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PHYTOTOXIN PRODUCTION BY ALTERNARIA SPECIES

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PHYTOTOXIN PRODUCTION BY *ALTERNARIA* SPECIES

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Peter John Cotty

entitled Phytotoxin production by Alternaria species

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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	Date 11 July 84

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director — Date 11 July 84

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SIGNED: John Colley

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ABSTRACT

Alternaria tagetica is capable of producing phytotoxins in vitro. One toxin was identified as zinniol with: 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.

The distribution of zinniol among *Alternaria* species was studied. Thirty-one isolates of 10 pathogenic *Alternaria* spp. were tested for their ability to produce zinniol. Analyses were performed by gas-liquid and thin-layer chromatography. Of the seven pathogenic large-spored, long-beaked species tested *A. carthami*, *A. macrospora*, *A. porri*, *A. solani*, *A. tagetica*, and an unnamed isolate from *Phaseolus vulgaris* pods produced zinniol. *A. brassicae*, a non-pathogenic isolate of *A. zinniae*, and three pathogenic species lacking large-spores and long-beaks (*A. alternata*, *A. citri*, and *A. raphani*) did not produce zinniol. The quantity of zinniol produced varied greatly among species, among 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 to 200 micrograms/ml. Conservation of zinniol in pathogenic large-spored *Alternaria* spp.

may be indicative of its importance in pathogenesis.

Light affects the behavior of Alternaria tagetica in vitro and in vivo. In vitro zinniol production occurred only during active fungus growth in the light; in the dark zinniol production occured primarily after growth stopped. In all filtrates, the quantity of zinniol rapidly declined once zinniol production ceased. Fungus growth was inhibited by both continuous and alternating light and sporulation occurred on one of three test media and only under alternating light. More lesions were produced on inoculated plants kept in dark humidity chambers than in illuminated humidity chambers. Low illuminance was more conducive to lesion development than high illuminance and more lesions developed on plants exposed to low illuminance for 48 hr prior to inoculation than on those exposed to high illuminance. The limitations of studies in which the effect of light has been overlooked are discussed.

CHAPTER 1

PHYTOTOXIN PRODUCTION BY *ALTERNARIA TAGETICA*

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 occured 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. This observation was the basis

for the speculation that toxins may play a role in disease. Toxin production in vitro is a common feature of many Alternaria species. Host-selective toxins are known to be produced by A. kikuchiana (29), A. mali (30), and A. alternata f. sp. lycopersici (13). Nonselective toxins such as alternariols, alternaric acid, and zinniol also are produced by a number of Alternaria species (41).

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 flourescent 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 spray-inoculated to runoff with spore suspensions (20,000 spores per ml) 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 (47). After 21 days of incubation at 24 C without illumination, the culture medium was passed through a 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 potassium phosphate monobasic, pH 4.4, three times. This extraction procedure was modified from that reported by Barash et al (3). 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 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-micrometer 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. (3) with either vanillin-sulfuric acid or anisaldehyde-sulfuric acid.

Silylated derivatives of fraction I and authentic zinniol were analyzed by gas-liquid chromatography (GLC). Trimethylsilyl derivatives were prepared with Tri-Sil Z (Pierce 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 i.d. 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.

Ulraviolet 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 5930 A 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. Subsequently 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 hersutum 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 fraction II were dialyzable.

One component in fraction I had the same Rf 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 Rf 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, =C2[Me]), 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-C2[H]-O), 4.63 and 4.71 (singlets of two protons each, -C2[H]OH), 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 micrograms per milliliter concentration, but not at 50 micrograms per milliliter. 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 under investigation.

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. (3, 38, 40). 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 (38), A. dauci (3), and A. solani (40) 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.

CHAPTER 2

ZINNIOL PRODUCTION BY *ALTERNARIA* SPECIES

Zinniol, 3-methoxy-4-methyl-5(3-methyl-2-butenyl-oxy)-1,2-benzenedimethanol, was first described as a major phytotoxic metabolite of *A. zinniae* in 1968 (38) and was only very recently demonstrated as a product of four other *Alternaria* species as well (3, 8, 40, 42, 44). The possibility that zinniol is an important factor in the pathogenicity of *A. zinniae* (38, 47), *A. dauci* (3), and *A. tagetica* (8) has been suggested. However, in these studies often only single isolates have been tested for toxin production (8, 38, 40, 42, 47). In some cases where more than one isolate was tested, quantification of zinniol production was performed on only one isolate (3, 40) or was not reported at all (8, 42, 44).

The production of zinniol by five *Alternaria* spp. and the low metabolic cost of its biosynthesis (42) compared to other *Alternaria* phytotoxins led us to the supposition that zinniol production may be a highly conserved characteristic of some plant pathogenic *Alternaria* spp. during coevolution with their hosts. Our interest in the possible conservation of zinniol 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.

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 lemmon 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. While most test fungi sporulated adequately under these conditions, *A. solani* and *A. zinniae* did not. Sporulation of these two fungi was induced with Kilpatrick's filter paper technique (21).

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 8 X 16 mesh fiberglass screen on moistened filter paper in 9-cm plastic petri dishes. Ten microliters of 0.005% Triton X-100 solution containing 1 to 50 spores was placed on the leaf surface. Controls were treated with 10 microliters 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/microliter and incubated in a non-illuminated humidity chamber (100% RH) 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 which caused black fruit rot (Table 1) were tested by injecting 200 microliters 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-to-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 Grogan, Kimble, and Misaghi on tomato (Lycopersicon esculentum Mill., cultivar Early Pak 7), A. brassicae (Berk.) Sacc. on broccoli (Brassica oleracea L. var. botrytis L., cultivar Italian Green Sprouting) and turnip (Brassica rapa L., cultivar 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., cultivar Yellow Sweet Spanish), A. tagetica Shome and Mustafee on marigold (Tagetes erecta L., cultivar Sparky), A. zinniae Pape on zinnia (Zinnia elegans Jacq., cultivars Giants Cactus Flowered, Giant Double, and Cherrytime), Alternaria sp. isolated from pinto bean pods on pinto bean (Phaseolus vulgaris L., cultivars Oliathe, Pinamerpa, and Canary 101). Pathogenicity tests were repeated at least twice with a minimum of five replications, except for the A. citri isolates which were tested once.

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

Species	Isolate	Disease	Host	Origin	# Trials[e]	Zinniol[f]
alternata f.sp. *lycopersici*	TA-1[b]	Stem canker	Tomato	Davis, CA	2	-
brassicae[a]	W1	Leaf spot	Wild Brassica	Litchfield, AZ	2	-
	W7				2	-
	W14				2	-
carthami[a]	S1	Blight	Safflower	Guasave, Mexico	4	+
	S2				2	+
	S3				2	+
citri	OR1	Black Rot	Orange	Tucson, AZ	2	-
	OR3				3	-
	OR4				2	-
citri	RL1[c]	Blight	Rough Lemon	Florida	2	-
macrospora[a]	C3	Leaf spot	Cotton	Cochise County, AZ	5	+
	C8				1	+
	C9				2	+
	C10				4	+
porri[a]	07	Purple blotch	Onion	Stuart, AZ	2	+
	010				2	+
	015				2	+
raphani	Tu1	Black spot	Turnip	Litchfield, AZ	4	-
	Tu2				3	-
	Tu3				2	-
solani[a]	T4	Early blight	Tomato	San Quintin, Mexico	5	+
	T9				2	+
	T15				2	+
tagetica[a]	16	Blight	Marigold	Los Mochis, Mexico	3	+
	17				5	+
	23				3	+
zinniae[a]	Z18[d]	Blight	Zinnia	Berkeley, CA	6	-
?[a]	B2	Pod canker	Bean	Ks. Settlement, AZ	4	+
	B3				3	+
	B5				3	+

? = Species name not assigned.

[a]Long-beaked, large-spored *Alternaria* sp.

[b]Received from Ken Kimble, Moran Seed Co., Davis, Calif. 95616

[c]Received from J. M. Gardner, AREC, University of Florida, Lake Alfred, FL 33850

[d]Received from R. B. Hine, Dept. of Plant Pathology, Univ. of Arizona, Tucson, AZ 85721

[e]Number of experiments in which zinniol production was tested.

[f]+ = zinniol production detected in at least 1 experiment.

[g]Fungi were incubated in a casamino acid enriched medium at 27 C for 35 days prior to filtrate analysis.

Zinniol Production

Hyphal tip- or single-spore-derived isolates were used for toxin production. A 2-cm-dia disk from a 2-wk-old V-8 agar culture was seeded into a 125-ml flask containing 25 ml of casamino acid-enriched medium (47). Cultures were incubated without shaking at 24 C in the dark for 35 days. The length of the incubation time was based on the curve for production of zinniol by A. dauci (3).

Zinniol was extracted by a modification of the procedure reported by White and Starratt (47). The culture filtrates from five flasks were pooled and passed though Whatman #1 filter paper, adjusted to pH 10 with 5.0 N and 1.0 N NaOH, and extracted 3 times with 50 ml chloroform. The alkaline pH permitted partitioning of all the pigments into the aqueous phase. The chloroform fraction was washed twice with 50 ml 1.0 N NaOH, pH 13, and three times with 50 ml potassium phosphate monobasic, 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 for zinniol production one to six times (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% SE-52 on Gas Chrom Q (8). The column, detector, and injector were kept at 190, 220, and 250 C, respectively, and the carrier gas

(nitrogen) flow rate was 30 ml/min. This column was superior to the 2% SE-30 column used by Barash et al (3).

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 manufacturer's instructions. A standard curve (micrograms zinniol per centimeter peak height) was constructed with silylated authentic zinniol (supplied by Dr. J.A. Martin, Roche Products Ltd., Welwyn Garden City, England).

Identification of zinniol was confirmed with thin-layer chromatography (TLC). Ten to 100 microliters of each of the fractions were co-chromatographed with authentic synthetic zinniol on prechanneled, 200 micrometer-thick-silica-coated-glass TLC plates with preadsorption zones (Analtech, Newark, DE 19711) using acetone-hexane-chloroform (1:1:1, V/V) as a solvent. Zinniol was visualized with vanillin-sulfuric acid (3,8). 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 (8).

Percent Recovery

To determine the percent recovery of zinniol from culture filtrates, a known quantity of zinniol was added to 80 ml of non-

inoculated 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 plastic petri dishes. Twenty-microliter aliquots of solutions of synthetic zinniol (50, 100, and 200 micrograms per milliliter) in 0.02 M phosphate buffer, pH 5.5, containing 10% ethanol were applied to 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. Leaves were kept at 25-27 C under 12 hr fluorescent light (4,500 lux) daily and after 3 days were rated for their sensitivity to the test materials under a dissecting microscope (30X) using transmitted light. Leaves exhibiting darkening around 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 *A. zinniae* isolate, which was non-pathogenic 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 (8) 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 greater than 2 micrograms per milliliter of zinniol. Zinniol spots on TLC plates stained violet-purple with vanillin-sulfuric acid and faded to red-brown within a month. In no case was a zinniol-like substance detected in filtrates of a non-zinniol-producer 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 micrograms per milliliter (Table 3). Onion appeared 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. Detec-

Table 2. Quantities[a] (ug) of zinniol detected per test[c] in filtrates of zinniol-producing Alternaria isolates.

Species	Isolate[b]	Highest	Lowest	Average[b]
carthami	S 2	36671	32263	34468
	S 3	17210	14905	16058
	S 1	30656	18316	23629
?	B 2	7113	4087	5989
	B 3	79	6	34
	B 5	1524	89	882
macrospora	C 8	d	d	40
	C 9	26	0	13
	C10	240	0	77
	C 3	1340	0	889
porri	07	9306	6316	7811
	010	8947	8582	8765
	015	43739	9868	26803
tagetica	16	1615	416	1068
	17	6687	771	3579
	23	785	547	700
solani	T 9	60	58	60
	T 4	3760	19	1044
	T15	50	24	37

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

Plant	Zinniol concentration (ug/ml)		
	50	100	200
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.

tion of in vitro toxin production by test organisms depends upon chemical, nutritional, and environmental factors which may influence toxin production, as well as, the sensitivity of the analytical techniques employed (32). Moreover, toxin production in vitro is not always correlated with that in vivo (32). 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 (38, 47). Therefore, the inability of one non-pathogenic isolate of A. zinniae to produce detectable 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 with small spores and short beaks and A. raphani, with medium-size spores and medium-length beaks did not produce detectable levels of zinniol in vitro. However, the number of species tested in the later two groups is not large enough to conclusively support the possibility that zinniol is not produced by small-spored, short-beaked Alternaria spp. (8). 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 and

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 found similar differences in production of alternaric acid and altersolanol-A among isolates of A. solani and between experiments using the same isolate (42).

The bioassay results agree with previous reports (3, 8, 47) that zinniol is a non-selective phytotoxin. This is in contrast to a number of Alternaria toxins which are highly host-selective. In the latter case, the pathogens generally attack newly developed, widely cultivated plant genotypes (15, 28, 29, 33). Most of the older genotypes are highly tolerant of these pathogens and, 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 (13, 35), first arose in the early 1970's as a pathogen of only a few tomato varieties which were abandoned almost immediately (15). In a similar manner, during long periods of host-pathogen coevolution, highly selective toxins may be 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 (18, 22).

The broader host range and relative stability of certain Alternaria spp. may be due to their ability to produce non-selective toxins. Host resistance to highly selective toxins would be acquired far more rapidly than to non-selective toxins. Therefore, non-selective toxins, particularly those synthesized with maximum metabolic economy, are more likely to be conserved in closely related species through evolutionary time. 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.

CHAPTER 3

EFFECT OF LIGHT ON THE BEHAVIOR OF *ALTERNARIA TAGETICA* IN VITRO AND IN VIVO

Even though the effect of light on sporulation of *Alternaria* spp. is well documented (43), little information is available on how light affects other aspects of *Alternaria* life cycles and their interactions with plants. Experiments designed to elucidate the effect of humidity on disease induction by *Alternaria* spp. have frequently been performed in the dark or under unregulated and/or unmeasured low light conditions (1,4,20,23). Similarly, light often has not been considered and/or strictly monitored in studies dealing with toxin production by *Alternaria* spp. Consequently, much of our current knowledge of plant pathogenic *Alternaria* spp. is based on experiments in which light was not regarded. The need for studies on the effects of light on disease development (7) and toxin production (24) has been emphasized.

We have studied the effect of light on marigold (*Tagetes erecta*) susceptibility to *A. tagetica*. We have also investigated the influence of light on the growth, sporulation, and spore germination of the fungus, and in vitro production of zinniol, a phytotoxin of possible importance in diseases caused by a number of *Alternaria* spp. (3,8,9,47).

Materials and Methods

Cultures

Isolate 23, a pathogenic isolate of *A. tagetica* already known to produce zinniol (9), was used in these studies. The fungus was maintained in the dark at 27 C on a modified V-8 juice medium (5/2) containing 5% V-8 vegetable juice (V/V) and 2% agar (W/V). When sporulating cultures were required, subcultures were placed under flourescent lights (5,200 lux) on a 12 hr diurnal cycle at 27 C. Illuminance was measured with a model 756 Weston sunlight illumination meter, (Weston Instruments, Inc. Newark, N.J. 07114).

Effect of Light on Fungus Growth

The fungus was grown under various light regimes on the following three solid media: Potato Dextrose Agar (PDA) (Difco Laboratories, Detroit, Michigan, 48201), the casamino acids-enriched medium (TOX) used for zinniol production (8,9,47) solidified with 2% agar, and 5/2 medium described above. Fungal disks, 4-mm in diameter, taken from the edge of advancing fungal colonies on medium 5/2, were placed in the center of 9-cm diam plastic petri plates containing various media. Cultures were incubated in either the light or the dark by placing seeded culture plates inside 14-cm diam clear plastic petri plates or inside identical 14-cm diam plates painted on the outer surfaces with a flat black enamel paint. The 14-cm plates were then placed under appropriate illumination. This method permitted free exchange of gases in both light and dark treatments and thus

was superior to wrapping plates with various materials (1,16,27).

Culture plates were maintained at 27-29 C under either 21,000 or 8,000 lux illuminance either continuously or on a 12 hr diurnal cycle. General Electric (General Electric, Cleveland, Ohio 44117) F48T12 and Sylvania (Sylvania Electric Products, Inc., Danvers, Mass. 01923) F20T12 cool white flourescent light provided the 21,000 and 8,000 lux illuminances, respectively. Radial growth of the fungus was measured at the widest colony diameter daily for 7 days. The values were used to generate a regression line by the least squares method. To prevent the initial lag period from skewing the data, the zero-time points were not included in the calculations. The slopes of the regression lines were used as estimates of the fungus growth rates. Differences among rates within experiments were determined by analysis of variance. Values with statistically significant differences were separated by the least significant difference method (LSD).

The following experiment was conducted to determine if the observed responses of A. tagetica to light could be duplicated by changes in the media induced by light prior to seeding, as reported for other fungi (48). Nine-cm-diameter culture plates containing 5/2 medium, on which the greatest light responses occurred, were exposed to light or darkness by placing them in either clear or blackened 14-cm plates under 8,000 lux continuous illuminance at 27-29 C for 72 hr. Light-treated and dark-treated culture plates were then seeded with the fungus as described earlier. Each group was split and

incubated at 27-29 C, half in the light (8,000 lux) and half in the dark, as previously described. Fungus growth rate was measured and values were analyzed as described above.

Effect of Light on Germination

Ten microliter spore suspensions were applied to microscope slides placed on moistened filter papers inside 9-cm plastic petri dishes. Spores were subjected to either continuous light (23,000 lux) or continous dark at 27-29 C by placing petri dishes inside either clear or blackened 14-cm plastic plates as described earlier. The number of germ tubes per spore and the maximum germ tube length per spore were determined with a compound microscope after 4 hr.

Effect of Light on Lesion Production

Marigold plants (Tagetes erecta L. cv orangeade) were grown in sand in 7-oz styrofoam cups under flourescent lights with 8,000 lux illuminance (Photosynthetically active radiation at 400-700 nm (PAR) = 96 microeinsteins/s/square meter) on a 12 hr diurnal cycle at 27-33 C. Cups were watered daily with distilled water and fertilized once every 10 days with 10 ml of a 4,000 ppm solution of Miracle-Gro (Stern's Nurseries, Inc., Geneva, N.Y. 14456). Five 6-wk-old plants (8 to 10 leaf stage) were trimmed of senescent and damaged leaves and sprayed to run-off with spore suspensions containing 600-1000 spores/ml and were immediately placed in humidity chambers. Similar individuals were paired and pairs were split between treatments. Inocula were prepared by flooding sporulating plates (prepared as

described above) with sterile distilled water and by passing the suspensions through two layers of cheesecloth.

Humidity chambers were constructed from disposable plastic 16-oz soda pop bottles which permitted illumination of plants during infection. These bottles, with the bottoms removed, fit over the individual marigold plants used in this experiment. For dark treatments plastic chambers were covered with two layers of aluminum foil. Glass thin layer chromatography tanks (30x27x10-cm) with clear glass lids containing 100 ml of distilled water also were used as humidity chambers to determine if the observed responses were due to factors unique to the plastic chambers. Identical chambers covered with aluminum foil were used for dark treatments. All humidity chambers containing plants were placed in growth chambers (Environmental Growth Chambers, Chagrin Falls, Ohio 44022) under flourescent lights (General electric cool white bulbs, F48T12) in various illuminances for various periods as described below. Plants were removed from the humidity chambers after different periods and maintained at 27-29 C under 21,000 lux (PAR = 252 microeinstein-/s/square meter) illuminance on a 12-hr diurnal cycle. Lesions on the six oldest leaves of each plant were counted by illuminating the leaves with back lighting 48 hr after inoculation.

To compare the effect of light and dark on lesion production, inoculated plants inside either plastic or glass chambers were exposed to 21,000 lux light for 12, 20, 24, and 48 hr. Experiments on the effect of high verses low light on lesion production were

conducted within one environmental chamber, a portion of which was shaded with fiberglass screening to reduce the illuminance from 23,000 lux to 2,000 lux (PAR = 24 microeinsteins/s/ square meter). Plants were subjected to either high (23,000 lux) or low (2,000 lux) illuminance inside plastic humidity chambers (as above) for 20 hr and were examined for lesion production 48 hr after inoculations as described earlier. Experiments were repeated twice, each with 6 replications.

The effect of varying light intensity prior to infection on host susceptibility was tested by exposing plants for 48 hr prior to inoculation to either 23,000 lux or 2,000 lux illuminance on 12 hr diurnal cycles. Plants were then inoculated (as above) and placed in unilluminated humidity chambers for 16 hr after which they were removed and kept under 23,000 lux light for an additional 32 hr and examined for lesion production. The experiment was repeated twice with 6 replications.

Effect of Light on Zinniol Production

Erylenmeyer flasks (125 ml) containing 25 ml of a casamino acids enriched medium, used previously for zinniol production (8,9,47), were seeded with 2-cm-diam disks from two-wk-old sporulating cultures of A. tagetica on 5/2 medium. For dark treatment, flasks were wrapped with two layers of aluminum foil. Wrapped and unwrapped flasks were arranged in a complete block design and incubated without shaking under 23,000 lux continuous light at 27-29 C.

After 10, 20, and 30 days culture filtrates were pooled in

groups of five and passed through Whatman #1 filter paper. The fungus colonies were dried on preweighed filter papers at 106 C for 48 hr and weighed to the nearest 10 mg. The filtrate was extracted and analyzed for zinniol content as described earlier (9). Filtrates were adjusted to pH 10 and extracted three times with 50 ml of chloroform. 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 potassium phosphate monobasic, pH 4.5, and evaporated under reduced pressure. Zinniol was silylated and quantified by gas-liquid chromatography as previously described (8). 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% SE-52 on Gas Chrom Q. The column, detector, and injector were kept at 190, 220, and 250 C, respectively, and the carrier gas (nitrogen) flow rate was 30 ml/min. A standard curve (micrograms zinniol/cm peak height) was constructed with silylated synthetic zinniol (synthetic zinniol was supplied by Dr. J.A. Martin, Roche Products Ltd., Welwyn Garden City, England). Each of the two experiments consisted of 6 treatments replicated twice. Each replicate consisted of one analysis of five filtrates pooled.

Results

Effect of Light on the Growth of the Fungus

The growth of the fungus on the three tested media was inhibited

by both continuous and alternating light (Table 4). Although the growth rates varied between experiments, the differences between the growth rates of light-grown cultures were in most cases statistically significant ($p = 0.01$ by the LSD method).

Exposure of media for 72 hr to either continuous 8,000 lux illuminance or dark prior to seeding did not result in statistically significant differences ($P = 0.2$ by the LSD method) in the rate of growth. This held for growth both in the dark and within 8,000 lux illuminance.

Effect of Light on Lesion Production

More lesions were produced on inoculated plants kept in the dark than on those kept in the light in both glass and plastic humidity chambers (Table 5). More lesions also developed on inoculated plants kept under low illuminance (2,000 lux) than on those kept under high illuminance (23,000 lux) (Table 6).

The light intensity to which plants were exposed prior to inoculation also affected the number of lesions. More lesions developed on plants grown under low illuminance (2,000 lux on a 12 hr diurnal cycle) for 48 hr prior to inoculation than on plants grown under high illuminance (23,000 lux on a 12 hr diurnal cycle) (Table 6).

Effect of Light on Spore Production and Germination

Spores were not produced on TOX or PDA media. No spores were produced under continuous light or continuous dark on any medium.

Table 4. Growth rates of Alternaria tagetica on solid media under various light regimes at 27-29 C

Experiment[a] number	Illuminance (lux)	Medium[b]	Diurnal light[c] period (hr)	Rate[d] (um/hr)
1	23,000	PDA	24	293 ± 14 A
			0	373 ± 17 B
		5/2	24	292 ± 16 A
			0	369 ± 42 B
		TOX	24	177 ± 4 C
			0	212 ± 3 C
2	23,000	PDA	12	346 ± 2 A
			0	402 ± 3 B
		5/2	12	343 ± 9 A
			0	437 ± 9 C
		TOX	12	261 ± 6 D
			0	289 ± 7 E
3	8,500	5/2	24	202 ± 40 A
			0	416 ± 10 B
		TOX	24	154 ± 3 C
			0	214 ± 3 A
4	8,500	PDA	24	239 ± 61 A
			0	473 ± 3 B
		TOX	24	157 ± 1 C
			0	212 ± 3 D
5	8,500	5/2	12	447 ± 4 A
			0	467 ± 3 B
		TOX	12	211 ± 5 C
			0	260 ± 3 D
6	8,500	PDA	12	410 ± 14 A
			0	430 ± 6 B
		TOX	12	215 ± 2 C
			0	243 ± 4 D

[a] Treatments were replicated three times. Each replicate consisted of one culture; radial growth was measured seven times at 20 to 30 hour intervals. All experiments were repeated twice with similar results; data from one repetition is presented here.

[b] PDA = potato dextrose agar; 5/2 = 5% V-8 vegetable juice and 2% agar; TOX = the casamino acids enriched medium used for zinniol production plus 2% agar.

[c] The fungus was exposed to light at the indicated illuminance for 0, 12 or 24 hr daily.

[d] Growth rate ± standard deviation. Growth rate was determined by calculating the slope of the best fit line for the seven measured points with the least squares method. The initial point was not included to avoid slope skewing by the lag period. Statistical comparisons were made only within experiments. Values not followed by the same letter are significantly different (P=0.01 except experiment 6 for which P=0.05) by the LSD method.

Table 5. Effect of light (23,000 lux) during high humidity periods on the number of lesions produced by Alternaria tagetica on 30-40 day old marigold plants.

Experiment[a] number	Humidity[b] period	Number of lesions[c]	
		Light	Dark
1	12	0	2 ± 1
2	12	0	14 ± 11
3	20	0	11 ± 5
4	20	2 ± 2	58 ± 21
5	24	2 ± 2	25 ± 14
6	24	0	11 ± 7
7	48	6 ± 4	51 ± 22
8	48	34 ± 11	86 ± 45

[a] Light and dark treatments were replicated 4 times each in experiments 1-2 and 5-8, and 3 times in experiments 3-4.

[b] Duration of period immediately following inoculations during which plants were maintained in humidity chambers.

[c] Mean number of lesions on the six oldest leaves per plant. The differences between light and dark treatments were significant at the 5% level by analysis of variance in experiments 1-7 and at the 7% level in experiment 8.

Table 6. The number of lesions produced on 35 day old marigold plants exposed to different light intensities prior to and after inoculation with A. tagetica.

Experiment[a] number	Illuminance (lux)[b] prior to inoculation	Humidity[c] period (hr)	Illuminance (lux)[d] during humidity period	Number of[e] lesions	
1	8,000	20	23,000	9.5 ± 5.8	A
	8,000	20	2,000	50.7 ± 23.5	B
2	8,000	20	23,000	1.6 ± 1.6	A
	8,000	20	2,000	26.3 ± 16.0	B
3	23,000	16	0	12.7 ± 7.4	*
	2,000	16	0	23.3 ± 10.0	*
4	23,000	16	0	17.5 ± 4.4	A
	2,000	16	0	45.2 ± 14.7	B

[a] Experiments consisted of two treatments, each with six replicates.

[b] Light intensity on a 12 hr diurnal cycle under which plants were grown for 48 hr prior to inoculation.

[c] Duration of period immediately following inoculations during which plants were maintained in humidity chambers.

[d] Continuous illuminance.

[e] Mean number of lesions on the six oldest leaves per plant ± the standard deviation. Statistical comparisons were made only within experiments. Numbers not followed by the same letter are significantly different by analysis of variance, (p=0.01). * = significantly different by analysis of variance (p=0.06).

Abundant spores were produced on 5/2 medium under light (8,000 lux and 23,000 lux) on a 12 hr diurnal cycle at 27-29 C. Light condition affected neither the number of germ tubes per spore nor the maximum germ tube length per spore.

Effect of Light on Zinniol Production

Zinniol production was greatly affected by light. The zinniol production pattern in the light and in the dark differed and probably followed the curves in Figure 1 which were extrapolated from the data in Table 7. Zinniol production in the light coincided with the active growth of the fungus and stopped as the fungal growth ceased. In the dark only small amounts of zinniol were produced during active fungus growth while large quantities were rapidly produced for a short period after cessation of growth. In both the light and the dark, the quantity of zinniol in filtrates rapidly declined once zinniol production ceased.

Discussion

Sporulation of many Alternaria spp. is influenced by light intensity and duration (28). Results of this study have shown that A. tagetica is not an exception and that a diurnal light cycle is required for sporulation. In addition, the growth rate of A. tagetica was decreased by exposure to light on all media and under all light regimes tested. The growth of A. brassicicola (45) and A. brassicae (15, 27) are also decreased by light while that of A. carthami is increased (1). Griffin (14) has pointed out that light

Table 7. Effect of light on growth and zinniol production by Alternaria tagetica in a casamino-acids enriched liquid medium at 27 to 28 C.

Experiment number[a]	Illuminance (lux)[b]	Growth period	Growth (g)[c]	Zinniol (ug)[d]
1	0	10 days	2.5 ± 0.55	210 ± 142
	0	20 days	2.5 0.02	9856 ± 113
	0	30 days	2.6 ± 0.06	2748 ± 573
	23,000	10 days	2.1 ± 0.16	1827 ± 113
	23,000	20 days	2.5 ± 0.08	4034 ± 956
	23,000	30 days	2.6 ± 0.13	418 ± 90
2	0	10 days	2.5 ± 0.17	2179 ± 313
	0	20 days	2.6 ± 0.09	3869 ± 1047
	0	30 days	2.6 ± 0.08	402 ± 176
	23,000	10 days	2.4 ± 0.09	6031 ± 81
	23,000	20 days	2.7 ± 0.14	784 ± 119
	23,000	30 days	2.7 ± 0.13	185 ± 6

[a] Each experiment consisted of 6 treatments replicated twice. Each replicate consisted of five filtrates (25 ml each) which were pooled and batch analyzed.

[b] Continuous illuminance.

[c] Dry weight ± standard deviation.

[d] Micrograms zinniol ± standard deviation. Quantities have been divided by 0.62 to compensate for the average percent recovery as previously described (9).

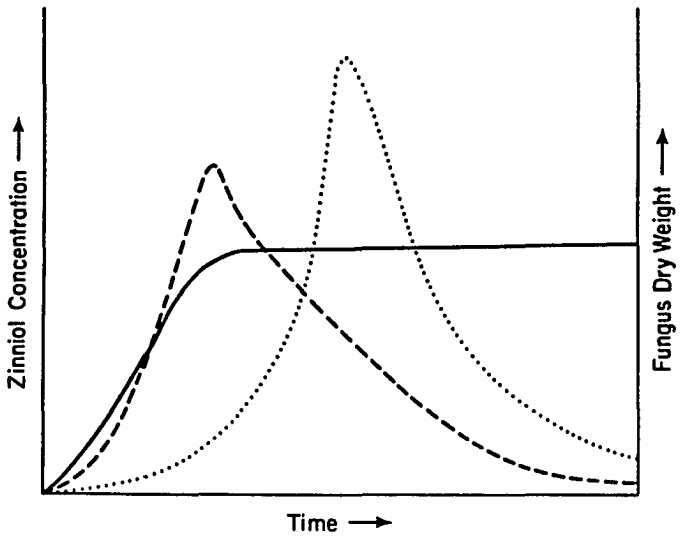

Fig. 1. Zinniol production under continuous light (dashed line) and continuous dark (dotted line) as a function of growth (solid line) extrapolated from data in Table 4.

modulation of in vitro fungus growth must be cautiously interpreted because preexposure of media to light can result in growth inhibition (48). In our study, no differences were found between the growth rates of A. tagetica on media preexposed to 8,000 lux for 3 days and on media preexposed to dark.

We found that light also influences production of zinniol, a non-selective phytotoxin which may play a role in a number of Alternaria diseases including that caused by A. tagetica on marigold (3, 8, 9, 47). Results summarized in Table 7 show that zinniol is predominantly produced during the active growth phase of the fungus in the light and during the stationary growth phase in the dark. The relationships between growth phase and zinniol production in the light and in the dark are more clearly seen in the extrapolated curves in Fig. 1. The patterns of zinniol production by A. tagetica in the light and in the dark differ greatly from that reported for A. dauci. The quantity of zinniol present in filtrates of A. dauci increased with increases in fungus dry weight and remained unchanged once zinniol production ceased. In contrast, the quantity of zinniol in culture filtrates of A. tagetica rapidly declined once zinniol production stopped. A. tagetica may thus produce an extracellular system, not produced by A. dauci, that alters zinniol once production stops.

Light-mediated inhibition of toxin production by A. alternata has been reported (17, 31, 36). The light-induced alterations in the zinniol production pattern of A. tagetica exemplifies difficulties of

evaluating toxin production data in studies where toxin levels are measured at one time (9, 17, 31) or during one growth phase (9, 17, 31, 36) (exponential or stationary) of the fungus. A reevaluation of the earlier studies (9, 17, 31, 36) may be of value.

Results presented here show that the number of lesions produced on A. tagetica inoculated marigold plants is decreased by increased light intensity in the humidity chamber. Similarly, dark exposure increases lesion diameter on bean leaves (Phaseolus vulgaris cv. Irene and Corene) inoculated with A. zinniae (46). However, in that study, light intensity was neither measured nor carefully regulated and the effect of light on the number of lesions produced was not determined.

The observation that light modulates disease production by A. tagetica and a number of other pathogens (2, 5, 11, 12, 37, 46) has profound implications on the interpretation of the results of studies on various aspects of Alternaria diseases. For example, light-dependent resistance mechanisms may be overlooked in laboratory screenings conducted under inappropriate light conditions (20, 25, 26). Such studies may lead to an unrealistic assessment of relative tolerance of cultivars and/or species to certain Alternaria spp. Moreover, incidence and severity of diseases caused by Alternaria and some other pathogens in the field may be expected to be influenced by factors such as canopy position, cloud cover, and shading which modulate the quality and the quantity of light which plant parts receive. Shading has been implicated in at least one Alternaria

disease (2) and canopy position may explain in part the higher susceptibility of older leaves to Alternaria spp. (25, 39).

In studies designed to assess the role of humidity on disease development, light condition has generally not been taken into account as an important parameter. Inoculated plants are often placed in humidity chambers under no light or low light conditions, for periods longer than 12 hr (1, 4, 6, 19, 20). The observed influence of light on disease development underscores the need for considering the effect of light on disease during exposure of plants to high humidity. Careful consideration of both light and humidity may help to separate the influences of these two variables on the disease.

The mechanism by which light intensity influences host susceptibility to A. tagetica is not known. Preliminary observations using the tissue clearing method of Diener (10) and staining with a fast green/clove oil mixture showed that A. tagetica penetrates marigold leaves predominantly by direct means. Therefore, a simple effect on stomata opening is inadequate to explain the light effect on the disease. The growth of the fungus in vitro is somewhat reduced in the presence of light. However, it is rather unlikely that the increased susceptibility of marigold to A. tagetica under low light intensity is conditioned solely by a concomitant increased rate of colonization of the host by the fungus. The results indicate that the light response also is not due to inhibition of spore germination.

Our demonstration that exposure of marigolds to low light

intensity prior to inoculation leads to an increase in susceptibility suggests that the light-induced changes in susceptibility may be due to the effect of light on the host rather than on the pathogen. Changes in susceptibility to disease were observed in plants subjected to low light intensity for as little as 12 hr. Thus, alterations in susceptibility may indeed be a natural phenomenon because plants in the field are diurnally subjected to low light intensity for comparable periods.

Zinniol production in vitro is modulated by both light and the growth phase of the fungus (Fig. 1). It is, therefore, tempting to ascribe a role for zinniol in the low light-high susceptibility equation. However, since zinniol production in vivo has not been demonstrated, the role of this toxin in disease and in light-induced responses remains highly speculative.

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