Production of Zinniol by *Alternaria tagetica* and its Phytotoxic Effect on *Tagetes erecta*

P. J. Cotty, I. J. Misaghi, and R. B. Hine

Department of Plant Pathology, University of Arizona, Tucson 85721.
Journal Series Paper 3747 of the Arizona Agricultural Experiment Station, Tucson.
The authors would like to thank J. A. Martin for supplying synthetic zinniol.
Accepted for publication 13 April 1983.

ABSTRACT

Cotty, P. J., Misaghi, I. J., and Hine, R. B. 1983. Production of zinniol by *Alternaria tagetica* and its phytotoxic effect on *Tagetes erecta*. Phytopathology 73:1326-1328.

Alternaria tagetica, the causal agent of stem, leaf, and flower blight of marigold, is capable of producing phytotoxins in vitro. One of the toxins was identified as zinniol by comparing it with synthetic zinniol in: bioassays; thin-layer and gas chromatography; staining properties; ultraviolet and mass spectrometry, and nuclear magnetic resonance spectroscopy. The identity of the other toxin(s) has not been established. Symptoms similar to those caused by infection developed on detached marigold leaves treated with either of the toxins or synthetic zinniol. The toxins are not host selective and are capable of causing injury to zinnia, cotton, okra, corn, tomato, watermelon, and sunflower.

Marigolds (*Tagetes erecta* L.) are grown commercially on approximately 3,500 ha of land near Los Mochis, Sinaloa, Mexico, from October to July. Xanthophyll pigments extracted from the flowers are used in poultry feed. *Alternaria tagetica* Shome and Mustafee was identified during the winter of 1979 as the causal agent of a serious stem, leaf, and flower blight of marigold. The initial symptoms of the disease are dark-brown necrotic spots on stems, leaves, and flowers. During the early stages of disease development, leaf spots are often surrounded by chlorotic halos. Subsequently, leaf spots expand and coalesce, which leads to wilting and drying of the leaves and plant death. Damage to the flowers is particularly extensive resulting in total darkening and shriveling of petals during the late stages of the disease.

Serious outbreaks of flower blight occurred during the winters of 1979 and 1980 when foggy, wet conditions prevailed. The disease caused approximately 50–60% reduction in flower yield.

Results of our preliminary studies showed that cell-free culture filtrates of the fungus contained a factor which, when introduced into leaves through wounds, caused symptoms on marigold leaves similar to those caused by infection (2). This observation was the basis for the speculation that toxins may play a role in this disease. Toxin production in vitro is a common feature of many *Alternaria* species. Host-selective toxins are known to be produced by *A. kikuchiana* (4), *A. mali* (5), and *A. alternata* f. sp. *lycopersici* (3). Nonselective toxins such as alternariols, alternaric acid, and zinniol also are produced by a number of *Alternaria* species (8).

The objectives of this study were to determine the identity of the toxic components in culture filtrates of *A. tagetica* which exhibit toxicity on marigold leaves and to establish a possible relation between the toxins and those reported from other *Alternaria* species.

MATERIALS AND METHODS

Pathogenicity test. Cultures of the fungus isolated from diseased marigolds collected in Los Mochis, Mexico, were maintained on a modified V-8 medium (5% V-8 juice [v/v] and 4% agar [w/v]) under 12 hr of fluorescent light (5,200 lux) daily at 24 C. Seven-wk-old marigold plants (cultivars Golden Rooster, Orangeade, and Hawaii) and zinnia plants (*Zinnia elegans* Jacq., cultivars Giant Fantasy, Giant Double, Tapestry, Lilliput, and Giant Flowered Zinnia), grown in an environmental chamber at 24 C under 16 hr of fluorescent light (5,800 lux) daily, were used. Plants were sprayed inoculated to runoff with spore suspensions (2.0×10^4 spores per milliliter) obtained from 2-wk-old cultures of *Alternaria tagetica* grown on the modified V-8 medium. Plants were incubated in a nonilluminated humidity chamber (100% RH) at 24 C and examined for typical disease symptoms after 24 and 48 hr. The disease was allowed to progress by maintaining plants at 24 C under 16 hr of light (5,800 lux) daily until infected plants shriveled and died. Pathogenicity tests were repeated at least twice.

Preparation and partial purification of toxins. A highly virulent single-spore-derived isolate (17E) was used for toxin production. A disk (2 cm in diameter) of a 1-wk-old culture was seeded into each 125-ml flask containing 25 ml of casamino acid-enriched medium (9). After 21 days of incubation at 24 C without illumination, the culture medium was passed through Whatman No. 1 filter paper, adjusted to pH 8.5 with 1.0 N NaOH, and extracted three times with equal volumes of chloroform. The chloroform fraction referred to hereafter as (fraction I) was washed with one-third volume of 0.1 M KH_2PO_4, pH 4.4, three times. This extraction procedure was modified from that reported by Barash et al (1). Chloroform was then evaporated under reduced pressure, and the residue was dissolved in 2 ml of chloroform and stored at 4 C.

The residual chloroform was removed from the aqueous fraction (fraction II) under reduced pressure. The aqueous fraction was then adjusted to pH 5.5 with 0.1 N HCl, filter sterilized, and stored at 4 C.

Characterization of the fraction I toxin. The solubility of fraction I in water and in a number of organic solvents was similar to that of zinniol, a phytotoxin produced by *A. zinniae*. Therefore, the fraction was cochromatographed with authentic synthetic zinniol (supplied by J. A. Martin, Roche Products Ltd., Welwyn Garden City, England) on 200-μm-thick, silica-coated plastic TLC plates (Silica Gel 60 F_{254}, E. Merck, Darmstadt, W. Germany) and developed with the following solvent systems: acetone-hexane-chloroform (1:1:1, v/v), hexane-acetone (3:1, v/v), or acetone-chloroform (1:1, v/v). Zinniol was visualized on plates as described by Barash et al (1) with either vanillin-sulfuric acid or anisaldehyde-sulfuric acid.

Silylated derivatives of fraction I and authentic zinniol were analyzed by gas-liquid chromatography (GLC). Trimethylsily derivatives were prepared with Tri-Sil Z (Pierre Chemical Co., Rockford, IL 61105) according to manufacturer's instructions. Analyses were performed with a Varian series 1700 dual flame ionization gas chromatograph (Varian Associates, Inc., Palo Alto, CA 94306) with a 110 cm long, 2.1-mm ID stainless steel column of 3.0% SE-52 on Gas Chrom Q. The column, detector, and injector were kept at 190, 220, and 250 C, respectively, and the flow rate was 30 ml/min.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. § 1734 solely to indicate this fact.

Ultraviolet spectra of zinniol and of fraction I (before and after TLC separation) in chloroform were determined with a Gilford model 2600 computerized spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH 44074) with a 0.1 nm step size and three readings per point.

Mass spectra of the silylated compounds were determined after gas chromatography with a Hewlett-Packard 5930A quadrapole mass spectrometer, (Hewlett-Packard, Palo Alto, CA 94304). To determine the NMR spectrum of the toxin in fraction I, TLC plates were spotted with zinniol and with fraction I with and without added authentic zinniol and developed with acetone-chloroform-hexane (1:1:1, v/v). Migrated spots of fraction I were scraped from the plates and eluted with chloroform. Chloroform was evaporated under nitrogen, residues were dissolved in deuterated chloroform and analyzed with a WM 250 NMR spectrometer (Bruker Instruments, Inc., Manning Park, Billerica, MA 01821). H-NMR data were compared with those of synthetic zinniol. Preparation and purification of fraction I through TLC were performed numerous times; H-NMR and mass spectrometry were done once.

Bioassay of toxin fractions. The following preparations were bioassayed: crude extracts, fraction I (before and after TLC separation), fraction II, and fractions from sterilized, noninoculated media prepared by the procedures used to fractionate the culture filtrates. The chloroform from fraction I was removed under partial pressure and the residue was dissolved in 1 ml of ethanol and diluted with 0.02 M phosphate buffer, pH 5.5, to a final concentration of 10% ethanol. Subsequent dilutions were made with 10% ethanol in the above buffer.

For the preliminary bioassay 6-wk-old zinnia and marigold plants were excised at the soil line and placed in vials containing 5.0 ml of nondiluted test solutions. Plants were kept at 24 C under 12 hr of fluorescent light (5,200 lux) daily throughout the bioassay and were evaluated after 48 hr. This bioassay was repeated twice. For leaf bioassays excised leaves of marigold and nonhost plants (*Zinnia elegans* Jacq., *Gossypium hirsutum* L., *Hibiscus esculentus* L., *Zea mays* L., *Lycopersicon esculentum* Mill., *Citrullus vulgaris* Schrad, and *Helianthus annuus* L.) were placed on moistened filter papers in plastic petri dishes. Twenty microliters of different dilutions of a test solution were applied to the adaxial surfaces of leaflets on one side of the leaf midrib. Opposite leaflets were treated with fractions from uninoculated media. Shortly after application of the test solutions, leaflets were pricked once through the solution with a hypodermic needle and incubated at 25 C under 12 hr of fluorescent light (5,200 lux) daily and rated for symptoms after 48 hr. The dilution end point (the highest dilution of a fraction active in the bioassay) of each toxic fraction was determined on marigold and zinnia by diluting the test fractions up to 50 times. Other hosts were assayed at a 1:1 dilution only. A 1:2 dilution is equivalent to one half the concentration of the original filtrate.

RESULTS

Pathogenicity tests. All marigold cultivars tested were susceptible to *A. tagetica*. Lesions were apparent within 48 hr in the humidity chamber and progressed rapidly upon removal from the humidity chamber. Plants shriveled and dried in 3–10 days when placed on greenhouse benches. The symptoms of the disease were identical to those observed in the field. None of the zinnia cultivars tested was susceptible.

Sporulation. The fungus sporulated profusely on the modified V-8 medium after 5 days at 25 C under 12 hr of fluorescent light (5,200 lux) daily.

Isolation and characterization of the toxin. Toxin concentrations sufficient to be detected by the leaf bioassay were accumulated in the liquid culture medium after 21 days of incubation. The crude culture filtrate was separated into a chloroform fraction (fraction I) and aqueous fraction (fraction II) following chloroform extraction. The toxic components of both fraction I and II were dialyzable.

One component in fraction I had the same R_f values as synthetic zinniol in three different solvents and exhibited color reactions identical to those of zinniol with both vanillin-sulfuric acid and anisaldehyde-sulfuric acid spray reagents. The R_f values were 0.4, 0.24, and 0.6 in acetone-hexane-chloroform (1:1:1, v/v), hexane-acetone (3:1, v/v), and acetone-chloroform (1:1, v/v), respectively.

Zinniol and an active component in fraction I had identical retention times (7 min) in GLC. The ultraviolet spectrum of zinniol in chloroform had a major peak at 241.5 nm and minor peaks at 274 nm and 281 nm. The active component of fraction I separated by TLC had the same ultraviolet spectrum as synthetic zinniol in chloroform. The 241.5-nm peak was predominant in fraction I before TLC.

The mass spectra of the silylated phytotoxic component of fraction I and silylated zinniol were identical. The major peaks were (m/e): 320, 252, 237, 169, 163, and 147.

The H-NMR spectrum of zinniol agreed with that reported by Starratt (6): 1.71 and 1.77 (singlets of three protons each, $=CMe_2$), 2.13 (three protons, singlet aromatic Me), 3.21 and 3.38 (broad singlets, hydroxyl protons), 3.73 (three protons, singlet, -OMe), 4.49 (two protons, doublet, J = 6.5 Hz, $=C\text{-}CH_2\text{-}O$), 4.63 and 4.71 (singlets of two protons each, $\text{-}CH_2OH$), 5.46 (one proton, triplet, =CH-), and 6.65 (one proton, singlet, aromatic). In addition to the above peaks assigned to zinniol, the following contaminant peaks also were present in the active fraction eluted from TLC plates: 0.86, 1.09, 1.23, 1.39, 1.53, and 8.08.

Bioassay of toxic fractions. Fractions I and II were phytotoxic to all plants tested. Exposure of excised marigold and zinnia plants to fraction I resulted in wilting and the associated symptoms of darkening of the tissue adjacent to midribs and veins within 48 hr. These symptoms were similar to those reported for zinniol (9). Wilting also was induced in excised plants subjected to fraction II within 48 hr but no darkening occurred. In bioassays involving detached, wounded leaves, fraction I recovered from TLC plates, as well as zinniol, caused dark-brown lesions in 48 hr typical of the primary lesions of the disease. Fraction II produced lighter and more diffuse, gray-brown lesions than fraction I. The dilution-end-points of the toxic fractions were the same on both zinnia and marigold and ranged from 2:1 to 1:2 for fraction I and from 1:1 to 1:25 for fraction II. Synthetic zinniol produced symptoms on detached wounded leaves at a 100 μg/ml concentration, but not at 50 μg/ml. Fractions from sterilized, uninoculated media prepared by the procedure used to fractionate culture filtrates were not phytotoxic.

Results of thin-layer and gas chromatography, staining of TLC plates, UV, MS, and NMR spectra and bioassays show that the active component in fraction I is zinniol. The identity of the toxin(s) in fraction II is currently being determined.

DISCUSSION

Results of our study show that *A. tagetica* produces at least two toxins. One was identified as zinniol, a phytotoxin produced by three other *Alternaria* spp. (1,6,7). The identity of the other toxin(s) has not been established yet. Each toxin is capable of producing some of the symptoms of the disease on marigold, the host of the fungus. These toxins are not host-selective since they also are active against zinnia, a non-host, at the same concentrations which caused damage to marigold.

Zinniol may be considered a potentially important factor in pathogenesis since it is produced by *A. tagetica* (this report), *A. zinniae* (6), *A. dauci* (1), and *A. solani* (7) and causes pertinent symptoms on the respective hosts of at least the first three fungi. In the *A. tagetica*-marigold system, in addition to zinniol, at least one other toxin may be involved in pathogenesis.

Our attempts to isolate zinniol from diseased tissue have not yet been successful. The failure could be due to degradation of the toxin prior to or during extraction or to the insensitivity of the analytical methods employed.

To our knowledge, zinniol has only been recovered from large-spored *Alternaria* spp. with long beaks. Furthermore, we have not been able to detect it in filtrates from *A. alternata* f. sp. *lycopersici*, which is a small-spored species. Thus, the production of zinniol by the four large-spored species with long beaks may be merely another indication of their taxonomic relatedness.

LITERATURE CITED

1. Barash, I., Mor, H., Netzer, D., and Kashman, Y. 1981. Production of zinniol by *Alternaria dauci* and its phytotoxic effect on carrot. Physiol. Plant Pathol. 19:7-16.
2. Cotty, P. J., Misaghi, I. J., and Hine, R. B. 1982. Phytotoxin production by *Alternaria tagetica*. (Abstr.) Phytopathology 72:942.
3. Gilchrist, D. G., and Grogan, R. G. 1976. Production and nature of a host-specific toxin from *Alternaria alternata* f. sp. *lycopersici*. Phytopathology 66:165-171.
4. Nishimura, S., Kohmoto, K., and Otani, H. 1979. The role of host-specific toxins in saprophytic pathogens. Pages 133-146 in: Recognition and Specificity In Plant Host-Parasite Interactions. J. M. Daly and I. Uritani, eds. Japan Scientific Soc. Press, Tokyo, and University Park Press, Baltimore. 355 pp.
5. Okuno, T., Ishida, T. Sawai, K., and Matsumoto, T. 1974. Characterization of alternariolide, a host-specific toxin produced by *Alternaria mali*. Chem. Lett. (Chem. Soc. Jpn.) 1974:635-638.
6. Starratt, A. N. 1968. Zinniol: A major metabolite of *Alternaria zinniae*. Can. J. Chem. 46:767-770.
7. Stoessl, A., Unwin, C. H., and Stothers, J. B. 1979. Metabolites of *Alternaria solani* V: Biosynthesis of altersolanol A and incorporation of naltersolanol A-$^{13}C_x$ into altersolanol B and macrosporin. Tetrahedron Lett. 27:2481-2484.
8. Stoessl, A. 1981. Structure and biogenetic relations: Fungal non-host specific. Pages 109-219 in: Toxins in Plant Disease. R. D. Durbin, ed. Academic Press, New York. 536 pp.
9. White, G. A., and Starratt, A. N. 1967. The production of a phytotoxic substance by *Alternaria zinniae*. Can. J. Bot. 45:2087-2090.