Influence of Infection by Phytophthora drechsleri on Inhibitory Materials in Resistant and Susceptible Safflower Hypocotyls

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ABSTRACT

Resistant Biggs safflower (*Carthamus tinctorius*) hypocotyls stab-inoculated with *Phytophthora drechsleri* mycelium showed a greater susceptibility around inoculation wounds flushed with water by string wicks than did inoculated controls without wicks, suggesting that a fungistatic material might have been removed in the water. Extracts of inoculated Biggs hypocotyls were generally less inhibitory, however, than extracts from inoculated susceptible Nebraska 10 (N10) safflower, and no positive evidence was obtained for a phytoalexin.

Hypocotyl exudates, obtained by spotting the cut ends of hypocotyls directly on silica-gel thin-layer sheets, were chromatographed in carbon tetrachloride or hexane. One spot which fluoresced red under ultraviolet was generally larger on chromatograms of Biggs exudates spotted 24 hr after inoculation than with inoculated N10 exudates. Ethyl acetate extracts showed little difference between resistant and susceptible inoculated hypocotyls in the amount of this substance. The solubility of the substance, its apparent polymerization under ultraviolet light, and its ultraviolet and infrared spectra all indicated that it was a polyacetylene, though its exact structure was not determined. Preliminary assays indicated that the substance was inhibitory to zoospore germination and to a lesser extent to mycelial growth of *P. drechsleri*. The physiologic significance of the increase of this substance is uncertain, but the substance appears different from others previously reported to increase in plant tissues with disease development. Phytopathology 60:1000-1004.

The physiological and biochemical mechanisms involved in resistance of safflower (*Carthamus tinctorius* L.) to root rot caused by *Phytophthora drechsleri* Tucker are poorly understood. There appears to be no morphological basis for the difference between the resistant breeding line Biggs and the susceptible variety Nebraska 10 (N10) in disease reactions of hypocotyls to the pathogen (9). Biggs exhibits a rapid hypersensitive response to the pathogen and is killed only by high inoculum concentrations under adverse environmental conditions (6, 7, 12). N10 reacts more slowly, with a water-soaking and browning followed by hypocotyl collapse and plant death (9). Growth of the fungus is sparse and difficult to detect in Biggs hypocotyls, whereas in N10 there is massive intercellular and intracellular spread of the hyphae (9).

In a study of safflower plants grown under different environments, the hypocotyl contents of water-insoluble calcium and pectic substances were related to *Phytophthora* resistance on a fresh-wt but not a dry-wt basis, suggesting a possible relation of cell wall hydration to resistance (12). Other mechanisms of resistance may be present, however. The hypersensitive necrosis itself does not appear to explain resistance (6, 7).

The possible loss of a resistance factor from excised hypocotyls could explain both the loss of resistance in excised immersed hypocotyls (6) and the relationship of prolonged surface sterilization to improved isolation of the fungus from inoculated Biggs hypocotyls (9). The research reported herein was prompted by recent successes of others in demonstrating phytoalexin production, as initially reviewed by Cruickshank (4).

MATERIALS AND METHODS.—Safflower plants were grown in a greenhouse unless otherwise specified, in 0.95-liter crocks, 7-8 plants/crock, as described previously (9).

Zoospores of *P. drechsleri* (isolate 201) were obtained from lima bean agar-glucose plates flooded with soil extract and placed at 15 C (9). Safflower plants, 3-4 weeks old, were usually inoculated by flooding the crocks and adding zoospores ($1.0\text{-}2.0 \times 10^5$/crock, unless otherwise specified). Crocks were drained 22-24 hr after inoculation. Mycelium from lima bean agar-glucose plates was scraped up for hypocotyl stab inoculations as described previously (7).

The wound-leaching experiments with stab-inoculated and healthy plants were done as described by Klarman & Gerdemann (8). Four plants/crock for a given treatment were leached by running a string through the hypocotyl wound in each seedling. One end of the string was placed in a beaker of water and the other end was placed over the edge of the crock, where the water evaporated or dripped from the string end. Wounds were sealed with petroleum jelly.

Various solvents were used in hypocotyl extractions, as footnoted in Table 1. In experiment 2, hypocotyl sections were dropped in boiling 95% ethanol for 5 min. The ethanol extract was then filtered through Whatman No. 2 filter paper before concentration. In experiment 3, hypocotyls were triturated in a Waring Blendor, whereas in experiments 1 and 4, hypocotyls were ground with a mortar and pestle with acid-washed sand. Extracts in experiments 1, 3, and 4 were then centrifuged at 1,000 *g*, and the pellets discarded.

Bioassays were performed on the hypocotyl extracts

TABLE 1. Effect of incorporation of extracts of healthy (H) and inoculated (I) Biggs and N10 safflower hypocotyls into lima bean agar on the growth of *Phytophthora drechsleri* colonies in dam tubes

Experiment[a]	Equivalent tissue concn.	Glucose in agar	Colony age	Biggs H	Biggs I	N10 H	N10 I	Check[b]
	g/ml agar		*days*	*Growth in mm at 24 C*				
1	0.7	+	13	103	81	110	78	99
2	1.0	+	10	74	69	89	43	74
3a	1.6	+	9	46	49	50	45	58
b	1.6	+	9	67	49	63	49	79
4	1.2	—	10	33	34	34	12	76

[a] Hypocotyls in experiments 1 and 4 were extracted 3 days after inoculation. Hypocotyls in experiments 2 and 3 were extracted 2 days after inoculation. Extractions in experiment 1 were in water; solutions were concentrated under air at 37 C, 0.45 μ millipore-filtered, and added to melted agar. In experiment 2, tissues were extracted with boiling ethanol, evaporated to dryness, and resuspended in water before agar was added. In experiment 3, extraction was with 70% ethanol (v/v); samples were then evaporated under vacuum to dryness at 50 C. Then 3a was obtained by washing the residue with absolute ethanol and 3b by following the ethanol wash with a water wash; 3a was added to heated agar, while 3b was sterilized with the agar by autoclaving. Experiment 4 extracts were in ethyl acetate. They were evaporated to dryness under vacuum at 50 C, resuspended in 95% ethanol, and added to sterile agar.

[b] Checks contained an amount of solvent equal to that used to add the tissue extract in 5 ml of agar. Checks 1, 2, and 3b received water, whereas 3a and 4, respectively, received 0.2 ml absolute and 95% ethanol.

by adding 0.2 ml of a concentrated extract fraction to 5.0 ml of lima-bean agar, except in experiments 3 and 3b, where the agar was added to the sample (5.2-ml total), and the sample plus agar were autoclaved together for 15 min at 121 C. The agar-sample mixtures were poured into dam tubes, and after solidification a section of agar containing *P. drechsleri* mycelium was placed at one end of the tube. Linear growth down the tube was measured at intervals for 9-13 days.

Thin-layer chromatography was done with Eastman silica-gel sheets and thin-layer-chromatography developing apparatus. Ascending paper chromatography was done with Whatman 3MM paper. Solvents used included butanol:acetic acid:water (65:10:25, v/v); 2 and 10% acetic acid (v/v); and isopropanol:ammonia:water (200:10:20, v/v).

Ultraviolet spectra were obtained with a Beckman DK-2A ratio recording spectrophotometer, and infrared spectra with a Perkin-Elmer 421-grating infrared spectrophotometer.

Ultraviolet and infrared spectra of the unidentified polyacetylene were obtained on material isolated from 8.0 g Biggs hypocotyls 2 days after inoculation by grinding with a mortar and pestle in 3 volumes of petroleum ether. The pellet was spun down at 1,610 *g* and discarded. The petroleum-ether supernatant was partitioned in a separatory funnel against an equal volume of 5% aqueous sodium bicarbonate (w/v) and then evaporated to 1.5 ml under a stream of N. Then 1.0 ml was streaked onto a Chromar 1000 sheet, and, following chromatography with carbon tetrachloride, a streak with an R_F of 0.63, located by brief long-ultraviolet exposure, was eluted in absolute ethanol. This eluate was concentrated and rechromatographed with hexane, and a streak with R_F 0.21 was eluted with hexane. An ultraviolet spectrum was then obtained. The material was then concentrated under N, rechromatographed with carbon tetrachloride, and eluted with carbon tetrachloride. The eluate was then filtered through sintered glass and evaporated under N, and an infrared spectrum was obtained. All chromatography was done with spectral-grade reagents.

RESULTS.—Phytoalexin production in resistant soybeans in response to *Phytophthora* infection was initially demonstrated by leaching the material from stab-inoculation wounds with wet strings (8). I did comparable experiments using resistant Biggs and susceptible N10 safflower plants approximately 5 weeks old. Hypocotyls were examined 2 weeks after inoculation. Results were as follows in a total of 2 experiments. When strings through the hypocotyls were allowed to flush water through the wounds in the absence of *P. drechsleri*, 0 of 8 of either Biggs or N10 was killed, and necrosis was limited to the area immediately adjoining the wound. If hypocotyls were stab-inoculated with *P. drechsleri* and the strings omitted, 0 of 8 Biggs and 8 of 8 N10 were killed. Biggs hypocotyls showed some necrosis in the central pith, with most of the cortex remaining green. N10 hypocotyls became completely necrotic and collapsed, and the plant tops wilted. If inoculation wounds of Biggs were leached with strings, 7 of 12 Biggs were completely necrotic in cross section, although there was no wilting. The other five showed necrosis at least comparable to that of the inoculated Biggs without wicks. Inoculated N10 (7 of 7) showed complete necrosis of hypocotyl cross sections, and wilting occurred eventually, although the presence of the wet string seemed to delay it. Thus, results were consistent with the hypothesis that an inhibitory material was being drained from the resistant inoculated Biggs.

The string technique has several disadvantages in that Biggs hypocotyls were quite small, a wound effect might be introduced, and the possibility of microbial contamination was great. Hence, initial experiments to locate an inhibitor were done by extraction of hypocotyls by various techniques (Table 1). Extracts of inoculated Biggs showed considerable inhibition over healthy control extracts in experiments 1 and 3b. However, 4 of 5 extracts of inoculated N10 were obviously inhibitory, and inhibition was equal to or greater than that caused by extracts of inoculated Biggs.

Most extracts were also examined, by both thin-layer and paper chromatography, with the solvents that are

listed under MATERIALS AND METHODS. No new spots were detected on chromatograms of inoculated Biggs extracts under long or short ultraviolet, either with or without fuming with ammonia, or after spraying with ferric chloride-potassium ferricyanide reagent (5), when they were compared to chromatograms of inoculated N10 and healthy Biggs and N10 extracts. Hence, the material from the chromatograms was not eluted and bioassayed.

Ethanol extraction, concentration, and successive petroleum ether and ethyl acetate partition allowed spectrophotometric detection in these fractions of two phytoalexins in *Rhizoctonia*-infected bean hypocotyls (10). Comparable procedures with healthy and infected Biggs and N10 hypocotyls showed no evidence of new ultraviolet-absorbing peaks that were unique to inoculated Biggs.

One possible explanation for the failure to demonstrate a phytoalexin by the previous techniques was that a phytoalexin was produced that was unstable and was lost during extraction, concentration, and bioassay. As an exploratory rapid assay technique, healthy and inoculated Biggs and N10 hypocotyls of uniform size were successively sectioned and spotted 6-8 times on thin-layer silica-gel sheets. This resulted in an orange-red spot on the chromatogram. The colored material appeared to exude from canals running longitudinally through the hypocotyl. These canals have been described as primordial laticifers, and are located in the perivascular ring between the cortex and the vascular region (14). The chromatogram sheets were then placed in numerous solvent systems and examined after chromatography for visible and ultraviolet-absorbing spots.

When very nonpolar solvents such as carbon tetrachloride and hexane were used during chromatography on silica-gel sheets, an initially colorless spot, appearing red under long and short ultraviolet, was consistently larger in inoculated Biggs exudates (Fig. 1). With short exposure to ultraviolet or a longer exposure to visible light and/or air, the spot turned visibly tan, whereas under ultraviolet it became orange-brown. R_F values were generally about 0.55-0.68 for carbon tetrachloride and 0.20-0.25 for hexane. The increase in intensity of this spot was demonstrable with exudates from plants 24 hr after inoculation (the earliest time studied) and for 5 days or more afterward. This increase also occurred in sections of the hypocotyl not showing obvious disease symptoms, suggesting that the increase was systemic throughout the hypocotyl.

Small amounts of this substance were present in healthy Biggs and N10 exudates. The amount of this substance in exudates was generally greater from inoculated N10 than from healthy N10. Its amount sometimes visually appeared equal to that in inoculated Biggs during the first 24-48 hr, but was generally less. The amount appeared to diminish after this time.

In two ethyl acetate extractions of healthy and inoculated Biggs and N10 hypocotyls the amount of this substance, as visually estimated from the chromatogram, was close in the two inoculated treatments, and much less in their healthy counterparts.

The chemical nature of this substance was further studied by ultraviolet and infrared spectrophotometry. The ultraviolet absorption spectrum (Fig. 2) showed numerous absorbance peaks, with a series of low-intensity peaks in the region of 290-380 mμ and a series of high-intensity peaks in the lower ultraviolet regions. The ultraviolet chromophore of this substance appears characteristic of an ene-tetrayne structure. The polyacetylenic nature of the substance is further confirmed by the infrared spectrum (Fig. 3), as indicated by the small transmittance peaks between 4.4 and 4.7 μ. The

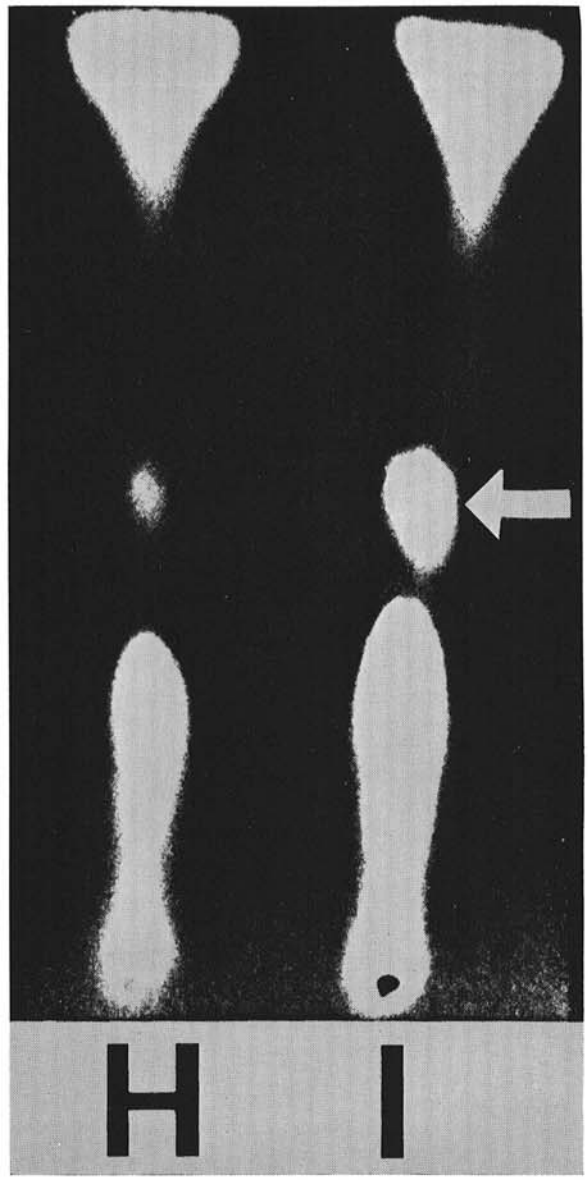

Fig. 1. Hypocotyl exudates of healthy (H) and inoculated (I) Biggs safflower plants 48 hr after inoculation with a zoospore suspension of *Phytophthora drechsleri*. Exudates from paired hypocotyls were spotted on a silica-gel sheet and chromatographed with carbon tetrachloride. Direction of solvent migration was from bottom to top. The initial picture was obtained with a Gevacopy negative, with illumination by a long ultraviolet light. The compound of interest is marked by an arrow.

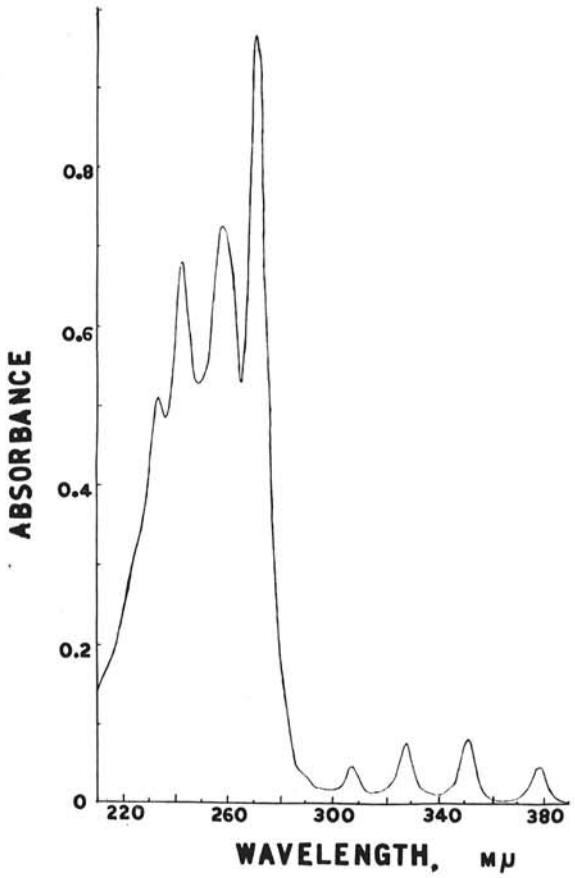

Fig. 2. Ultraviolet absorbance spectrum in hexane of the unidentified polyacetylenic compound found at the arrowed location in the chromatograph shown in Fig. 1. See MATERIALS AND METHODS for description of isolation procedures.

substance may also be an ester (increased transmittance at about 5.7 μ, although the presence of impurities is possible.

A single experiment was run to ascertain the influence of this substance on *P. drechsleri*. A streak obtained by spotting approximately 20 inoculated Biggs hypocotyls on a silica-gel sheet was chromatographed in carbon tetrachloride, and the streak (R_F 0.68) containing this substance was eluted in 95% ethanol and concentrated under N to 0.3 ml. The substance moved as a single spot when rechromatographed in either carbon tetrachloride or hexane, and had an absorbance of 0.230 at 271 mμ, the absorbance maximum. A microsyringe was used to add either 3 or 5 μliters of this substance to 0.1 ml of a zoospore suspension containing approximately 500 zoospores. Comparable controls received 3 or 5 μliters of ethanol. Zoospore germination in the center portion of the drops was counted at 5 hr after addition of the ethanol. The 3- and 5-μliter controls, respectively, showed 38 of 39 and 40 of 42 zoospores germinated, whereas the zoospores receiving 3 and 5 μliters of the polyacetylenic unknown showed 12 of 40 and 0 of 34 zoospores germinated. When 0.1 ml of this substance was added to 4.9 ml of sterile lima bean agar with glucose in a dam tube, fungus growth was reduced 20-30% for the first 2 days but thereafter was near normal as compared with an ethanol control.

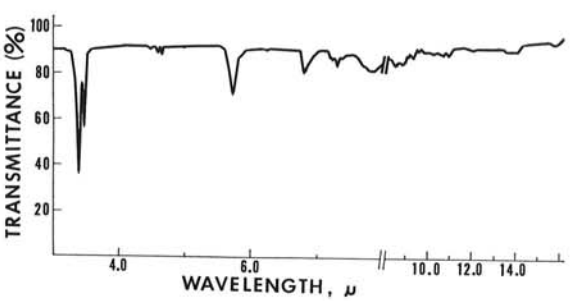

Fig. 3. Infrared spectrum in carbon tetrachloride of the unidentified polyacetylenic compound. The preparative procedures are described in MATERIALS AND METHODS.

DISCUSSION.—The reasons are uncertain why a bona fide phytoalexin was not located by initial extraction experiments after the string technique suggested that one might be present. One may have been present but unstable, or its presence masked by other materials. Alternatively, of course, there may have been no phytoalexin present, although two recent abstracts (1, 13) suggest that there was.

The role of the polyacetylenic substance increasing in response to infection is uncertain in that both susceptible and resistant hypocotyls showed a striking increase. Hypocotyl spotting directly on silica gel usually suggested a greater increase in the resistant Biggs than in the susceptible N10. The difference in results between spotting and extraction may reflect only differences in the hypocotyl anatomy of the two safflower lines, since N10 usually exhibited a much tougher pith tissue at this stage and is more difficult to cut and spot. Also, the susceptible N10 rapidly develops water-soaking symptoms, which may influence the spotting of the relatively water-insoluble polyacetylenes.

Polyacetylenes have been reported in healthy plants of the genus *Carthamus* by Bohlmann, Köhn, and Arndt (2), who described 17 different compounds, including two which were ene-tetraynes. Although no published report was found at the completion of this work suggesting an increase in any polyacetylene as a result of plant disease development, a potential role of these compounds in plant disease resistance was suggested by their occurrence in most tribes of the *Compositae* (11), and by their potential fungistatic or fungitoxic properties as observed here and as reported elsewhere (3). Little had been reported previously on possible functions of these compounds in plants (11). It now appears that one or more polyacetylenic substances in safflower, different from the one described here, may actually be functioning as phytoalexins (1, 13). If, as it appears from this work, this unidentified polyacetylene is confined primarily to the laticifers (as its systemic response throughout the hypocotyl might suggest), there may be a biochemical basis to the possible exclusionary role of the perivascular ring in

safflower resistance to the rust fungus *Puccinia carthami* Cda. This polyacetylene, or other polyacetylenes present here, could conceivably prevent further penetration into the vascular region.

The unidentified polyacetylene described here showed an increase in quantity within 24 hr of infection. In Biggs, the fungus generally fails to reach the perivascular ring, being restricted to the cortex (9). This suggests that the polyacetylene increases in response to materials released from the fungus mycelium, and does not require direct hyphal contact with the laticifers. Whether the increase of this polyacetylene represents an increase in synthesis, its release from a bound form, or its production by modification of a pre-existing compound, was not determined. However, the rapidity of the response (24 hr, or possibly earlier) indicates that this may be one of the early responses to infection. The hypersensitive fleck normally appears at about 12 hr, and ceases enlargement by 16-24 hr (9). Thus, the rapidity of response also suggests that polyacetylenes may have some basic role in disease resistance in safflower. This area needs further study.

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