its pathogenicity. *A. alternata* f. sp. *lycopersici* and *A. citri* that have small spores and short beaks, and *A. raphani*, that has medium-sized spores and medium-length beaks, did not produce detectable levels of zinniol in vitro. However, the number of species tested in the latter two groups is not large enough to conclusively support the possibility that zinniol is not produced by small-spored, short-beaked *Alternaria* spp. (2). The inability of *A. brassicae* to produce zinniol may reflect a phylogeny divergent from those of other large-spored and long-beaked *Alternaria* spp. However, the dynamics of in vitro toxin production and degradation may vary even among isolates of the same species (12); therefore, zinniol production by some species may have been missed by the single sampling time used in this study.

Quantities of zinniol produced by different *Alternaria* species, and by different isolates of one species, were quite variable (Table 2). The level of zinniol production by a single isolate also varied among tests. Stoessl et al (16) found similar differences in production of alternaric acid and altersolanol-A among isolates of *A. solani* and between experiments with the same isolate.

The bioassay results agree with previous reports (1,2,18) that zinniol is a nonselective phytotoxin. This is in contrast to a number of toxins of *Alternaria* that are highly host-selective. In the latter case, the pathogens generally attack newly developed, widely cultivated plant genotypes (3,8,9,11). Most of the older genotypes are highly tolerant of these pathogens; therefore, diseases caused by host-selective, toxin-producing fungi are easily managed by elimination of a few susceptible genotypes. For example, *A. alternata* f. sp. *lycopersici*, which produces highly selective toxins (3,13), first arose in the early 1970s as a pathogen of only a few tomato cultivars that were abandoned almost immediately (4). In a similar manner, during long periods of host-pathogen coevolution, highly selective toxins may have been readily eliminated as significant factors due to the minor host changes that would result in resistance. On the other hand, some *Alternaria* spp. are not as highly host-specific. These *Alternaria* spp. are typified by *A. solani*, the causal agent of early blight of tomato, potato, and other solanaceous plants. Compared to the Alternaria diseases in which highly host-selective toxins have been implicated, early blight has a very long history. One possible annotation to the history of early blight is that highly selective toxins are not vital to the pathogenicity of *A. solani*. It should be noted that a host-selective fraction has been detected in culture filtrates of *A. solani* (5,7).

The broader host range and relative stability of certain *Alternaria* spp. may be due to their ability to produce nonselective toxins. Host resistance to highly selective toxins would be acquired far more rapidly than to nonselective toxins. Therefore, nonselective toxins, particularly those synthesized with maximum metabolic economy, are more likely to be conserved in closely related species during evolution. Our results indicate that zinniol may be an example of such a conserved metabolite. However, conservation of zinniol may be due to some aspect other than its involvement in the disease.

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