An Antifungal Polyacetylene Compound from Phytophthora-infected Safflower

C. A. Thomas and E. H. Allen

Plant Pathologist and Biochemist, respectively, Crops Research Division, ARS, USDA, Beltsville, Maryland 20705.

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ABSTRACT

A virulent isolate of *Phytophthora drechsleri* caused necrotic lesions when placed in hypocotyl wounds of 8-week-old *Carthamus tinctorius* plants. An aqueous solution of the methanol-soluble substances extracted from infected hypocotyls was very inhibitory to mycelial growth of the fungus, whereas similar solutions from healthy hypocotyls were not inhibitory. The inhibitory factor, extractable from the aqueous solution by diethyl ether, was isolated by thin-layer chromatography by using three successive solvent systems. Ultraviolet, infrared, and mass spectral data indicated that the compound is 3,11-tridecadiene-5,7,9-triyne-1,2-diol (safynol).

Considering amounts of safynol lost during chromatography, healthy hypocotyls contained 55 μg/100 g fresh tissue. The concentration approximately doubled during 4 days after wounding. A 20-fold increase in concentration occurred in hypocotyls during 4 days after inoculation with *P. drechsleri*. The median effective dose (ED_{50}) of the compound to inhibit mycelial growth of *P. drechsleri* was 12 μg/ml. Safynol accounted for as much as 75% of the inhibitory activity of alcohol extracts from infected hypocotyls. Phytopathology 60:261-263.

Varieties and selections of safflower (*Carthamus tinctorius* L.) differ in their resistance to *Phytophthora* root rot incited by *Phytophthora drechsleri* Tucker. The safflower selection Biggs possesses a high level of resistance to known races of the pathogen (9). Wound-inoculation of hypocotyls of adult greenhouse plants differentiates the Biggs resistance from reactions of more susceptible varieties.

Klisiewicz & Johnson (5) reported that morphology is not a factor in resistance of Biggs. Spore germination, growth of germ tubes, and penetration occurred both in Biggs and in susceptible varieties; substances toxic to germ tube growth were not found in fresh wound sap of healthy Biggs hypocotyls. They suggested that dead tissues of infected Biggs may be unsuitable for continued growth of *P. drechsleri* if toxic substances are released.

This study was begun to determine if compounds inhibitory to mycelial growth of *P. drechsleri* develop in Biggs hypocotyls in response to infection. The identity, concentration, and antibiotic activity of the major fungitoxic compound found in infected safflower hypocotyls are described.

MATERIALS AND METHODS.—The pathogenic reactions on safflower cultivars of isolate 201 of *P. drechsleri* used in this study have been described (9). Plants of the safflower selection Biggs were grown and inoculated in the greenhouse. Plants (four/pot) were grown in steamed soil in porous 8-inch clay pots. Inoculum consisted of 5-mm square plugs cut from lima bean agar plate cultures incubated 8 days at 27°C. Plants were inoculated 7 weeks after emergence by smearing inoculum into a 7-mm incision made approximately one-fourth of the way through the hypocotyl just above the soil surface. The inoculated area was covered with a plastic tube. Inoculated plants and wounded and unwounded control plants were held at 30°C in the greenhouse.

Hypocotyl sections were harvested 4 days after inoculation. Sections were cut at the vertical edges of the necrotic lesions which were approximately 1.8 to 2.3 cm long and extended one-fourth to one-half the way around the hypocotyl. Sections of similar dimensions were cut from hypocotyls of control plants. The harvested tissue sections were immediately extracted four times with methanol, 3 ml/g fresh tissue, in a blender for 5 min. Tissue fragments were recovered after each extraction by filtration. Alcohol was removed from the combined extracts by distillation under reduced pressure at 28°C. Water was added to the remaining aqueous solution to make a volume of 1 ml/g fresh tissue. Ether soluble substances in the aqueous solution were removed by shaking three times with diethyl ether and partitioning in a separatory funnel. Traces of ether in the aqueous phase were removed by evaporation under reduced pressure. Solvents were ACS certified, suitable for electronic, spectral, or microanalysis use. The ethyl ether was either anhydrous (Fisher Co.) containing 5×10^{-6}% sodium diethyldithiocarbamate or freshly distilled over $FeSO_4$.

Thin-layer chromatography (TLC) was employed to isolate the inhibitory compound present in the ether-soluble portion of aqueous solutions prepared from infected hypocotyls. The absorbant consisted of one part SilicAR (TLC-4GF, Mallinckrodt) mixed with forty parts Kiesel-Gel (D5, A. H. Thomas). The following solvents were used: (A) benzene:ethyl acetate:formic acid, 75:24:1, v/v; (B) chloroform:acetone:formic acid, 95:4:1, v/v; and (C) ethyl ether:petroleum ether:formic acid, 80:19:1, v/v. The locations of compounds on the plates were revealed as bands that quenched the fluorescence of the SilicAR under an ultraviolet (254 nm) lamp.

Mycelial growth tests were used to detect antifungal activity. Five-mm discs from lima bean-agar plate cultures of isolate 201 of *P. drechsleri* incubated 8 days

at 27°C were placed in 5 ml of test solution in 3 × 1 cm dishes. Measurements of the radial growth of the mycelium were made after 24 hr at 25°C in the dark.

In preparation for antifungal tests of ether soluble substances separated by TLC, the silica gel from quenching as well as nonquench-bands was eluted with ethyl ether. The ether eluates were washed with water and evaporated to dryness. Residues were mixed by stirring with an assay medium which consisted of sterile, aqueous lima bean extract, pH 5.7.

Due to the chemical reactivity of the compound isolated, all extractions, chromatographic procedures, and assays were performed either in the dark or with as little light as necessary.

RESULTS.—*Inhibitory activity of hypocotyl extracts.*—Three tests were run to determine the inhibitory activity of aqueous solutions of the alcohol-extractable substances. In each test, solutions containing 1 ml/g fresh tissue were prepared from 45 or more fresh-infected and control hypocotyls. The solutions were adjusted to about pH 5.0. The mycelial growth of *P. drechsleri* in the aqueous solution of alcohol-soluble compounds from infected hypocotyls was 80% less than the growth in the aqueous solution from healthy hypocotyls. Extraction of the aqueous solution (of alcohol-soluble compounds from infected hypocotyls) with ethyl ether increased mycelial growth 75%. When the residue from the ether-soluble fraction was incorporated into the ether-extracted aqueous solution, inhibitory activity similar to that of the unextracted aqueous solution resulted. Similar incorporation of ether-soluble compounds from healthy hypocotyls into ether-extracted aqueous solutions from healthy hypocotyls resulted in no inhibition of mycelial growth. No inhibition was detectable in controls in which the residue from a similar quantity of ethyl ether evaporated to dryness, was mixed with either the healthy-hypocotyl aqueous solution, or the ether-extracted solution from infected hypocotyls.

Isolation and identification of the antifungal compound.—These tests were replicated four times. When concentrated ether solutions (prepared from 100 g fresh-infected hypocotyls) were streaked across plates in a narrow band 2 cm from the edge, and were developed by using solvent A, compounds eluted from a band at R_F .21 completely inhibited mycelial growth. Compounds from all other bands showed little or no inhibition. Compounds obtained from R_F .21 in solvent A were then chromatographed with solvent B. Although many compounds separated with solvent B, only those at R_F .17 completely inhibited mycelial growth. The inhibitory compounds from R_F .17 on plates developed with solvent B showed additional separation when chromatographed with solvent C. With solvent C, a compound from a band at R_F .5 completely inhibited mycelial growth. Compounds from all other bands showed no inhibitory activity. The inhibitory compound from plates developed with solvent C showed no further separation when rechromatographed with solvents A, B, or C or when chromatographed with several other solvents.

The inhibitory compound from plates developed with solvent C was rechromatographed with this solvent. Eluates of the single band were evaporated to dryness, and the residue was dissolved in absolute ethanol for ultraviolet spectral studies. Maxima were found in the ultraviolet spectrum at 354, 330, 309, 290, 269, 255, 246, 235, 225, and 215 nm. The infrared spectrum, in CCl_4, revealed the presence of hydroxyl groups (3610 cm^{-1}) and acetylenic bonds (2,190 and 2,175 cm^{-1}). The mass spectrum showed that the compound has a molecular wt of 200. The ultraviolet, infrared, and mass spectral data, to be reported elsewhere in detail, indicate that the compound is 3,11-tridecadiene-5,7,9-triyne-1,2-diol (1, 2). We propose the name "safynol" to simplify referral to this compound. The concentrations of safynol in absolute ethanol were determined by optical density at 269 nm ($\varepsilon = 61{,}600$).

Loss of safynol during chromatography.—Since the compound is highly reactive, it was presumed that considerable loss occurs during chromatography. In three separate tests, 186, 765, and 798 μg of safynol each were added to water. The aqueous solutions were extracted with ether and successively chromatographed with the solvents A, B, and C with all procedures the same as those described. The amounts recovered in the three tests were 120, 472, and 544 μg, or an average of 65%.

Concentration of safynol in healthy, wounded and unwounded, and infected hypocotyls.—Two or more samples of 50-100 μg each of (i) fresh unwounded; (ii) wounded but noninoculated; and (iii) *P. drechsleri*-inoculated hypocotyl tissues were extracted with alcohol. Purification of safynol was achieved with TLC by using solvents A, B, and C consecutively. Considering loss during purification, the average amounts of safynol recovered from the unwounded, wounded, and infected tissues were, respectively, 55, 118, and 1,080 μg/100 g (Table 1).

Inhibition of mycelial growth of P. drechsleri with safynol.—Assays were performed, using an aqueous solution of the alcohol-soluble substances from fresh healthy hypocotyls, 1 ml/g, at pH 5.0. Absolute ethanol containing a known amount of safynol was added to the assay medium, and the ethanol was subsequently removed by distillation under reduced pressure. Inhi-

TABLE 1. Safynol[a] content of safflower hypocotyls 4 days after wounding and wound-inoculation with *Phytophthora drechsleri* at 30°C

Hypocotyl tissue	Fresh wt content		Dry wt content
	Determined	Corrected[b]	Corrected[b]
	μg/100 g	μg/100 g	μg/10 g
Healthy	36	55	32
Wounded only	77	118	68
Wounded and inoculated	700	1080	620

[a] 3,11-tridecadiene-5,7,9-triyne-1,2-diol (safynol), average of two tests.

[b] Corrected for 35% loss during purification by TLC (see text).

bition of mycelial growth was determined by comparing the growth with that obtained in a control medium to which pure absolute ethanol had been added and subsequently removed. In two tests, the median effective dose (ED_{50}) of safynol required to inhibit the mycelial growth was 12 μg/ml. Mycelial growth was completely inhibited by safynol at 30 μg/ml.

DISCUSSION.—Bohlmann et al. (1) described 10 naturally occurring polyacetylenes from safflower (*C. tinctorius*), including the one identified in this study. Although acetylenic compounds occur in many plants and are highly toxic to fungi, bacteria, and nematodes (8), their role in disease resistance has received little attention. Marx (7) reported production of diatretyne nitrile by a mycorrhizal fungus, and his studies suggest that this antibiotic affects the resistance of pine roots to pathogenic infections. Van Fleet (10) found that plants not recognized as containing polyacetylenes may produce such compounds if wounded. Although production of fungitoxic compounds by plant tissues in response to infection is well known (6), we are not aware of reports concerning such production of an identified toxic polyacetylene.

The accurate measurement of the toxicity of a polyacetylene is difficult, since it is probably breaking down during the antibiotic assay. For example, crystalline mycomycin has a "half-life" of 3 hr when stored at 27°C under nitrogen or in vacuo (4). Polyacetylenes are known to undergo polymerization in the presence of light (3) and oxygen (4). We found it imperative to conduct assays with safynol in total darkness, and to use as short a time period as possible to measure the inhibitory activity.

The antibiotic activity of safynol is not surprising, since other polyacetylene alcohols have been shown to be antibiotic (8). Due to loss of compound during extraction, purification, and assay, and to the presence of considerable healthy tissue in the infected hypocotyls extracted, the data presented tend to underestimate the amount and toxicity of safynol present in infected cells. The amount of safynol in alcohol extracts from infected hypocotyls appears to account for as much as approximately 75% of the inhibitory activity of the extract, considering loss during chromatography. We consider safynol to be the major, but not the only, antibiotic compound that accumulates in *Phytophthora*-infected safflower hypocotyls. The relative rates of accumulation of safynol in uniformly infected tissues of resistant, moderately resistant, and susceptible varieties must be determined before the role of this antibiotic polyacetylene in plant resistance can be assessed.

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