Resistance of Fungi to the Photosensitizing Toxin, Cercosporin

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ABSTRACT

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Isolates of 18 different species of fungi were tested for sensitivity to the toxin, cercosporin, which is toxic to plants, mice, and bacteria, but not to *Cercospora* species which produce it. Oomycetes were very sensitive to cercosporin, whereas yeasts and some of the other mycelial fungi tested were resistant. Analysis of the fatty acid composition of *Cercospora nicotianae* indicated that the predominant fatty acid produced by the fungus was linoleic acid, which is susceptible to lipid peroxidation caused by cercosporin. *C. nicotianae* grown in the presence or absence of cercosporin showed no change in fatty acid composition, indicating that toxin resistance is not due to the production of fatty acids that are resistant to peroxidation. Differences in levels of oxidative enzymes that quench oxygen free radicals also do not appear to be important in fungal resistance. Levels of superoxide dismutase did not differ in extracts from *C. nicotianae* and from the cercosporin-sensitive fungus *Phytophthora cinnamomi*. Catalase activity was considerably higher in *P. cinnamomi*, although no peroxidase activity could be detected in this fungus. Levels of lipid and water-soluble antioxidants differed slightly between *C. nicotianae* and *P. cinnamomi*, with *C. nicotianae* having higher levels of antioxidants. None of these factors appear to be responsible for the major level of cercosporin resistance found in *Cercospora* spp.

Cercosporin is a nonspecific toxin produced by members of the genus *Cercospora*. It was first isolated in 1957 (34) from *Cercospora kikuchii* T. Matsu & Tomoyasu, a soybean pathogen, and has since been isolated from many *Cercospora* spp. (3,4,20, 38,39,42) and from plants infected with *Cercospora* (20,34). Its characterization and structure were reported independently by Lousberg and co-workers (37) and Yamazaki and Ogawa (53). When purified cercosporin is applied to a wide range of host species, it reproduces typical necrotic symptoms of the disease.

Cercosporin appears to be unique among plant pathogen toxins because it is a photosensitizing compound, i.e., a compound that sensitizes cells to visible light (10). Photosensitizers themselves are not toxic to cells, but in the presence of light, they absorb light energy and are converted to an electronically excited triplet state (21,46). The triplet sensitizer may then react in two ways. It may be reduced by a reducing substrate and then, in turn, donate the electron to oxygen to produce superoxide ions. Alternatively, the triplet sensitizer may react directly with oxygen by an energy transfer process yielding the electronically excited singlet state of oxygen. Previous studies demonstrated that cercosporin produces both singlet oxygen and superoxide when irradiated with light (13,17).

Singlet oxygen and superoxide are toxic to living cells, causing oxidation of cellular constituents including lipids, proteins, carbohydrates, and nucleic acids (46). In plant cells, cercosporin causes the peroxidation of membrane fatty acids, leading to major changes in the structure of the membranes and leakage of nutrients out of the cells (11,12). As would be expected, cercosporin shows generalized toxicity to many organisms. All plants that have been tested are sensitive to cercosporin, including those resistant to the disease (4,20). Mice and bacteria are also sensitive to cercosporin (54). Further, it has not been possible to select for cercosporin-resistant tobacco and sugar beet protoplasts and cell cultures by mutagenesis and selection of cells with cercosporin in culture (Daub, *unpublished*). *Cercospora* species, however, produce high concentrations of cercosporin in the light and are apparently unaffected by it. This resistance is surprising considering the generalized toxicity of singlet oxygen and superoxide.

Two lines of evidence suggest that cercosporin plays an important role in diseases caused by *Cercospora* spp. First, cercosporin is light activated, and light has been shown to be important for the development of disease symptoms in several *Cercospora* diseases (7–9). Second, treatment of sugar beet leaf tissue with cercosporin results in ultrastructural changes similar to those seen in plants infected by *Cercospora beticola* Sacc. (47,48), and these changes (particularly membrane damage) are consistent with the known mode of action of cercosporin. Thus an understanding of resistance mechanisms to cercosporin may allow for the future development of novel control measures for these damaging diseases. Because cercosporin resistance has not been found in plants, the purpose of this work was to study the resistance mechanisms of *Cercospora* spp. Specifically, the purpose was to determine if the resistance was specific to *Cercospora* spp. or was expressed by other fungi as well, if the composition of the *Cercospora* cell membranes was the basis of the resistance, and if oxidative enzymes and antioxidants played a role in resistance.

MATERIALS AND METHODS

Isolation of cercosporin. Cercosporin was isolated and purified from cultures of *C. beticola* (ATCC 24080) as previously described (10). Stock solutions were prepared in acetone and stored at −20 C in the dark. Concentration of the acetone stock was adjusted so that final acetone concentrations in all assays did not exceed 0.5% (v/v).

Fungal cultures. Fungal isolates used and their sources are as follows: *Aphanomyces eutiches* Drechsler, *Pythium aphanidermatum* (Edson) Fitzpatrick (A. Filonow, Michigan State University); *Phytophthora parasitica* Dast. var. *nicotianae* (Breda de Haan), *Phytophthora cinnamomi* Rands (H. D. Shew, North Carolina State University); *Cochliobolus heterostrophus* Drechs. HMD-80-2, *Cochliobolus carbonum* Nelson 81-64, *Setosphaeria turcica* (Luttrell) Leonard & Suggs 2207-5r2, *Verticillium* sp. T-16, *Fusarium oxysporum* Schlecht. f.sp. *lycopersici* (Sacc.) Snyd. & Hans. R56 (R. P. Scheffer, Michigan State University); *Colletotrichum lagenarium* (Pass.) Ell. & Halst. r1, *Cladosporium cucumerinum* Ell. & Arth. No. 2, *Cladosporium fulvum* Cke., *Alternaria solani* (Ell. & Martin) Sor. (R. Hammerschmidt, Michigan State University); *Saccharomyces cerevisiae* Meyen DC5 (R. Malmberg, Cold Spring Harbor Laboratory);

Sporobolomyces sp. (K. J. Leonard, North Carolina State University); *Cercospora beticola* (ATCC 24080); *Cercospora nicotianae* Ell. & Ev. (ATCC 18366); and *Cercospora zeae-maydis* Tehon & Daniels Troy (Type A) (F. M. Latterell, USDA).

Fungal sensitivity. Sensitivity of fungal isolates to cercosporin was tested by plating the fungi on potato-dextrose agar (PDA) (Difco) containing 1 and 10 μM cercosporin. Cercosporin as an acetone stock was added to the medium after autoclaving. Mycelial plugs were cut with a cork borer and placed mycelial side down on the agar medium. Cultures were incubated in a lighted 25 C growth chamber (80 $\mu E \cdot m^{-2} \cdot sec^{-1}$). At 24-hr intervals, radial growth was measured. Sensitivity of yeast cultures was tested by colony counts. A colony was dispersed in water, and 10-fold dilutions of that suspension were plated on the cercosporin-containing media. The number of colonies on treated and control plates was determined at 4 days. In all cases, controls were plated on PDA containing 0.5% acetone and incubated in the light. Fungi were also tested for growth on cercosporin-containing media when incubated in the dark to ensure that no nonspecific inhibition was occurring. No differences were seen in the amount of growth in the dark on cercosporin-containing media as compared with controls for all isolates tested. Each experiment consisted of four plates per treatment, and the experiment was performed twice.

Fungal sensitivity was also tested in shake culture. Five plugs of agar cultures of *Cercospora nicotianae* and *Phytophthora cinnamomi* were inoculated into liquid malt medium (34) containing 10 μM cercosporin or 0.5% acetone. Cultures were incubated on a shaker (125 rpm) under lights (150 $\mu E \cdot m^{-2} \cdot sec^{-1}$) for 3 days. Growth was measured by filtering the cultures, drying them for 2 days at 80 C, and weighing them.

Toxicity of hematoporhyrin and methylene blue. *C. nicotianae* and *P. cinnamoni* were tested for inhibition by the singlet oxygen-generating sensitizers methylene blue and hematoporphyrin. Hematoporhyrin and methylene blue (Sigma Chemical Co., St. Louis, MO) were added to PDA after autoclaving as acetone and water stocks, respectively. Fungal cultures were plated on the media and tested as described above. Hematoporphyrin was tested at a concentration of 10 μM and methylene blue at concentrations of 10, 50, and 100 μM.

Fatty acid analysis. *C. nicotianae* was grown in liquid stationary culture on V-8 juice broth (V8B) or potato-dextrose broth (PDB) for 2 wk either under fluorescent lights or in the dark. The mycelial mats were harvested, washed, ground in 2-propanol in a Sorvall omnimixer, and extracted twice with boiling 2-propanol as described by Kates (33). The residue was extracted twice with chloroform: 2-propanol (1:1, v/v) followed by extraction with chloroform. The chloroform-propanol extracts were combined, evaporated to dryness under N_2, redissolved in chloroform, washed with 1% NaCl, and again evaporated to dryness under N_2. The residue was dissolved in methanolic NaOH (4 mg/ml) and hydrolyzed at 95 C for 2 hr under N_2. Water was added to the extracts, and they were washed with hexane, acidified by the addition of HCl, and extracted with hexane to remove the fatty acids.

Cercosporin produced by cultures is extracted along with the fatty acids. To minimize differences that might be caused by oxidation of the fatty acids by cercosporin during extraction, all cultures were protected from light throughout the procedure. Also, the approximate amount of cercosporin present in extracts from toxin-producing cultures was determined spectrophotometrically at 565 nm (a wavelength where interference from other fungal pigments was minimal), and an equivalent amount of purified cercosporin was added to extracts of nonproducing cultures. A comparison of extracts of non-cercosporin-producing cultures with and without added cercosporin showed no significant differences in the composition of fatty acids.

Fatty acid methyl esters were prepared by the boron trifluoride method described by McKersie and Thompson (41) and were separated and identified on a Varian 3700 gas chromatograph equipped with a 3.66-m × 3.3-cm glass column packed with 10% DEGS-PS on 80/100 Supelcoport (Supelco, Inc., Bellefonte, PA) maintained at 200 C. The methyl esters were identified by reference to standards, and peak areas were computed from the peak height × width at half height. Data presented are the result of four separate experiments.

Superoxide dismutase assay. *C. nicotianae* and *P. cinnamomi* were grown in liquid shake culture in malt medium for 3 days. The mycelial mats were harvested, washed with water, and ground (2 ml/g fresh weight) in 0.1 M potassium phosphate buffer (pH 7.8) in a Sorvall omnimixer. The suspension was centrifuged and the supernatant dialyzed overnight in the cold before being tested for superoxide dismutase activity.

Superoxide dismutase activity was measured using a modification of the photochemical assay described by Giannopolitis and Ries (24) and Beauchamp and Fridovich (5). The reaction mixture consisted of 1 ml of fungal extract, 13 mM methionine, 1.3 μM riboflavin, 63 μM *p*-nitroblue tetrazolium (NBT), and 0.1 M potassium phosphate buffer (pH 7.8) in a final volume of 3 ml. Fungal extracts boiled for 10 min were used as controls. The reaction mixtures were irradiated from above with a high intensity photo flood lamp. Light intensity at the top of the reaction tubes was approximately 1,200 $\mu E \cdot m^{-2} \cdot sec^{-1}$. Superoxide production (reduction of NBT) was monitored by following the increase in absorbance at 560 nm against a nonirradiated blank. Each experiment contained two replications and the experiment was performed three times.

Catalase assay. Cultures of *C. nicotianae* and *P. cinnamomi* were grown and harvested as described for the superoxide dismutase assays. The mycelium was ground in 50 mM potassium phosphate buffer (pH 7.0), and the mycelial extract frozen and thawed three times before being centrifuged and the supernatant used for the catalase assay.

Catalase activity was determined by measuring the decomposition of H_2O_2 in a spectrophotometric assay described by Aebi (1). The reaction mixture contained 10 mM H_2O_2, 500 μl of fungal extract supernatant, and potassium phosphate buffer (50 mM, pH 7.0), in a final reaction volume of 3 ml. Decomposition of H_2O_2 (decrease in absorbance at 240 nm) was monitored for 30 sec against a blank lacking H_2O_2. Purified catalase (Sigma Chemical Company, St. Louis, MO) and boiled fungal extracts were also tested. Each experiment contained four replications, and the experiment was performed twice.

Peroxidase activity. Acetone powders were prepared by the methods of Hammerschmidt et al (29) from cultures of *C. nicotianae* and *P. cinnamomi* grown and harvested as described for the superoxide dismutase assay. Acetone powders were extracted overnight (50 mg of powder per milliliter) in 0.01 M potassium phosphate buffer (pH 6.0). The suspensions were centrifuged, and the supernatants were used immediately for peroxidase assays or stored frozen. Extracts were also dialyzed against 0.01 M potassium phosphate buffer (pH 6.0) overnight at 4 C.

Peroxidase activity was assayed by a spectrophotometric assay described by Putter and Becker (44). The reaction mixture contained fungal extract, H_2O_2, and 2,2′-azino-di-(3-ethyl-benzothiazoline-[6]-sulphonic acid) diammonium salt (ABTS), all in 0.05 M potassium phosphate buffer (pH 6.0). Absorbance was measured at 405 nm at 1 and 6 min after the addition of the fungal extract. The change in absorbance over the 5-min period (ΔA_5) was used as a measure of peroxidase activity. Peroxidase activity was also assayed by the *O*-dianisidine method (52).

Discontinuous gel electrophoresis was carried out in 7.5% polyacrylamide slab gels at pH 9.5 (27). Extracts (0.3 mg of protein per sample) were loaded in 0.5 M sucrose with bromphenol blue as a tracking dye. Purified horseradish peroxidase (Sigma Type VI, 300 purpurogallin units per milligram solid) was run as a comparison. The gels were run at constant voltage (200 V) until the tracking dye reached 1 cm from the end of the gel. Peroxidase bands were visualized by the methods of Hammerschmidt et al (29).

Antioxidants. *C. nicotianae* and *P. cinnamomi* were grown and harvested as described for the superoxide dismutase assays. The mycelium was frozen, lyophilized, ground to a powder, and extracted with chloroform:methanol:H_2O (5:10:4, v/v/v) as described by McKersie et al (40). The extract was filtered and

mixed with equal volumes of chloroform and water, and aqueous and organic phases were separated. Aqueous and organic fractions were washed, evaporated to dryness, and redissolved in, respectively, methanol and ethyl acetate.

Antioxidant activity was measured using the coupled oxidation of β-carotene and linoleic acid in two assays as described by Hammerschmidt and Pratt (28). Initially, extracts were spotted on silica gel thin-layer chromatography (TLC) plates, and the plates were sprayed with a mixture of 6 mg of β-carotene and two drops of linoleic acid in 90 ml of chloroform:ethanol (1:2). The chromatograms were exposed to daylight until the background color was bleached (1–2 hr). Extracts showing antioxidant activity were then tested in a spectrophotometric assay. The assay mixture was prepared by mixing 0.1 mg of β-carotene, 1 ml of chloroform, 20 mg of linoleic acid, and 200 mg of Tween 40. The chloroform was removed in a rotary evaporator, and 50 ml of oxygenated distilled water was added. Five milliliters of this mixture was mixed with 0.2 ml of the fungal extracts. The tubes were incubated in a water bath at 50 C, and the amount of antioxidant activity was determined by measuring the change in absorbance at 470 nm at 15-min intervals. Controls contained 0.2 ml of methanol, and readings were taken against a blank lacking β-carotene.

Protein. Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

RESULTS

Fungal sensitivity. Several fungi other than *Cercospora* species were highly resistant to cercosporin (Table 1). In general, Oomycetes were sensitive to cercosporin, whereas other mycelial fungi, with some exceptions, were resistant. Yeasts, both *Saccharomyces cerevisiae* and three different isolates of the Basidiomycete yeast *Sporobolomyces*, were also resistant to cercosporin. This differential sensitivity was also expressed when fungi were grown in liquid shake culture in malt medium. Dry weights of 3-day-old *C. nicotianae* cultures grown in the presence and absence of 10 μM cercosporin were 100 ± 6 and 110 ± 6 g, respectively, whereas for *P. cinnamomi* they were 81 ± 3 g without cercosporin and 16 ± 8 g with cercosporin.

TABLE 1. Percent growth inhibition of fungal species grown on cercosporin-containing medium[a]

Fungus	Cercosporin concentration	
	1 μM	10 μM
Aphanomyces euteiches	62	97
Pythium aphanidermatum	64	94
Phytophthora cinnamomi	46	100
Phytophthora parasitica	59	100
Alternaria solani	0	0
Cercospora beticola	0	0
Cercospora nicotianae	0	0
Cercospora zeae-maydis	0	0
Cladosporium cucumerinum	0	0
Cladosporium fulvum	0	0
Cochliobolus carbonum	0	35
Cochliobolus heterostrophus	48	77
Colletotrichum lagenarium	0	0
Fusarium oxysporum	24	52
Setosphaeria turcica	21	50
Verticillium sp.	0	0
Saccharomyces cerevisiae[b]	0	0
Sporobolomyces sp.[b]	0	0

[a] Fungal cultures plated on potato-dextrose agar containing cercosporin. Growth measured as the radius of colony at 2 or 3 days.
[b] Sensitivity assayed by colony counts.

Resistance to hematoporphyrin and methylene blue. Hematoporhyrin and methylene blue, two singlet oxygen-generating photosensitizers (32), were tested for toxicity to *C. nicotianae* and *P. cinnamomi*. Hematoporphyrin (10 μM) completely inhibited growth of *P. cinnamomi* but had no effect on *C. nicotianae* when these cultures were incubated in the light, a result identical to that found for cercosporin. By contrast, methylene blue (10, 50, and 100 μM) had no inhibitory effect on either fungus.

Fatty acid analysis. Cercosporin kills cells by peroxidation of polyunsaturated fatty acids in membrane lipids (11,12), thus organisms with highly saturated membranes would be expected to have resistance to cercosporin. Most fungi (including the Deuteromycetes) have fairly saturated membranes (49,50). The fatty acid composition of *C. nicotianae* was investigated to see if it was composed primarily of saturated and monoenoic fatty acids (which are resistant to peroxidation) and if the composition differed depending on whether or not the fungus was actively producing the toxin.

Fatty acids extracted from *C. nicotianae* were not highly unsaturated, containing predominantly palmitic (16:0), stearic (18:0), oleic (18:1), and linoleic (18:2) acids (Table 2). However, linoleic acid, which is susceptible to peroxidation, composed over 50% of the fatty acids. Further, fatty acid composition did not differ significantly when the fungus was grown under conditions either favoring or suppressing cercosporin production. *C. nicotianae* produced a higher percentage of polyunsaturated fatty acids when grown on V8B (a medium on which this isolate will not produce cercosporin) as compared with PDB. However, no differences were seen in fatty acid composition when the fungus was grown on PDB in the light and in the dark, suggesting that differences in the composition of fatty acids were due to different nutrient conditions, and that the fungus does not shift its fatty acid composition to protect itself when actively producing cercosporin.

Superoxide dismutase assay. Extracts from *C. nicotianae* and *P. cinnamomi* had comparable superoxide dismutase activity, with the *C. nicotianae* and *P. cinnamomi* extracts causing, respectively, a 43 and 46% reduction overall in the levels of superoxide present (Fig. 1). By comparison, the addition of 0.03 mg/ml of purified superoxide dismutase (Sigma Chemical Co., St. Louis, MO) to boiled extracts of *C. nicotianae* and *P. cinnamomi* caused a 74 and 68% reduction, respectively, in the amount of superoxide produced.

Catalase activity. Extracts of *P. cinnamomi* showed high catalase activity, comparable to levels seen with 15 μg of purified catalase (Table 3). By contrast, very low levels of activity were found in extracts from *C. nicotianae*. Extracting the fungal cultures in the presence of 0.25% sodium cholate, dialyzing the

TABLE 2. Fatty acid composition[a] of *Cercospora nicotianae* grown under cercosporin-producing and nonproducing conditions

Growth conditions[b]	Production of cercosporin	Fatty acids (% of total)					
		Palmitic (16:0)	Palmitoleic (16:1)	Stearic (18:0)	Oleic (18:1)	Linoleic (18:2)	Linolenic (18:3)
PDB Light	+	22 ± 2[c]	trace[d]	5 ± 1	21 ± 4	52 ± 5	trace
PDB Dark	−	22 ± 0	trace	5 ± 1	24 ± 4	49 ± 5	trace
V8B Light	−	16 ± 1	trace	5 ± 1	19 ± 3	58 ± 5	2 ± 0
V8B Dark	−	17 ± 1	trace	5 ± 1	23 ± 3	53 ± 6	2 ± 1

[a] Determined by gas chromatography of the fatty acid methyl esters as compared with standards.
[b] Cultures grown in the light and in the dark on potato-dextrose broth (PDB) and V-8 juice broth (V8B).
[c] ± Standard error.
[d] Less than 1%.

extracts, or using fresh extracts reduced the level of catalase activity in *P. cinnamomi* extracts as compared with that obtained when the ground fungus was frozen and thawed, and these treatments did not increase activity from *C. nicotianae*. No activity was found when extracts were boiled for 10 min.

Peroxidase activity. Acetone powder extracts of *C. nicotianae* and *P. cinnamomi* were assayed for peroxidase activity by both the ABTS and *O*-dianisidine assays. Extracts from *P. cinnamomi* had no activity in concentrations in the range of 8 mg of protein down to 0.1 mg of protein per 5-ml assay volume. Extracts from *C. nicotianae* had very low activity when added to the assay mixture at 0.1 mg of protein ($\Delta A = 0.02$) but had no activity with greater amounts of extract. This lack of activity was apparently due to the presence of inhibitors in the crude extracts (Table 4). When increasing amounts of these extracts were added to purified horseradish peroxidase, the activity of this enzyme was inhibited. Dialyzing the extracts removed the inhibitory compound(s) from *P. cinnamomi* extracts (Table 4), but peroxidase activity was still not detectable when these dialyzed extracts were subsequently assayed (data not shown). Dialysis had no effect on the inhibitory activity of *C. nicotianae* extracts.

To purify the peroxidase enzymes from inhibitors in the crude extracts, samples were run on polyacrylamide gels. No peroxidase bands were detected in *P. cinnamomi* extracts with gels loaded with up to 300 mg of protein. All peroxidase activity in *C. nicotianae* extracts was localized in a single band, which had the same mobility as purified horseradish peroxidase (data not shown). The intensity of staining obtained from loading 300 μg of protein of the crude extract appeared visually to be equivalent to approximately 0.05 μg of purified peroxidase.

Antioxidant activity. Aqueous and organic fractions extracted from lyophilized cultures of both *C. nicotianae* and *P. cinnamomi* had antioxidant activity when spotted onto TLC plates and sprayed with β-carotene and linoleic acid. Visually, the organic fractions appeared to have greater activity than aqueous fractions. No differences were noted between aqueous and organic fractions from the two species.

Aqueous and organic fractions of extracts from both *C. nicotianae* and *P. cinnamomi* also showed substantial antioxidant activity in the spectrophotometric assay (Fig. 2). There was little difference in antioxidant activity of aqueous and organic fractions from the same fungus, but activity of fractions differed between the two fungi, with *C. nicotianae* extracts showing higher activity than those from *P. cinnamomi*.

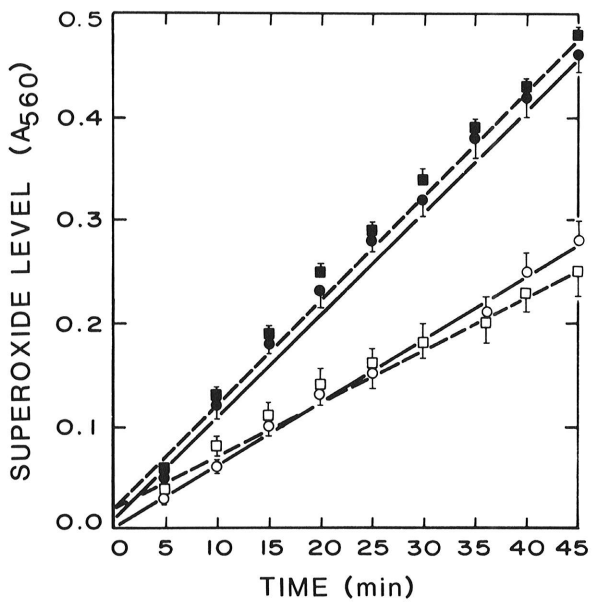

Fig. 1. Superoxide dismutase activity of extracts of *Cercospora nicotianae* (—o-●—) and *Phytophthora cinnamomi* (–□-■–) as measured by the quenching of superoxide produced in a photochemical reaction between riboflavin and methionine. The amount of superoxide present was determined by measuring the increase in absorbance at 560 nm due to the reduction of *p*-nitroblue tetrazolium to blue formazan by superoxide. Open symbols: active extracts; closed symbols: boiled extracts (control).

DISCUSSION

Differences were found in the sensitivity of fungal species to cercosporin. In general, Oomycetes were very sensitive, whereas yeasts and some of the other mycelial fungi were resistant. Similar results were obtained by diCosmo et al (15), who studied the photoinduced toxicity of α-terthienyl and several thiophene derivatives on certain fungi. *Pythium* and *Saprolegnia* species were very sensitive, whereas species of *Alternaria*, *Aspergillus*, *Cladosporium*, *Colletotrichum*, and *Rhizopus* were more resistant, particularly *Alternaria* and *Cladosporium*. α-Terthienyl, like cercosporin, is an oxygen-requiring photosensitizer that appears to generate both singlet oxygen and superoxide (2). Resistance of several fungal spp. to these compounds is interesting considering the generalized toxicity of photosensitizers. It suggests that resistance is caused by natural components of the fungal cell rather than specialized adaptations, because only a few of these species are known to produce similar toxins (43,45,55).

Hematoporphyrin showed the same spectrum of toxicity as cercosporin to fungal isolates. By contrast, methylene blue, which is also a potent singlet oxygen producer, was not toxic to either *C. nicotianae* or *P. cinnamomi*. This difference may be due to differences in localization of these compounds in the cell. Although virtually all photosensitizers act by generating active oxygen species, their effects on cells differ because of differential localization in cells. In general, the site where the sensitizer is located determines the primary site of damage (32). Hematoporphyrin is a membrane sensitizer (32). Cercosporin presumably also localizes in membranes, because it is lipid-soluble and has the

TABLE 3. Catalase activity[a] in extracts of *Cercospora nicotianae* and *Phytophthora cinnamomi*

Extracts	Catalase activity (ΔA_{30})	Protein concentration (mg)[b]
Purified catalase[c]	0.15 ± 0.005[d]	0.015
C. nicotianae		
Experiment 1	0.02 ± 0.004	3.2
Experiment 2	0.02 ± 0.000	2.7
P. cinnamomi		
Experiment 1	0.14 ± 0.003	2.8
Experiment 2	0.15 ± 0.01	3.0

[a]Catalase activity measured as the decomposition of H_2O_2 (decrease in absorbance at 240 nm) over 30 sec (ΔA_{30}).
[b]In the 3-ml reaction mixture.
[c]Sigma, 24,000 units/mg of protein.
[d]± Standard error.

TABLE 4. Inhibition of purified horseradish peroxidase activity[a] by acetone powder extracts of *Cercospora nicotianae* and *Phytophthora cinnamomi*

Extract concentrations (mg protein/ assay volume)	Percent inhibition[b]			
	P. cinnamomi		*C. nicotianae*	
	Undialyzed	Dialyzed	Undialyzed	Dialyzed
0.1	21	4	28	4
1.0	87	31	100	96
2.0	100	23	100	100

[a](Sigma, type VI) assayed by the ABTS assay as described in the Materials and Methods. Peroxidase concentration = 0.025 μg per 5-ml assay volume.
[b]Peroxidase activity was measured as the change in absorbance at 405 nm over 5 min. (ΔA_5). ΔA_5 values of controls (no extracts added) were 0.58 ± 0.05 and 0.48 ± 0.02 for experiments with the undialyzed and dialyzed extracts, respectively.

primary effect of causing lipid peroxidation. Methylene blue, by contrast, is highly water soluble and does not appear to penetrate cells very well (32). Thus, it is possible that methylene blue is not toxic because it does not penetrate, or, alternatively, it may penetrate but localizes in a region where different defense mechanisms are able to control the toxicity.

The difference seen in the sensitivity of Oomycetes and other fungi to cercosporin and other photosensitizers allows a study of factors that may be involved in resistance by looking at differences between these groups of fungi. One difference between Oomycetes and many other fungi is the composition of their membranes. Membranes of Oomycetes contain long-chain, highly unsaturated fatty acids (49,50). By contrast, most yeasts do not have polyunsaturated fatty acids, and mycelial Ascomycetes and Deuteromycetes have predominantly short-chain fatty acids which are not highly unsaturated (49,50). Because cercosporin damages cells by causing peroxidation of polyunsaturated fatty acids, it was possible that the fatty acid composition of the membrane was the basis of resistance. An analysis of fatty acids in *C. nicotianae* demonstrated, as expected, that the predominant fatty acids were short chain and not highly unsaturated. Fatty acid composition is unlikely to be the sole basis of resistance, for over 50% of the fatty acids present are susceptible to peroxidation, and *C. nicotianae* does not shift its fatty acid composition in response to toxin production. However, the degree of multiplication of free radicals in a system increases with an increase in the number of conjugated double bonds (51). Thus, in the presence of other defense mechanisms, the more saturated membranes of *C. nicotianae* may contribute significantly to resistance.

Superoxide dismutase, catalase, and peroxidases are the major enzymes that protect cells from damage caused by activated oxygen (19,22). Superoxide dismutase scavenges superoxide, whereas catalase and peroxidases scavenge hydrogen peroxide, which is formed through the dismutation of superoxide radicals and can further react with superoxide radicals to form the toxic hydroxyl radical (22). Increased levels of these enzymes have been implicated in the resistance of plants to free-radical generating agents such as ozone, SO_2, and paraquat (19,30,31,35). In the case of cercosporin toxicity, studies with paraquat-resistant plants suggest that superoxide dismutase may play a protective role in plants. Leaves of paraquat-resistant tobacco plants having elevated superoxide dismutase levels (23) showed no symptoms when sprayed with a cerosporin solution and showed less electrolyte leakage following cercosporin treatment than did normal plants (K. Tanaka, *personal communication*). In fungi, however, superoxide dismutase does not appear to be important in cercosporin resistance, because there was no difference in activity of this enzyme in extracts of *C. nicotianae* and *P. cinnamomi*. The role of catalase and of peroxidase is not clear. No peroxidase activity was detected in extracts of the cercosporin-sensitive fungus *P. cinnamomi*, but catalase activity was significantly greater in *P. cinnamomi* than in *C. nicotianae*. Because these enzymes perform the same function, either one should be able to exert a protective effect. In plants, catalase and peroxidases do not appear to be important in protection against cercosporin. A paraquat-resistant tobacco mutant having elevated levels of catalase and proxidase (31) was just as sensitive to cercosporin as the paraquat-sensitive control (Daub, *unpublished*).

Enzymatic defenses provide the first line of defense against oxygen radicals, but they are only effective against nonlipid free radicals (51). Lipid free radical reactions, such as those that occur in membranes damaged by photosensitizers, are terminated by the action of antioxidants. α-Tocopherol (vitamin E), probably the most important antioxidant in plant and animal systems, is found in fungi (16,36) but in extremely low concentrations as compared with plants and algae (18,36). Polyphenols are also potent antioxidants and, unlike tocopherols, are common constituents of fungi (6) and thus may be important in protection against cercosporin. Extracts from both *P. cinnamomi* and *C. nicotianae* had significant antioxidant activity, but activity in extracts from *C. nicotianae* was significantly greater than that from *P. cinnamomi*. Whether the increased activity is sufficient to provide cercosporin protection in vivo is not known.

It is probable that fungal defense against cercosporin is caused by a combination of factors. None of the possible defenses investigated here appear sufficient to provide protection on their own. Enzymatic defenses do not appear to be important, and differences in fatty acid composition and in levels of antioxidants would only provide partial protection. We presently have data supporting a role of carotenoids (which are potent singlet oxygen quenchers) and of the fungal cell wall in resistance (14,25,26). Further studies are needed to elucidate the contribution of all of these factors.

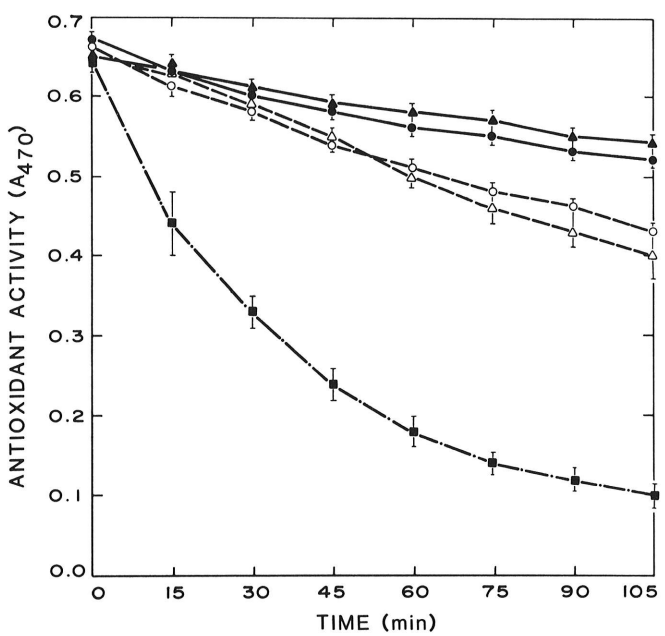

Fig. 2. Antioxidant activity of aqueous (Δ, ▲) and organic (o, •) extracts from *Cercospora nicotianae* (—) and *Phytophthora cinnamomi* (----) as compared with controls without extracts (–·–·–). Antioxidant activity was determined by measuring the inhibition of β-carotene destruction resulting from the coupled oxidation of β-carotene and linoleic acid.

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