Cercosporin, a Photosensitizing Toxin from *Cercospora* species

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ABSTRACT

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Cercosporin, a nonspecific toxin produced by members of the genus *Cercospora*, is structurally related to the photosensitizing compound hypericin. Cercosporin was isolated from cultures of *Cercospora beticola* and *Cercospora nicotianae* and tested for toxic effects on suspension-cultured cells of tobacco. Cercosporin was toxic to tobacco cells only when they were incubated in the light. The action spectrum of the killing response agreed with the absorption spectrum of cercosporin. Dabco and bixin, two compounds that quench singlet oxygen, delayed killing of cells by cercosporin. Cercosporin is thus acting as a photosensitizing agent in host plants. The possible role of photosensitizing compounds in plant diseases is discussed.

Additional key words: plant cell culture.

Cercosporin is a nonspecific toxin produced by members of the fungal genus *Cercospora*. It was first isolated in 1957 (12) from dried mycelia of *Cercospora kikuchii*, a soybean pathogen. Its characterization and structure (Fig. 1) were reported independently by Lousberg and co-workers (13) and Yamazaki and Ogawa (25). Cercosporin has since been isolated from a large number of *Cercospora* species (1,2,6,14,16,23) and from cercospora-infected plants (6,12,23). When the isolated toxin is purified and applied to a wide range of host species, it reproduces many symptoms of the disease.

Cercosporin is structurally related to several photosensitizing compounds, ie, compounds that sensitize cells to visible light. The best known of these is hypericin, which is produced by members of the genus *Hypericum* (11). Photosensitizing compounds such as hypericin absorb light to form electronically excited states and then transfer this energy to oxygen, producing either singlet oxygen ($^1O_2$) or superoxide ions ($O_2\cdot^-$) (7,20). Both $^1O_2$ and $O_2\cdot^-$ are extremely toxic to cells, causing the oxidation of fatty acids, sugars, cellulosic materials, guanine, and several amino acids (cysteine, histidine, methionine, tryptophan, and tyrosine), which in turn results in damage to DNA, inactivation of enzymes, and destruction of cell membranes (7,20). Yamazaki and co-workers (26) noted the structural similarities between the compounds and tested the photodynamic action of cercosporin. Mice and bacteria were killed by cercosporin only when they were exposed to light, and oxygen was involved in the reaction. Cercosporin induces ion leakage from potato, carrot, beet, and corn tissue when these tissues are irradiated with an incandescent bulb (15).

The purpose of this work was to provide definitive evidence that cercosporin is acting as a photosensitizing agent in host plants. This paper reports the kinetics of the killing of plant cells by cercosporin, an action spectrum for the killing response, and the inhibition of the killing response by singlet oxygen quenchers.

MATERIALS AND METHODS

Isolation of cercosporin. Cercosporin was isolated by a modification of the methods of Kuyama and Tamura (12). Cultures of *Cercospora beticola* (ATCC #24080) and *C. nicotianae* (ATCC #18366) were grown on malt agar (15 g Difco malt extract, 3 g peptone, 30 g glucose, and 9 g agar per liter) under a daily regime of 16 hr light and 8 hr dark for 4 wk. The mycelium was harvested, air-dried, crushed, and extracted with ether in a Soxhlet apparatus. The ether extract was evaporated to dryness, dissolved in chloroform, purified on a column of dry calcium phosphate, and eluted fractionally with 1.5% methanol in chloroform. Cercosporin was crystallized from the red fractions by adding pentane and placing the extract in the cold (−20 C). The resulting cercosporin preparations were tested for purity by thin-layer chromatography and by absorption- and mass spectroscopy. The absorption- and mass spectra of the toxin preparations were in complete agreement with those presented by Yamazaki and Ogawa (25) for purified cercosporin. No differences were found between the toxin preparations from *C. beticola* and *C. nicotianae*.

Cell cultures. The *Nicotiana tabacum* 'Wisconsin 38' cell line (NT575) used was kindly provided by R. Malmberg. Liquid suspension stock cultures were maintained in the dark in Murashige and Skoog's medium (17) with 3 mg indoleacetic acid and 0.3 mg kinetin per liter by diluting fourfold into fresh medium twice per week. The cell density immediately following subculture was approximately 5×10^6 cells per 50 ml of suspension culture.

Toxin assays. All light intensity measurements were made with an LDC Kettering Radiant Power Meter. Experimental cultures were incubated on a rotary shaker (125 rpm) at 25 C and illuminated

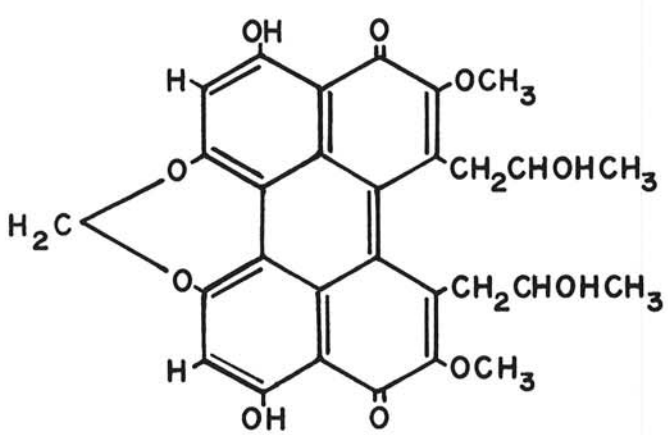

Fig. 1. Structure of cercosporin.

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with fluorescent lights at an intensity of 2.0×10^4 ergs·cm^{-2}·sec^{-1}. Flasks of dark-grown cultures were wrapped in black electrical tape and incubated on the same shaker.

Cercosporin was dissolved in 0.01N KOH, adjusted to the desired concentration spectrophotometrically, filter sterilized, and added (1-ml volume) to suspension cultures immediately after subculture. Control cultures received the same amount of KOH.

The toxicity of cercosporin was assayed two ways: in the first of these, the percentage of dead cells in a culture was determined by counting a sample of cells after staining them with bromphenol blue, which stains dead cells but not living cells. Aliquots of cell suspensions were removed from test flasks at 4-hr intervals and mixed with an equal volume of a 0.1% aqueous solution of bromphenol blue. Stained and unstained cells were counted under a microscope at ×160. Over 2,000 cells, from four separate aliquots, were counted for each treatment. The second method was to monitor the growth of the suspension cultures daily by measuring the volume of cells settled after 20 min in a graduated centrifuge tube.

Action spectrum. Suspension cultures were mixed with 5 μM cercosporin and incubated in flat-sided disposable tissue culture flasks (Corning Glass Works) on a reciprocal shaker at 25 C. Cultures were exposed for exactly 1 hr to different wavelengths and intensities of light obtained by using Baird Atomic Interference filters (9–12 nm half-band pass) in combination with a General Electric incandescent projector lamp. Light intensity was controlled by varying the voltage on the lamp. After the 1-hr light exposure, the cultures were shaken in the dark for 23 hr before the proportion of dead cells in each sample was determined.

Inhibitors. The $^1O_2$ quenchers used for inhibitor studies were dabco (1,4-diazabicyclo octane; Aldrich Chemical Co., Milwaukee, WI 53233) and bixin (a carotenoid carboxylic acid; Chemicals Procurement Laboratories, Inc., College Pt., NY 11356). Dabco is soluble in water. Bixin was dissolved in ethanol and dilute KOH; the ethanol was removed prior to use. The compounds by themselves had no measurable effect on suspension-cultured cells at the concentrations used. They were added to cultures immediately before the addition of cercosporin. Cultures were incubated as previously described (toxin assays). Aliquots were removed at 4-hr intervals, and the percentage of dead cells was determined.

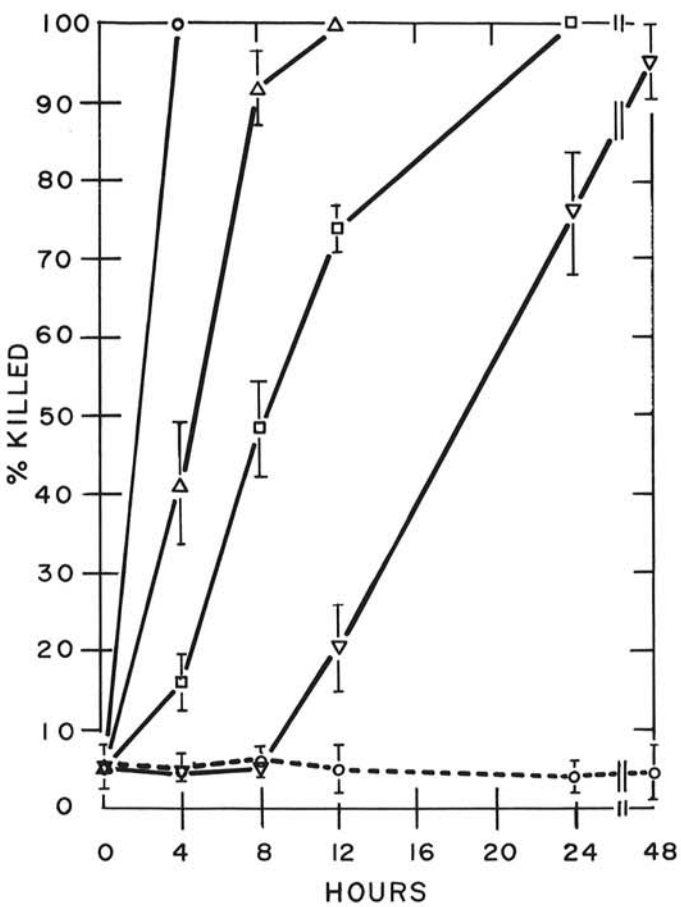

Fig. 2. Killing of suspension-cultured tobacco cells by cercosporin as a function of time in cultures incubated at a concentration of 40 μM in the dark (--o--) and at 5μM (o), 1μM (Δ), 0.5 μM (□), and 0.2 μM (▽) in the light (——). Control cultures of NT575 have approximately 5% dead cells. Light intensity at the base of the culture flasks was approximately 2.0×10^4 ergs·cm^{-2}·sec^{-1}.

RESULTS

Cercosporin rapidly killed suspension-cultured tobacco cells when cultures were incubated in the light (Fig. 2). At a cercosporin concentration of 5 μM, every cell in the culture (approximately 5×10^6 cells) was killed within 4 hr. With decreasing concentrations of cercosporin, there was an increase in the killing lag time and a decrease in the rate of killing. But even at 0.2 μM, all cells were killed within 48 hr. By contrast, in dark-incubated cultures, there was no significant increase in the percent of dead cells as compared to control cultures. This was true for cercosporin concentrations as high as 40 μM for as long as 7 days.

High concentrations of cercosporin did affect the growth rate of dark-incubated cultures. In the dark, control cultures and cultures with cercosporin concentrations up to 1 μM multiplied at a rate of approximately 0.59 doublings per day (dd). The growth rate dropped to 0.40 dd with 5 μM cercosporin, to 0.025 dd at 20 μM, and to zero at 40 μM. The effect of cercosporin was also tested on a suspension culture of sugar beets (*Beta vulgaris*). The killing response was the same as with the tobacco cells. Furthermore, detached leaves of beet and tobacco injected with solutions of cercosporin (5–100 μM) developed symptoms only when incubated in the light.

The action spectrum was derived from dosage-response curves constructed at each wavelength. There was a linear relationship between light intensity and killing response at each wavelength (Fig. 3). The light intensity required to give a 50% killing response

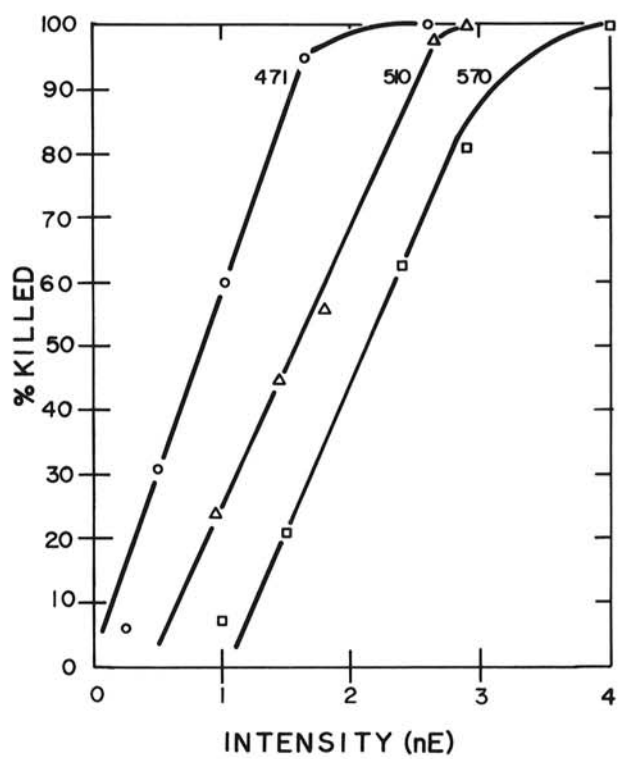

Fig. 3. Dosage response curves for the killing of suspension-cultured tobacco cells by cercosporin as a function of light intensity (nanoEinsteins) at different wavelengths. Cells were incubated with 5 μM cercosporin and exposed to light for exactly 1 hr. Shown are three representative curves, for wavelengths 471, 510, and 570 nm.

was used in constructing the action spectrum. Cultures without cercosporin were not affected by the light treatment.

The absorption spectrum and action spectrum of cercosporin are in close agreement (Fig. 4). The peaks correspond, and the ratios of peak heights are the same. Results of the reciprocity experiment, where the exposure times were varied along with the light intensity to give a constant light dose (intensity × time), are shown in Table 1. Reciprocity held over the exposure times and intensities used in the experiment.

To determine if cercosporin results in the production of $^1O_2$ or $O_2^{\cdot -}$, two compounds were tested for their inhibitory effect on the cercosporin killing response. Dabco, which is known to quench $^1O_2$ (18), and bixin, which should also quench $^1O_2$, both delayed the killing of cells by cerosporin (Figs. 5 and 6). Bixin inhibited the killing response more effectively than dabco. Bixin at 100 μM prevented killing by 0.5 μM cercosporin for the first 8–12 hr. In contrast, 1 mM dabco provided equivalent protection for only 4–8 hr. Both compounds acted by causing an increase in the length of the lag period before killing commenced. Killing proceeded at similar rates in the dabco-treated, the bixin-treated, and the untreated cultures after the lag period.

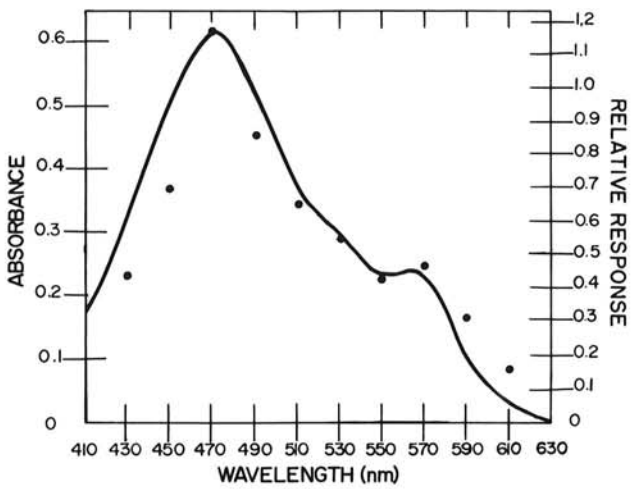

Fig. 4. Absorption spectrum (——) and action spectrum (●) of cercosporin. "Relative response" is the inverse of the light intensity required to give a 50% killing response at a particular wavelength as determined from the dosage-response curves.

TABLE 1. The killing of suspension-cultured tobacco cells by cercosporin as a function of light intensity, wavelength, and exposure time

Light intensity[a] (nEin)	Exposure time (min)	Intensity × time	Cells killed (%)
3.90	15	58.5	67.8 ± 4.3[b]
1.95	30	58.5	65.3 ± 3.9
0.98	60	58.8	68.3 ± 7.1
0.49	120	58.8	64.4 ± 2.0

[a] Wavelength, 471 nm.
[b] Standard error of mean.

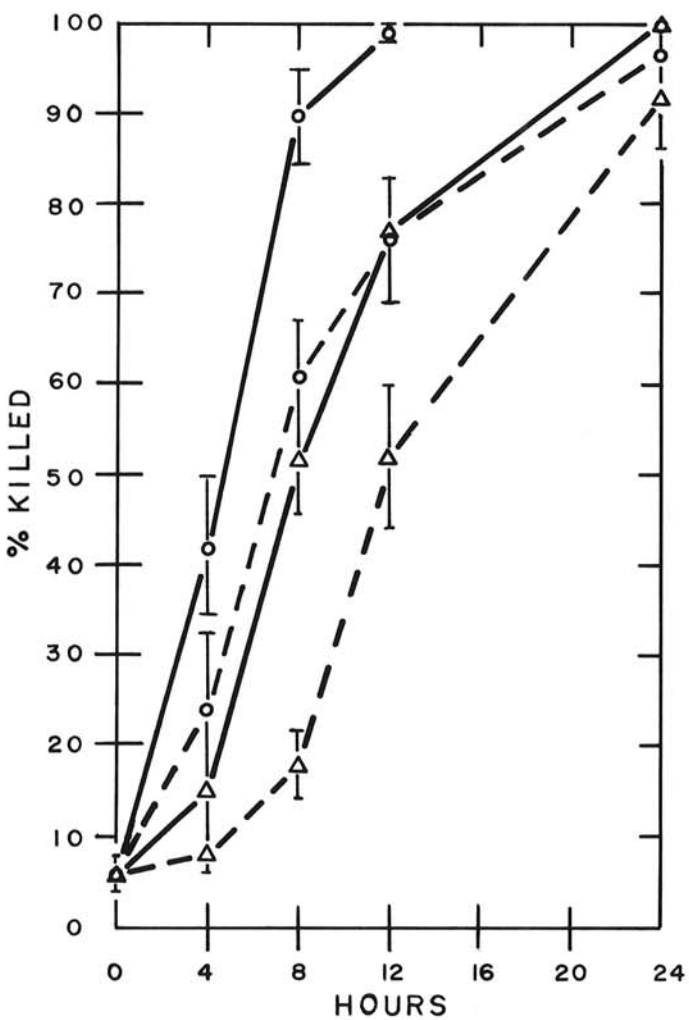

Fig. 5. Delay of killing of suspension-cultured tobacco cells by cercosporin in the presence of 1 mM dabco. Cercosporin concentrations are 1 μM (o) and 0.5 μM (Δ); with dabco added (----), and without dabco (——).

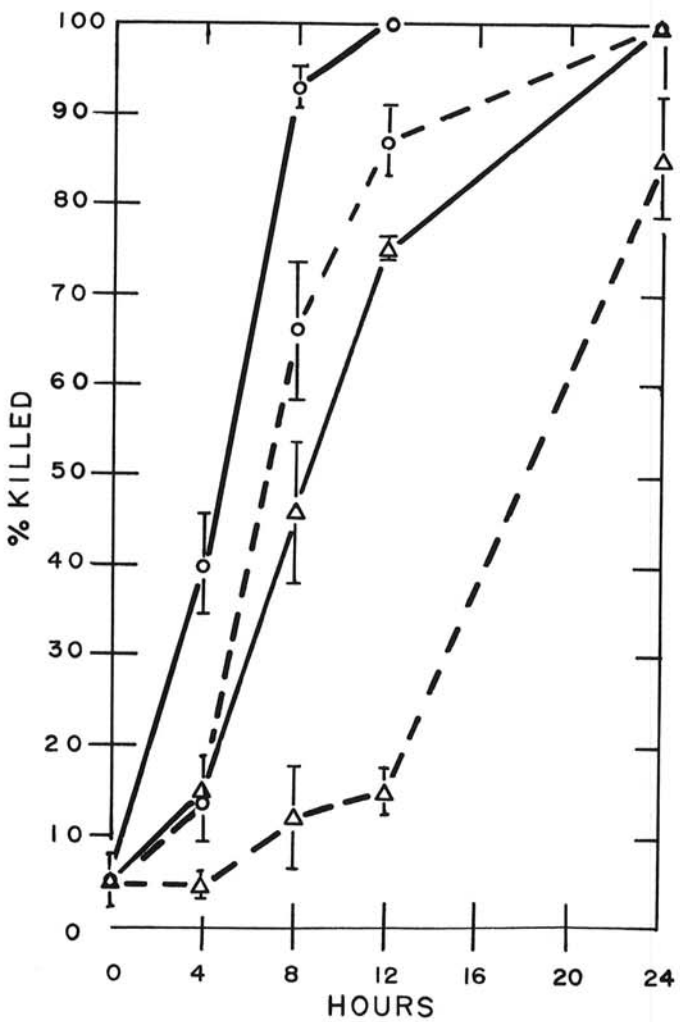

Fig. 6. Delay of killing of suspension-cultured tobacco cells by cercosporin in the presence of 100 μM bixin. Cercosporin concentrations are 1 μM (o) and 0.5 μM (Δ); with bixin added (----) and without bixin (——).

DISCUSSION

This paper presents evidence that cercosporin is acting as a photosensitizing agent in plants: it is able to kill plant cells only in the light; the action spectrum of the killing response agrees with the absorption spectrum of cercosporin; and compounds that quench $^1O_2$, one of the products of the photosensitizing reaction, delay the killing of cells by cercosporin.

The close agreement between the action spectrum and the absorption spectrum of cercosporin is probably due to the use of suspension-cultured cells as the host material. These cells had no chlorophyll and only minute amounts of carotenoids; therefore, very few pigments were present that could interfere with the light absorption by cercosporin.

Photosensitizers produce $^1O_2$ by an energy transfer reaction; the ground-state sensitizer is regenerated in the process (7,20). Dabco is a $^1O_2$ quencher (18) which is believed to quench by consuming $^1O_2$ (10). This can explain both the large amount of dabco required to inhibit the effects of cercosporin and the pattern of that inhibition. It may be that $^1O_2$ is quenched until the dabco is consumed (thus accounting for the extended lag period) and then killing proceeds at the same rate as in the control cultures.

Bixin has not been tested as a $^1O_2$ quencher. Studies with the related β-carotene, a very efficient $^1O_2$ quencher, have shown that the isoprenoid chain length is the critical factor in determining quenching ability (8). Bixin has the same chain length as β-carotene and is somewhat soluble in aqueous solutions. β-Carotene quenches $^1O_2$ by an energy transfer process and is therefore only slowly consumed in the reaction (9). Presumably, the same is true of bixin. It is not surprising, therefore, that bixin provided a more persistent protection of cells than did dabco. The absorption spectrum of bixin overlaps slightly with that of cercosporin, but the protective effect of bixin does not appear to be due to screening out the actinic light.

Although bixin decreased the toxicity of cercosporin in culture, plant cells apparently are not protected by their own carotenoids. Presumably, this is true because plant cell carotenoids are located in the chloroplasts and the chloroplasts, if they are a target of response cercosporin, are not of primary importance in the of the cells to cercosporin. First, the suspension cultures used had no chloroplasts, and they were killed very rapidly. Second, tobacco leaf disks exposed to cercosporin in the light leaked high concentrations of electrolytes within 1–2 min, suggesting that cercosporin is destroying the plasma membrane very rapidly (*unpublished*). If the plasma membrane is destroyed, the cells would not survive even if the chloroplasts were somewhat resistant. In this respect, cercosporin clearly differs from other light-requiring toxins such as tentoxin (21) and tabtoxin (4).

Cercosporin did not kill suspension-cultured cells in the dark, even at ×200, the lethal dose in the light. At such high concentrations, however, cercosporin did inhibit cell growth. The reasons for this are unknown. However, suspension cultures are extremely sensitive to environmental changes and are inhibited by high concentrations of many compounds. The inhibitory effect of cercosporin in the dark may be a secondary effect due to excessive concentration. This is supported by the observation that, in the dark, cercosporin at concentrations as high as 100 μM has no effect on leaves.

The effects of $^1O_2$ on cells are well known (20). Among the most significant of these effects, from the point of view of plant pathology, is the disruption of cell membranes. Changes in membrane permeability are often among the first detectable events in the onset of diseases caused by a variety of pathogens (24). These changes can be correlated with the susceptibility and resistance of the host to the disease and are believed to play a role in the availability of nutrients for the invading pathogen. Damage to the plasmalemma and to organelle membranes also appears to be a primary effect of some of the host-specific toxins that are known, by genetic and biochemical analysis, to play a role in disease (27).

Proof that cercosporin is acting as a photosensitizing agent centainly does not prove that it plays a causal role in diseases caused by *Cercospora* species. However, observations on disease progress in several hosts are consistent with the hypothesis that cercosporin does play such a role. For example, Cercospora blight of sugar beets is more severe under high light intensities than under low light intensities (3). Disruption of cellular membranes is one of the earliest ultrastructural changes observable in sugar beets infected by *C. beticola* (22). Furthermore, members of the genus *Cercospora* have an extremely broad host range, and cercosporin is produced by virtually all of them (1,5,14). The production of such a generalized and toxic compound as cercosporin may help to explain the almost universal pathogenicity found in the genus.

This is the first report describing the possible involvement of photosensitizing compounds in plant diseases. Similar compounds have been isolated from *Cladosporium phlei*, a pathogen of timothy (28), and from cucumbers infected with *Cladosporium cucumerinum* (19). Although the photosensitizing properties of the compounds produced by *Cladosporium* have not yet been tested, their production suggests that compounds such as these may be produced quite commonly among some groups of plant pathogens. Photosensitization may be an important mechanism of virulence or pathogenicity for these and other pathogens of higher plants.

LITERATURE CITED

1. Assante, G., Locci, L., Camarda, L., Merlini, L., and Nasini, G. 1977. Screening of the genus *Cercospora* for secondary metabolites. Phytochemistry 16:243-247.
2. Balis, C., and Payne, M. G. 1971. Triglycerides and cercosporin from *Cercospora beticola*: fungal growth and cercosporin production. Phytopathology 61:1477-1484.
3. Calpouzos, L., and Stalknecht, G. F. 1967. Symptoms of Cercospora leaf spot of sugar beets influenced by light intensity. Phytopathology 57:799-800.
4. Durbin, R. D. 1971. Chlorosis-inducing pseudomonad toxins: their mechanism of action and structure. Pages 369–385 in: S. Akai and S. Ouchi, eds. Morphological and Biochemical Events in Plant-Parasite Interaction. The Phytopathological Society of Japan, Tokyo.
5. Fajola, A. O. 1978. Cultural Studies in *Cercospora* Taxonomy: I. Interrelationships Between Some Species from Nigeria. Nova Hedwigia 29:912-921.
6. Fajola, A. O. 1978. Cercospsorin, a phytotoxin from *Cercospora* species. Physiol. Plant Pathol. 13:157-164.
7. Foote, C. S. 1976. Photosensitized oxidation and singlet oxygen: consequences in biological systems. Pages 85–133 in: W. A. Pryor, ed. Free Radicals in Biology. Vol. II. Academic Press, New York.
8. Foote, C. S., Chang, Y. C., and Denny, R. W. 1970. Chemistry of singlet oxygen. X. Carotenoid quenching parallels biological protection. J. Am. Chem. Soc. 92:5216-5218.
9. Foote, C. S., and Denny, R. W. 1968. Chemistry of singlet oxygen. VII. Qenching by β-carotene. J. Am. Chem. Soc. 90:6233-6235.
10. Foote, C. S., Denny, R. W., Weaver, L., Chang, Y., and Peters, J. 1970. Quenching of singlet oxygen. Ann. N.Y. Acad. Sci. 171:139-145.
11. Giese, A. C. 1980. Hypericism. Photochem. Photobiol. Rev. 5:229-255.
12. Kuyama, S., and Tamura, T. 1957. Cercosporin. A pigment of *Cercosporina kikuchii* Matsumoto et Tomoyasu. I. Cultivation of fungus, isolation and purification of pigment. J. Am. Chem. Soc. 79:5725-5726.
13. Lousberg, R. J. J. Ch., Weiss, U., Salemink, C. A., Arnone, A., Merlini, L., and Nasini, G. 1971. The structure of cercosporin, a naturally occurring quinone. Chem. Commun. 1463-1464.
14. Lynch, F. J., and Geoghegan, M. J. 1977. Production of cercosporin by *Cercospora* species. Trans. Br. Mycol. Soc. 69:496-498.
15. Macri, F., and Vianello, A. 1979. Photodynamic activity of cercosporin on plant tissues. Plant Cell Environ. 2:267-271.
16. Mumma, R. O., Lukezic, F. L., and Kelly, M. G. 1973. Cercosporin from *Cercospora hayii*. Phytochemistry 12:917-922.
17. Murashige, T., and Skoog, F. 1962. A revised medium for rapid growth and bioassay with tobacco tissue cultures. Physiol. Plant. 15:473-497.
18. Oannes, C., and Wilson, T. 1968. Quenching of singlet oxygen by tertiary aliphatic amines. Effect of Dabco. J. Am. Chem. Soc. 90:6527-6528.
19. Overeem, J. C., and Sijpesteijn, A. K. 1967. The formation of perylenequinones in etiolated cucumber seedlings infected with *Cladosporium cucumerinum*. Phytochemistry 6:99-105.
20. Spikes, J. D. 1977. Photosensitization. Pages 87–112 in: K. C. Smith, ed. The Science of Photobiology. Plenum Press, New York.
21. Steele, J. A., Uchytil, T. F., Durbin, R. D., Bhatnagar, P., and Rich, D. H. 1976. Chloroplast coupling factor 1: a species-specific receptor for

tentoxin. Proc. Nat. Acad. Sci. USA 73:2245-2248.
22. Steinkamp, M. P., Martin, S. S., Hoefert, L. L., and Ruppel, E. G. 1979. Ultrastructure of lesions produced by *Cercospora beticola* in leaves of *Beta vulgaris*. Physiol. Plant Pathol. 15:13-26.
23. Venkataramani, K. 1967. Isolation of cercosporin from *Cercospora personata*. Phytopathol. Z. 58:379-382.
24. Wheeler, H., and Hanchey, P. 1968. Permeability phenomena in plant disease. Annu. Rev. Phytopathol. 16:331-350.
25. Yamazaki, S., and Ogawa, T. 1972. The chemistry and stereochemistry of cercosporin. Agric. Biol. Chem. 36:1707-1718.
26. Yamazaki, S., Okube, A., Akiyama, Y., and Fuwa, K. 1975. Cercosporin, a novel photodynamic pigment isolated from *Cercospora kikuchii*. Agric. Biol. Chem. 39:287-288.
27. Yoder, O. C. 1980. Toxins in pathogenesis. Annu. Rev. Phytopathol. 18:103-129.
28. Yoshihara, T., Shimanuki, T., Araki, T., and Sakamura, S. 1975. Phleichrome: a new phytotoxic compound produced by *Cladosporium phlei*. Agric. Biol. Chem. 39:1683-1684.