Phytotoxic Substances Produced by Some Isolates of *Cercospora arachidicola* Are Not Cercosporin

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ABSTRACT

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Cercosporin is a red-colored, light-activated toxin produced by *Cercospora* species. It has been isolated from fungal cultures and from infected leaf tissue of several hosts at levels of 1–10 $\mu g/g$ of lesions or infected leaf tissue. We investigated the production of cercosporin by *C. arachidicola*, causal agent of peanut early leaf spot. No evidence was obtained for cercosporin production in vivo or in vitro by *C. arachidicola*. At an approximate detection limit of 12 pmoles of cercosporin/sample, cercosporin was not found in infected peanut leaves or lesions. Our procedures allowed extraction and detection of cercosporin concentrations below those that cause visible symptoms when purified cercosporin is injected into peanut leaves. Although the four isolates of *C. arachidicola* used for in vitro studies produced a red pigment in culture, this pigment was not cercosporin. Two major compounds with absorption maxima at 465 and 435 nm could be separated by thin-layer chromatography from extracts of cultures of four isolates of *C. arachidicola*. Crude culture extracts and both compounds of *C. arachidicola* were phytotoxic and exhibited antimicrobial activity in both the light and the dark. These compounds could not be detected in infected tissue. The identity and possible role of the phytotoxic substances isolated from *C. arachidicola* are not known.

Additional keyword: *Arachis hypogaea*.

Since Kuyama and Tamura (14) first isolated cercosporin from *Cercospora kikuchii* T. Matsu & Tomoyasu in 1957, production of cercosporin had been found to occur in a large number of *Cercospora* species (1–3,10,11,15–17,24). Cercosporin is a red-colored, nonspecific toxin. Although typical chlorotic and necrotic symptoms associated with disease are produced when purified cercosporin is applied to a wide range of host species (3,11), cercosporin has been isolated from only a few *Cercospora*-infected host plants (10,11,14,24).

Cercosporin is a light-activated toxin. Yamazaki et al (25) first reported the photodynamic action of cercosporin on mice and microbes. Plant cell damage is caused by membrane lipid peroxidation (7) and subsequent changes in membrane structure (9) when cercosporin-treated cells are exposed to light. In ultrastructural studies, membrane damage was found in early stages of infection of sugar beets by *Cercospora beticola* Sacc. (20). Because of its ability to damage membranes, cercosporin may have a role in initiating infection and/or lesion expansion.

Early leaf spot of peanut (*Arachis hypogaea* L.) is caused by *C. arachidicola* Hori. Melouk (16) found no correlation between red pigment production in culture and virulence of several isolates of *C. arachidicola*. However, it has been shown that cells in developing lesions on peanut leaves are killed in advance of mycelium of *C. arachidicola*, allowing inter- and intra-cellular

growth of the pathogen (13). Other toxins isolated from *C. arachidicola*, including dothistromin (21) and averufin (22), have not been investigated for their role in disease.

The possible production of cercosporin in vitro and in vivo by *C. arachidicola* was studied to determine its role in initation of infection and disease development. Phytotoxicity and antimicrobial activity of other partially characterized toxic substances produced by isolates of *C. arachidicola* are reported.

MATERIALS AND METHODS

Isolates of *C. arachidicola*. Isolates of *C. arachidicola* for studies of in vivo cercosporin production were obtained from infected leaf tissue collected from field-grown peanuts (cultivar Florigiant) in three locations in North Carolina in the late summer of 1985. Isolates were maintained on infected Florigiant plants in the greenhouse. Conidia for use as inoculum were collected from infected leaves by suction and then suspended in water with 0.01% Tween 80. The conidial suspensions were atomized onto test leaves, and inoculated plants were placed in the greenhouse in clear plastic bags or in humidity chambers.

Three North Carolina isolates plus an isolate of *C. arachidicola* from Oklahoma provided by H. Melouk were cultured on peanut oatmeal agar (POA) for studies of in vitro cercosporin production. The POA was made according to Smith (19) with the addition of 50 g of polyvinyl polypyrrolidone. Cultures were grown for 4 wk at 20–25 C.

Leaf tissue. Extractions were made from both field-grown Florigiant leaves and from leaves of Florigiant plants grown in the greenhouse. Greenhouse infections were initiated on intact plants or on detached leaves. Detached leaves with intact petioles were supported in 50-ml beakers containing washed quartz sand and deionized water. Both whole leaves with mature lesions and mature lesions cut from leaves were used for extractions. Lesion diameter ranged from 0.3 to 1 cm.

To test for possible interactive effects of isolate and genotype on cercosporin production, the three NC field isolates were inoculated onto peanut cultivars Florigiant, Florunner, and NC 6. All leaves from one plant of each isolate × genotype combination were collected 7 days after inoculation, freeze-dried, and subjected to extraction by the ethyl acetate procedure as described below. At 35 days after inoculation all leaves were collected from a second plant of each cultivar and extracts made as before. Isolate × genotype interactions were also tested by inoculating detached leaves supported in sand and preparing leaf extracts as described.

Leaf extraction. Infected leaf tissue was oven dried at 60 C or freeze-dried. Dried tissue was ground in a blender and extracted (1:4, w/v) in ethyl acetate for three days at -5 C; the solvent was changed daily. Solvent extracts were combined, filtered through Whatman No. 1 filter paper, and evaporated to dryness in a rotary evaporator. The residue was redissolved in a known quantity of ethyl acetate and was chromatographed on preparative silica gel plates, (Silica gel 60, E. Merk, Darmstadt) following the method for thin-layer chomatagraphy (TLC) (see below). After resolution, areas around R_f 0.28 were scraped, and the gel was eluted in ethyl acetate to partially purify cercosporin. Elution solvent was dried in a rotary evaporator, and the sample was redissolved in a small quantity of ethyl acetate and spotted on TLC plates.

Because we isolated no cercosporin from leaf tissue using the ethyl acetate procedure, a modification of the extraction procedure was tried. Dried tissue was extracted with chloroform:methanol (3:1, v/v). The chloroform:methanol extract was partitioned against water, and the organic phase was collected and evaporated to dryness. The residue was dissolved in ethyl acetate, and spotted on TLC plates.

Methods of detection. Cercosporin was separated and identified by TLC. A cercosporin standard was isolated from cultures of *C. beticola* (ATCC 24080) as previously described (6). Silica gel plates were pretreated in 2% H_3PO_4 and dried overnight at 100 C (3). Hexane:isopropanol (8:2) (14) was used as the developing solvent. Cercosporin could be detected as a red spot at R_f 0.28, which fluoresced red under long wave UV light.

Cercosporin was also separated and identified by high-performance liquid chromatography (HPLC) on a 0.8- × 10-cm μBondapak phenyl cartridge (Waters Chromatography Division, Milford, MA) with a mobile phase that consisted of acetonitrile:water (60:40) with 0.1% triflouroacetic acid at a flow rate of 1.5 ml/min. Cercosporin was detected by absorbance at 436 nm. Interference level of leaf components was determined by injecting healthy leaf tissue extracts with known amounts of a cercosporin standard.

Extraction efficiency. Two experiments were performed to determine the effectiveness of the extraction methods. In the first experiment, cercosporin (2 and 10 nmoles/g of leaf) was applied to the surface of peanut leaves. Part of the leaves were oven-dried at 60 C, and the rest were freeze-dried. Dried leaves were extracted by the ethyl acetate procedure. Each extract was spotted on a TLC silica gel plate next to the appropriate cercosporin standard of 2 and 10 nmoles. After the plates were developed, cercosporin-containing bands were scraped from the plate and eluted in ethyl acetate overnight. The absorbance of the solutions at 473 nm was measured to determine the percent loss of cercosporin.

In the second experiment, various concentrations of cercosporin in 20% acetone were injected into detached, fully expanded peanut leaves (cultivar Florigiant) using a syringe with a 26 gauge needle. Twenty percent acetone was injected into leaves as a control. Treated leaves were placed immediately under fluorescent light (39 μE·m^{-2}·sec^{-1}). After necrotic symptoms developed, leaves were dried at 60 C, and cercosporin was extracted by the ethyl acetate

procedure. Extracts were separated by TLC, and the TLC plates checked visually for detectable levels of cercosporin under UV light. Percent cercosporin recovery was not determined.

Soybean inoculations. Isolate PR of *C. kikuchii* (kindly provided by J. B. Sinclair, University of Illinois) was cultured on V-8 juice agar containing 2 g/L of dried, powdered soybean leaves under a 12-hr light/12-hr dark regime for 3 days at 25 C. Detached leaves of soybean (*Glycine max* (L.) Merr.) cultivar Lee were surface sterilized in 10% commercial bleach and placed in a Plexiglas box on water agar with 0.15 g/L of benzimidazole to retard leaf senescence. Leaves were inoculated with 100 μl of a 10^7 conidia/ml suspension of *C. kikuchii*. The Plexiglas box was sealed with Parafilm and incubated under lights (16 hr light/8 hr dark) for 2 wk. Leaves were dried and extracted as described above.

Culture extracts. Crude extracts for the detection of in vitro cercosporin production by cultures of *C. arachidicola* were prepared by methods previously described (6). Air-dried cultures were ground and their components extracted with ether in a Soxhlet apparatus. The ether extract was evaporated to dryness and redissolved in acetone. Extracts were separated by TLC and compared to a purified cercosporin standard. Culture extracts from four isolates of *C. arachidicola*, when resolved by TLC, had two major compounds in common with R_f values of 0.55 and 0.60. Compounds were partially purified by separation on preparative silica gel plates using the leaf extract purification procedure. The two compounds were scraped from the plates and reeluted in acetone. The elution solvent was filtered through Whatman No. 1 filter paper, evaporated in a rotary evaporator, and the sample was redissolved in a small volume of acetone. Compounds were designated Cpd1 and Cpd2 for R_f 0.55 and 0.60, respectively.

Toxicity experiments. Phytotoxicity of the two compounds was determined by injecting a dilution of each extract in 20% acetone into detached peanut leaves. Relative concentrations of the two compounds were determined spectrophotometrically at the absorbance maxima; absorbance of Cpd1 at 465 nm was 0.153 and Cpd2 at 435 nm was 0.115. As a control, 20% acetone alone was injected. Some of the treated leaves were placed in total darkness and others under fluorescent light.

Three microorganisms were tested for sensitivity to the crude culture extracts and to Cpd1 and Cpd2. One-centimeter-diameter plugs of 1-wk-old mycelial cultures of *Rhizoctonia solani* Kühn and *Phytophthora cinnamomi* Rands were transferred to the center of petri plates. Culture media used for the two fungi were water agar and malt agar (6), respectively. *Pseudomonas fluorescens* Migula was tested on nutrient agar (Difco). To form a lawn, 0.2 ml of a dense bacterial suspension was flooded across a petri plate. Five-millimeter disks of Whatman No. 1 filter paper were soaked in acetone, 1 mM cercosporin, crude culture extract of *C. arachidicola*, and solutions of Cpd1 and Cpd2 (absorbance of Cpd1 at 465 nm was 2.7 and Cpd2 at 435 nm was 2.8). Disks were allowed to dry and placed 1 cm from the fungal mycelial plugs and distributed evenly over the surface of the plates of *P. fluorescens*. Cultures were placed either in total darkness or under constant fluorescent light. Fungal inhibition was noted 2 days after treatment as reduced growth around the disks. Bacterial inhibition was noted as clearing in the lawn around disks 2 days after treatment.

RESULTS

Detection and extraction efficiency. In tests with cercosporin-spiked healthy leaf tissue, the best method of extraction was found to be the ethyl acetate procedure. The water wash in the chloroform extraction removed very little of the contaminating leaf components visible by TLC. The use of preparative silica gel plates to partially purify cercosporin from spiked leaf tissue was very effective in separating contaminating leaf components from the extracts.

The detection limit of the TLC method was determined to be 10 pmoles (5.34 ng) of purified cercosporin. Ultraviolet light was required to detect cercosporin concentrations below 500 pmoles.

The detection limit of cercosporin by HPLC was only 30 pmoles (16 ng). This low detection limit was probably due to the detection of cercosporin by HPLC at 432 nm, rather than at the absorption maxima of 205, 223, and 473 nm (25).

Cercosporin, when added to healthy leaves, was partially lost during extraction. Average cercosporin loss was 17% (average of 10 and 24% in two trials) when tissue was oven-dried and 27% (average of 16 and 39%) when tissue was freeze-dried. Thus with a detection limit of 10 pmoles and an approximate percent loss of 17%, the calculated minimum amount of cercosporin recoverable is approximately 12 pmoles/sample.

Necrotic symptoms were induced when 2 μM or greater solutions of cercosporin were injected into detached peanut leaves. Symptoms ranged from necrosis just at the point of injection with a 2 μM solution to confluent necrosis throughout the area injected with a 10 μM solution of cercosporin. If whole leaves were extracted (with one injection point per leaf), cercosporin could only be detected by TLC at levels great enough to cause macroscopic symptoms (2 μM and above). However, if the areas infiltrated with cercosporin were cut from the leaves and extracted, cercosporin could be detected at levels below those that cause symptoms (less than 2 μM).

Infected tissue extraction. Cercosporin was not detected in leaves inoculated with *C. arachidicola* in any of the extraction experiments. Up to 9 g of mature (0.3–1 cm diameter) or young (pinpoint necrotic spot) lesions were cut from field-grown peanuts, greenhouse-grown plants, detached leaves, and leaves in the genotype × isolate inoculations. As much as 56 g of whole leaves from the field or greenhouse, with lesions of variable or known age, were used for extraction. Results of typical extraction experiments following preparative and thin-layer chromatography are shown in Figure 1.

Using values for the cercosporin detection limit, approximate percent loss, and amount of tissue from which extracts were prepared in each experiment, we calculated estimates of the amount of cercosporin which would have to have been present to be detected (Table 1). The resulting calculations indicated that to detect cercosporin in any infected leaf tissue sample, cercosporin would have to have been present in levels ranging from 0.53 to 534

ng (1–100 pmoles). To compare these values to amounts of cercosporin that can be extracted from infected tissue where cercosporin is produced, extractions were made from leaves of soybeans inoculated with *C. kikuchii*. Cercosporin was extracted from the infected soybean leaves at a level of 1.4 μg/g of whole leaves. This value compares to previously reported values of 1–10 μg of cercosporin extracted per gram of lesion or infected leaf tissue (10,11).

Culture extraction. Exhaustive tests with one of the North Carolina isolates of *C. arachidicola* failed to demonstrate cercosporin production on a large variety of media. When grown on POA, this isolate and the other three isolates of *C. arachidicola* produced a red pigment in the medium, but no evidence of cercosporin production was found in extracts from the cultures. Culture extracts were found, however, to have two major compounds in common which were orange (Cpd1) and yellow-orange (Cpd2) in color (Fig. 2). When the silica gel plate was sprayed with 10 N KOH Cpd 1 and Cpd 2 turned purple and pink, respectively, and returned to orange and yellow-orange when sprayed with 10 N HCl. Cercosporin is red under acidic and neutral conditions and turns green in base. Cpd 1 had an absorption maximum in the visible range of 465 nm in acetone and

TABLE 1. Minimum detectable levels of cercosporin for various tissue extraction experiments[a]

Tissue extracted	Cercosporin/g (ng)
Mature field lesion	47.5
Young lesions from greenhouse	534
Entire leaf from field	4.8
Entire leaf from greenhouse	2.7
Isolate × genotype	
Detached leaves 7 and 35 days	8.0
Intact plant 7 days	1.1
35 days	0.53

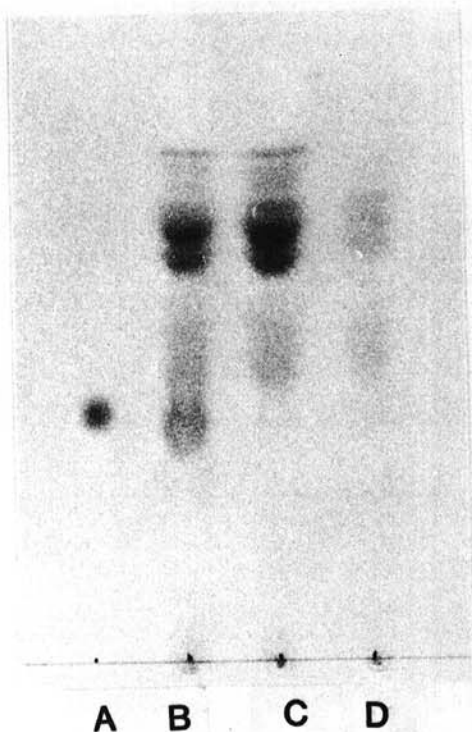[a] Procedures used described in Materials and Methods.

Fig. 1. Thin-layer chromatography of partially purified leaf extracts of infected tissue. A, cercosporin standard; B, infected tissue extract with the addition of cercosporin standard; C and D, infected tissue extracts.
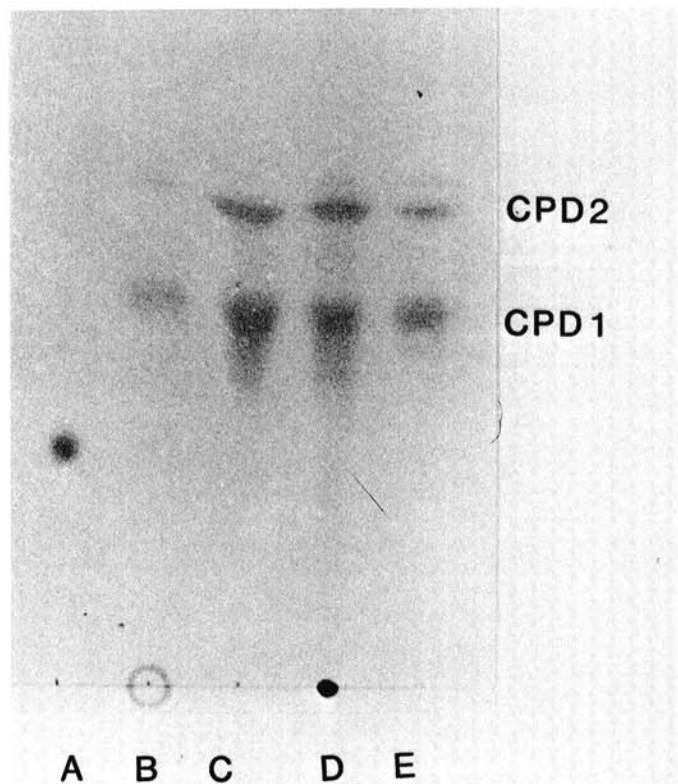

Fig. 2. Thin-layer chromatography of crude culture extracts of four isolates of *Cercospora arachidicola* (B–E). A, cercosporin standard. Cpd1 and Cpd2 have R_f values 0.55 and 0.6, respectively.

550 nm in KOH. The absorption maximum of Cpd 2 in the visible range was 435 nm in acetone and 515 nm in KOH. The two compounds could not be extracted using the ethyl acetate procedure from either whole infected leaves or lesions cut from leaves.

Toxicity experiments. Crude *C. arachidicola* culture extracts and the *C. arachidicola* compounds were both phytotoxic. Injected leaflets, placed either in the light or the dark, developed necrotic symptoms indicating that toxic action was not light dependent. These compounds could not be detected in extracts from necrotic areas.

The two compounds of *C. arachidicola* exhibited toxicity to microorganisms (Table 2). Both *R. solani* and *P. fluorescens* were inhibited by compounds 1 and 2 in both the light and the dark; neither organism was affected by the crude culture extracts. Presumably, the two compounds were not as concentrated in crude culture extracts and therefore did not inhibit growth even though the extract contained the compounds. Growth of *P. cinnamomi* was inhibited by the culture extract in both the light and the dark, but not by either compound 1 or 2. Cercosporin exhibited light-dependent inhibition of *P. cinnamomi* but not of *R. solani*, a result expected from previous studies on fungal sensitivity to cercosporin (8). Cercosporin also did not inhibit growth of *P. fluorescens*.

DISCUSSION

Evidence in this study suggests that cercosporin is not involved in the development of peanut early leaf spot. Cercosporin was not produced by four different isolates of *C. arachidicola* in culture nor could it be extracted from infected leaf tissue during initial infection or later stages of development.

Our methods allowed us to detect approximately 6.4 ng (12 pmoles) of cercosporin, including a loss factor during extraction of 17%. Purified cercosporin injected into leaves could be extracted and detected below concentrations that produce macroscopic symptoms (less than 2 μM). Also, we were able to extract cercosporin at a concentration of 1.4 μg/g of whole soybean leaves inoculated with *C. kikuchii*. This value agrees with those of Duvick (10) and Fajola (11), who reported extracting between 1 and 10 μg of cercosporin from 1 g of lesions or infected leaf tissue from several *Cercospora*-host systems. Our detection limit was at least 300 times more sensitive than these amounts. If cercosporin had been present in peanuts at a level of at least 1.1 ng/g of infected leaves, in vivo production could have been detected in our study.

In addition, no evidence was found for production of cercosporin in vitro. In previous reports, not all *Cercospora* species screened produced cercosporin. Assante et al (1) screened 61 species and only 24 produced cercosporin. Fajola (11) found that 12 of 20 species produced cercosporin. It is interesting that Fajola found in vivo production of cercosporin in those species that produced cercosporin in vitro, but cercosporin could not be extracted from tissue infected with isolates lacking in vitro production. Melouk and Schuh (16) have reported production of cercosporin by two of four isolates of *C. arachidicola*. By contrast, Stoessl (21) did not detect in vitro production of cercosporin in an isolate of *C. arachidicola*, although he did not rule out production in trace amounts. He found that both dothistromin (21) and averufin (22) were produced in large quanitities. Dothistromin inhibits RNA synthesis in *Chlorella pyrenoidosa* and *Bacillus megaterium* (12) and affects plant cell membrane permeability (18). Averufin is a biosynthetic precursor of aflatoxin B$_1$ (5). Unfortunately, little is known about the role of these two toxins in disease development. It appears that some factor other than cercosporin must be responsible for the necrosis and cell damage which has been observed in the advance of *C. arachidicola* mycelium growth in vivo (13). Cercosporin is a light-activated toxin, and shading has been recommended as a control measure for *Cercospora* leaf blight in bananas (23). In our lab, in contrast, infections occur more efficiently under diffuse or low light.

The crude culture extract and the two *C. arachidicola* compounds partially purified in our study were phytotoxic and did not require light for toxic effect. Absorption maxima of the two *C. arachidicola* compounds were different from those of dothistromin or averufin. Because the compounds could not be extracted from infected leaves or lesions, their importance to development of peanut leaf spot disease is unknown. The antimicrobial activity of the compounds suggests a possible involvement in antagonistic interactions or pathogen survival. Cole (4) reported that two unidentified compounds in *C. arachidicola*-infected peanut leaf extracts, which were not found in uninfected tissue, inhibited growth of *Phoma arachidicola*. The compounds were not sufficiently characterized for us to compare the *C. arachidicola* compounds isolated by us to those separated by Cole. Further work must be conducted to identify the toxic compounds produced in vitro by *C. arachidicola* and to determine their role in disease or survival of the pathogen.

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TABLE 2. Inhibition of microbes by crude *Cercospora arachidicola* culture extracts and by two *C. arachidicola* compounds in the light and the dark

Microbe	Crude extract	Cpd 1	Cpd 2	Cercosporin
R. solani	$\cdots$[a]	L/D	L/D	$\cdots$
P. fluorescens	$\cdots$	L/D	L/D	$\cdots$
P. cinnamomi	L/D	$\cdots$	$\cdots$	L

[a] L = inhibition in light, D = inhibition in dark, $\cdots$ = no inhibition.

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