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Peroxidation of Tobacco Membrane Lipids by the Photosensitizing Toxin, Cercosporin[1]

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ABSTRACT

Cercosporin, a nonspecific toxin from *Cercospora* species, is a photosensitizing compound which rapidly kills plant cells in the light. Cell death appears to be due to a cercosporin-mediated peroxidation of membrane lipids. Tobacco leaf discs treated with cercosporin showed a large increase in electrolyte leakage 1 to 2 minutes after irradiation with light. All tobacco protoplasts exposed to cercosporin in the light were damaged within 45 minutes. Chloroform:methanol extracts of toxin-treated suspension cultures gave positive reactions for lipid hydroperoxides in the thiobarbituric acid test. Cercosporin-treated leaf discs emitted high concentrations of ethane 12 to 24 hours after incubation in the light. Cercosporin also oxidized solutions of methyl linolenate as determined by the thiobarbituric acid assay and the emission of ethane. α-Tocopherol had an inhibitory effect on the cercosporin-mediated lipid peroxidation.

Cercosporin [1,12-bis(2-hydroxypropyl)-2,11-dimethoxy-6,7-methylenedioxy-4,9-dihydroxyperylene-3,10-quinone] is a nonspecific phytotoxin produced by members of the genus *Cercospora*. It was first isolated in 1957 (9) from *Cercospora kikuchii*, a soybean pathogen, and has since been isolated from a large number of *Cercospora* species (1, 2, 7, 11, 14, 23) and from *Cercospora*-infected plants (7, 9, 23). Its structure was determined independently by Lousberg *et al.* (10) and Yamazaki and Ogawa (25).

Cercosporin is a photosensitizing compound (5, 26), thus, it is not toxic to cells in the dark. In the presence of light, however, photosensitizers absorb the light energy to form electronically excited states and then transfer this energy to oxygen (8, 18). This results in the production of toxic oxygen species such as singlet oxygen or superoxide ions, which can damage living cells. Yamazaki *et al.* (26) showed that mice and bacteria were killed by cercosporin only when they were exposed to light, and that oxygen was involved in the process. Previous work in this laboratory (5) has determined that the action spectrum for the killing of tobacco cells by cercosporin is in close agreement with the absorption spectrum of cercosporin, and that the toxic effect of cercosporin can be inhibited by several quenchers of singlet oxygen.

The toxic effects of photosensitizing compounds are well known (8, 18). One of the most common of these effects is damage to cellular membranes. Macri and Vianello (12) reported that cercosporin induced ion leakage from several plant tissues, although a 30-min light exposure was required for any significant change to occur. Later, Cavallini *et al.* (4) showed that cercosporin could induce lipid peroxidation in several *in vitro* membrane systems, and that this lipid peroxidation could be inhibited by several single oxygen quenchers.

The purpose of this study was to investigate the effects of cercosporin on plant cells *in vivo* in order to test the hypothesis that cercosporin is able to kill plant cells by mediating peroxidation of membrane lipids. This paper reports results on the toxic activity of cercosporin on tobacco leaf tissue and suspension cultured cells by means of four separate assays: electrolyte leakage, damage to protoplasts, ethane emission, and the production of malondialdehyde (thiobarbituric acid assay).

MATERIALS AND METHODS

Cercosporin. Cercosporin was isolated and purified from cultures of *Cercospora nicotianae* as previously described (5). Stock solutions were prepared in either acetone (for assays that involved cell cultures) or methanol (for assays that utilized leaf tissues). Stock solutions were stored at −20°C in the dark. The final concentration of acetone or methanol in control and cercosporin-treated cultures did not exceed 1%.

Host Material. Tissue discs for electrolyte leakage and ethane studies were cut from fully expanded leaves of *Nicotiana tabacum* cv 'Wisconsin 38' grown in the greenhouse. The *N. tabacum* cv 'Wisconsin 38' cell culture (NT575) used for the protoplast and thiobarbituric acid assays was kindly provided by R. Malmberg. Liquid suspension cultures were maintained in the dark in Murashige and Skoog (15) medium with 3 mg IAA/L and 0.3 mg kinetin/L as previously described (5).

Protoplast Isolation. Protoplasts were isolated from NT575 suspension cultures 1 d after subculture. Five ml packed cells were mixed with 100 ml enzyme solution (3% Cellulysin, 1% Macerase, 500 mM mannitol [pH 5.7]) and incubated overnight in a rotary shaker at 20 rpm. The protoplasts were filtered through Miracloth, centrifuged at 150*g* for 15 min, and resuspended in protoplast buffer (5 mM Mes, 5 mM $CaCl_2 \cdot 2H_2O$, 450 mM mannitol [pH 6.0]). The protoplasts were washed twice and then purified by layering them onto 10% Ficoll (w/v in protoplast buffer) and centrifuging at 150*g*. Healthy protoplasts formed a band at the surface of the Ficoll solution. These protoplasts were harvested, washed, and suspended in a small volume of buffer.

Electrolyte Leakage. Electrolyte leakage was assayed by measuring the change in conductivity of a leaf disc bathing solution with a Markson Electromark Conductivity meter using a pipette type electrode. Conductivity is given as μmhos. Five 1-cm-diameter discs cut from tobacco leaves were washed in water, vacuum infiltrated with a cercosporin solution (5 μM in water with 1% methanol), and incubated with stirring in 5 ml of the same solution. Control discs were treated with water containing 1% methanol. The tissue discs were irradiated with light from a 750 w tungsten projector lamp filtered through a $CuSO_4$ solution, and the change in conductivity was monitored. Unless otherwise noted,

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the light intensity was approximately 800 J m^{-2} s^{-1}.

Protoplast Assay. Protoplasts suspended in buffer were mixed 1:1 with a 10 μM aqueous solution of cercosporin in 2% acetone or with 2% acetone, and placed in small wells in a disposable tissue culture plate. Protoplasts were irradiated with a Volpi Intralux Fiber Optics lamp at a light intensity of approximately 800 J m^{-2} s^{-1}. At 15-min intervals, samples of the cultures were observed under a microscope, and the number of healthy and damaged protoplasts was counted. Spherical protoplasts were scored as healthy; protoplasts which had visibly burst or were irregularly shaped with condensed cytoplasm were scored as damaged. This distinction had been previously confirmed by staining with bromphenol blue and fluorescein diacetate. Control protoplasts remained healthy for the duration of the experiment.

Ethane Assay. Five 1-cm-diameter leaf discs were vacuum infiltrated with a 10 μM aqueous solution of cercosporin in 1% methanol or with 1% methanol, and were placed in 25 ml flasks containing 5 ml of the infiltration solution. The flasks were sealed with serum stoppers and incubated under an incandescent lamp at a light intensity of approximately 100 J m^{-2} s^{-1}. At various intervals, 1-ml gas samples were removed from the flasks and analyzed for the presence of ethane with a Varian gas chromatograph as previously described (3). *In vitro* assays contained 100 μM methyl linolenate in water with 1% methanol. α-Tocopherol was also added as a solution in methanol.

Thiobarbituric Acid Assay. Ten ml NT575 cells in a 50-ml culture were treated with 10 μM cercosporin. Cultures were incubated on a rotary shaker at 125 rpm and irradiated with fluorescent lights at an intensity of approximately 20 J m^{-2} s^{-1}. Flasks of dark-incubated cultures were wrapped in aluminum foil and incubated on the same shaker. After incubation, cells were centrifuged at 500*g* for 5 min, and homogenized and extracted with 2 volumes of chloroform:methanol (2:1). The chloroform:methanol extract was evaporated to dryness under N_2, dissolved in 0.2 ml ethanol, and mixed with 2 ml of 0.2 M Tris-0.16 M KCl buffer (pH 7.4). The extract was analyzed for the presence of MDA[2] with the thiobarbituric acid assay as described by Placer *et al.* (17). Oxidation of methyl linolenate was assayed with 0.4 mg methyl linolenate in 2 ml Tris-KCl buffer and 10 μM cercosporin. These solutions were incubated and analyzed as above.

Chemicals. Cellulysin and Macerase were obtained from Calbiochem. Dialyzed and lyophilyzed Ficoll (type 400 DL), methyl linolenate (99% pure), and D,L-α-tocopherol were obtained from Sigma.

RESULTS

Electrolyte Leakage. Tobacco leaf discs treated with cercosporin showed a rapid increase in leakage of electrolytes after irradiation with light (Fig. 1). Leaf discs treated with cercosporin but not exposed to light, and untreated, light-exposed discs showed no increase in electrolyte leakage. The change in the rate of leakage after irradiation occurred very rapidly. Under the experimental conditions used (5 μM cercosporin, 800 J m^{-2} s^{-1}), there was a statistically significant increase in the rate of electrolyte leakage 1 to 2 min after irradiation as compared to the rate of leakage in the dark (Table I). A consistent increase in the rate was seen within the 1st min of light exposure, but the difference was not statistically significant.

Both light intensity and toxin concentration affected the rate of electrolyte leakage from the leaf discs. At 5 μM cercosporin and 800 J m^{-2} s^{-1}, the intial increase in the rate of leakage occurred 1 to 2 min after irradiation, and leakage occurred at a mean rate of 0.84 μmhos/min (average of the first 10 min after the initial increase). If the light intensity was reduced to 400 and 200 J m^{-2} s^{-1}, the initial rate increase occurred at 3 to 4 min and 6 to 7 min, respectively, and the mean rate of leakage decreased to 0.69 and 0.49 μmhos/min, respectively. Conversely, if the toxin concentration was reduced from 5 to 1 μM, 4 to 5 min were required for the rate of leakage to increase, and the mean rate was 0.41 μmhos/min.

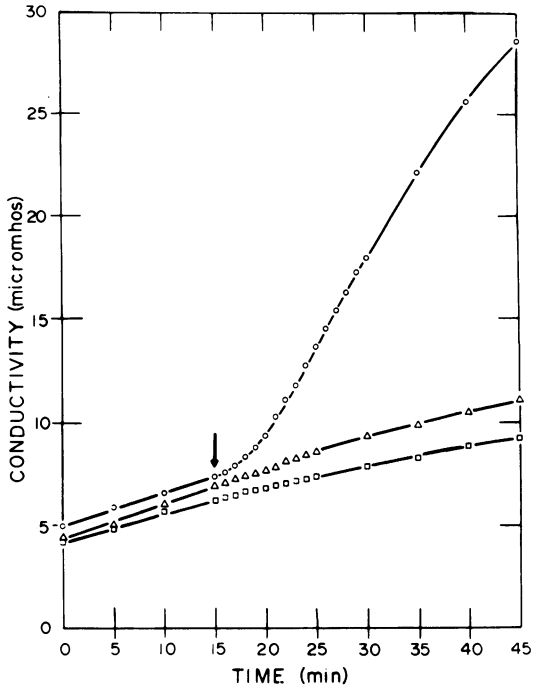

FIG. 1. Leakage of electrolytes (μmhos) from irradiated tobacco leaf discs treated wih cercosporin. Five 1-cm-diameter leaf discs were infiltrated with water containing 5 μM cercosporin. Leaf discs were incubated in the dark for 15 min to establish a background level of electrolyte leakage. At 15 min (arrow), two of the treatments were irradiated with 800 J m^{-2} s^{-1}. ○, treated with cercosporin, exposed to light after 15 min; □, water control, exposed to light after 15 min; △, treated with cercosporin, incubated in the dark throughout the experiment. Data represent the mean of six experiments.

Table I. *Rate of Electrolyte Leakage from Cercosporin-Treated Tobacco Leaf Discs Before and After Irradiation with Light*

Leaf discs were infiltrated with 5 μM cercosporin and irradiated with incandescent light (800 J m^{-2} s^{-1}) after 15 min incubation in the dark. Data represent the mean of six experiments.

Treatment	Time	Electrolyte Leakage[a]
	min	*μmhos/min*
Dark	0–5	0.17
	5–10	0.15
	10–15	0.15
Light	0–1	0.20
	1–2	0.28
	2–3	0.43
	3–4	0.55

[a] $LSD_{0.01}$ = 0.10, $LSD_{0.05}$ = 0.08.

Effects on Protoplasts. Cercosporin caused rapid damage to tobacco suspension cultured protoplasts in the light (Table II). After 45 min incubation with 5 μM cercosporin and 800 J m^{-2} s^{-1}, all protoplasts had either burst, or were irregularly shaped with condensed cytoplasm. Control (untreated) protoplasts were not affected by the high light intensities and remained healthy throughout the course of the experiment.

Ethane Emission. Volatile scission products such as ethane and

[2] Abbreviation: MDA, malondialdehyde.

Table II. *Effect of Cercosporin on Tobacco Suspension Culture Protoplasts in the Presence of Light*

Protoplasts were treated with 5 μM cercosporin and irradiated with 800 J m^{-2} s^{-1} of light. Control protoplasts were irradiated but not treated with cercosporin. The proportion of healthy protoplasts was determined from counts of approximately 500 protoplasts per treatment.

Time	Proportion of Healthy Protoplasts					
	Experiment I			Experiment II		
	Control	Treated	Treated / Control	Control	Treated	Treated / Control
min						
0	0.84	0.85	1.00	0.63	0.60	0.95
15	0.82	0.35	0.43	0.66	0.26	0.39
30	0.83	0.17	0.20	0.64	0.05	0.07
45	0.78	0	0	0.63	0	0

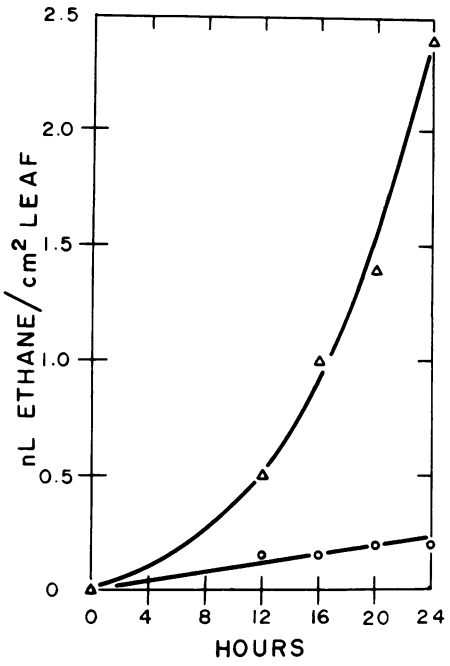

FIG. 2. Emission of ethane from irradiated tobacco leaf discs treated with cercosporin. Five 1-cm-diameter leaf discs were infiltrated with water containing 10 μM cercosporin, and were incubated under lights (100 J m^{-2} s^{-1}) in sealed flasks. Ethane emission was determined by gas chromatography. △, cercosporin-treated; ○, water control. Data represent the mean of nine experiments.

pentane are released during the breakdown of lipid hydroperoxides (6). Ethane was released by tobacco leaf discs treated with cercosporin 12 to 24 h after incubation in the light (Fig. 2). Untreated leaf discs released little or no ethane. Infiltration with 100 μM α-tocopherol did not alter the rates of ethane release from either the treated or control leaf discs.

Thiobarbituric Acid Assay. MDA, a breakdown product of lipid hydroperoxides (16), was detected colorimetrically by reaction with thiobarbituric acid. Over 4 times the amount of MDA could be detected in chloroform:methanol extracts of cercosporin-treated suspension cultured cells grown in the light as compared to dark-grown cells (Fig. 3). If 100 μM α-tocopherol was added to the cells, there was no detectable MDA after 3 h. There was also no detectable MDA in extracts of light-grown, untreated cells. Cercosporin is present in the chloroform:methanol extracts, and these extracts are unavoidably exposed to light during their preparation

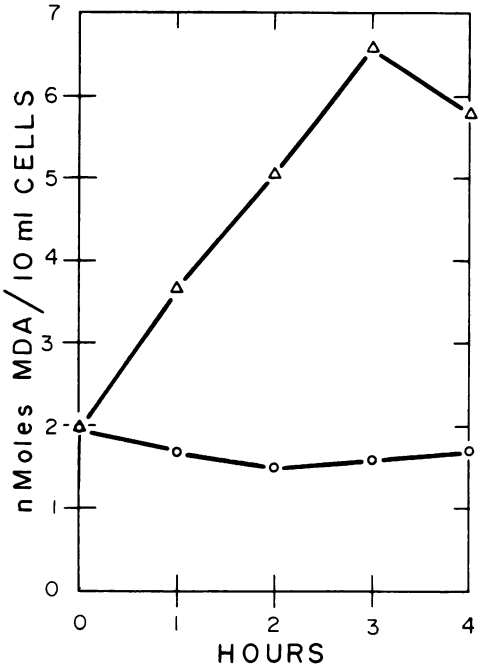

FIG. 3. Production of MDA by tobacco suspension cultured cells treated with 10 μM cercosporin. Cell extracts were assayed with thiobarbituric acid to determine the concentration of MDA. △, irradiated with fluorescent lights (20 J m^{-2} s^{-1}); ○, incubated in the dark. MDA was not detected in extracts from untreated cells after 4 h incubation in the light. Data represent the mean of three experiments.

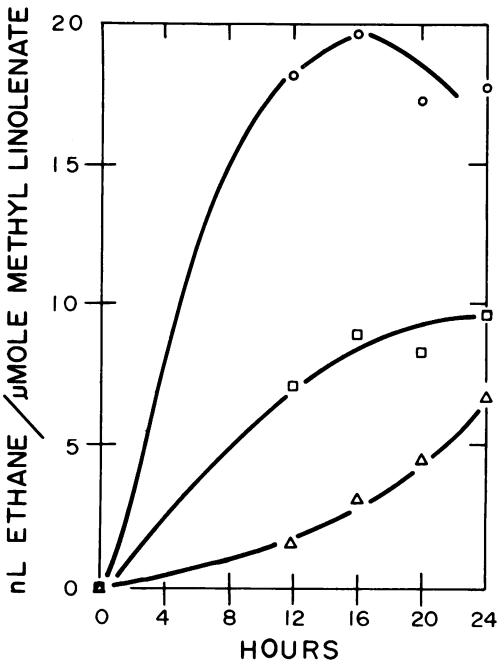

FIG. 4. Ethane emission from irradiated, cercosporin-treated solutions of methyl linolenate, and inhibition of ethane release by α-tocopherol. Reaction mixture contained 100 μM methyl linolenate and 10 μM cercosporin in H_2O. Cultures were irradiated with an incandescent light at 100 J m^{-2} s^{-1}. Ethane concntrations were determined by gas chromatography. ○, methyl linolenate treated with cercosporin; △, untreated methyl linolenate solution; □, cercosporin-treated methyl linolenate with 100 μM α-tocopherol. Data represent the mean of three experiments.

for assaying with thiobarbituric acid. The low, constant amount of MDA (approximately 1.6 nmol/10 ml cells) found in the dark-grown cells may have been due to the oxidation of the extract lipids by cercosporin during this time.

Methyl Linolenate. The ability of cercosporin to oxidize methyl linolenate was tested both by assaying with thiobarbituric acid and by measuring the release of ethane from methyl linolenate solutions. After 1 and 2 h of incubation in the light, 5.8 and 16.7 nmol, respectively, of MDA were detected in cercosporin-treated solutions of methyl linolenate. No MDA was detectable in dark-incubated solutions or in untreated methyl linolenate solutions incubated in the light. Ethane was released from cercosporin-treated methyl linolenate solutions during the first 12 to 16 h after incubation in the light (Fig. 4). Small amounts of ethane (approximately one-fourth the amount from cercosporin-treated solutions) were released from untreated solutions 16 to 24 h after incubation, presumably due to autoxidation of the methyl linolenate. In contrast to results obtained with leaf discs, α-tocopherol inhibited ethane release from cercosporin-treated methyl linolenate.

DISCUSSION

Cercosporin caused rapid injury to tobacco cell membranes as indicated by the bursting of protoplasts and leakage of electrolytes soon after toxin-treated tissues were exposed to light. Macri and Vianello (12) previously reported that cercosporin could induce ion leakage from plant tissues. However, significant differences were not seen until 30 min after irradiation, even with cercosporin concentrations 3 to 4 times those used in this study, suggesting that the toxic activity on membranes might be an indirect effect. The rapid response obtained in both assays reported here (1 to 2 min for a significant change in electrolyte leakage) suggests that cercosporin does have a direct effect on cell membranes.

The rapid membrane damage appears to be due to a peroxidation of membrane lipids. Cercosporin was able to induce the peroxidation of membrane lipids *in vivo* and to oxidize solutions of methyl linolenate as demonstrated by the emission of ethane and the detection of MDA. These results support the work of Cavallini *et al.* (4) who found that cercosporin induced lipid peroxidation in several *in vitro* membrane systems.

α-Tocopherol, a well-known inhibitor of radical chain oxidations (21), inhibited both MDA formation in cercosporin-treated suspension cultured cells and ethane release from methyl linolenate solutions. α-Tocopherol had no effect, however, on ethane release from cercosporin-treated tobacco leaf discs. The effectiveness of α-tocopherol is often tested in nutritional studies or model membrane systems (13), and studies have shown that α-tocopherol must be bound in the membrane in order to have effective chain terminating activity (22). Thus, adding α-tocopherol to a complex system (such as infiltrating tobacco leaf discs) may not be adequate for testing its inhibitory activity. Presumably, the suspension cultured cells were able to incorporate the added α-tocopherol into their membranes, because protection was seen in this case.

The membrane-damaging activity of cercosporin is consistent with the plant pathogenic role of *Cercospora* species. Early ultrastructural changes seen in *Cercospora*-infected and cercosporin-treated sugar beets include the disruption of cell membranes, including thickening and swelling of ER and mitochondrial membranes and disruption of the plasmalemma, tonoplast, and chloroplast membranes (19, 20). Changes in membrane permeability are often the first detectable events in diseases caused by a variety of plant pathogens, and such changes are believed to play a role in the availability of nutrients for the invading pathogen (24). Thus, cercosporin may play an important role in pathogenesis by providing the invading fungus with the nutrients required for growth and sporulation within the host tissues.

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