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Cell Surface Redox Potential as a Mechanism of Defense against Photosensitizers in Fungi

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The phytotoxin cercosporin, a singlet oxygen-generating photosensitizer, is toxic to plants, mice, and many fungi, yet the fungi that produce it, *Cercospora* spp., are resistant. We hypothesize that resistance to cercosporin may result from a reducing environment at the cell surface. Twenty tetrazolium dyes differing in redox potential were used as indicators of cell surface redox potential of seven fungal species differing in resistance to cercosporin. Resistant fungi were able to reduce significantly more dyes than were sensitive fungi. A correlation between dye reduction and cercosporin resistance was also observed when resistance levels of *Cercospora* species were manipulated by growth on different media. The addition of the reducing agents ascorbate, cysteine, and reduced glutathione (GSH) to growth media decreased cercosporin toxicity for sensitive fungi. None of these agents directly reduced cercosporin at the concentrations at which they protected fungi. Spectral and thin-layer chromatographic analyses of cercosporin solutions containing the different reducing agents indicated that GSH, but not cysteine or ascorbate, reacted with cercosporin. Resistant and sensitive fungi did not differ in endogenous levels of cysteine, GSH, or total thiols. On the basis of data from this and other studies, this report presents a model which proposes that cercosporin resistance results from the production of reducing power at the surfaces of resistant cells, leading to transient reduction and detoxification of the cercosporin molecule.

Cercospora spp. are a group of fungal plant pathogens that cause serious leaf-spotting diseases on a large number of host plants worldwide. One of the reasons for the success of this group of pathogens is their production of the highly toxic, photoactivated toxin cercosporin (7, 9, 29). Cercosporin is a lipid-soluble perylenequinone produced via the polyketide pathway (25). Cercosporin treatment of plant tissues results in fatty acid peroxidation and destruction of membrane lipids, leading to rupture of the plasma membrane and cell death (8, 10). It is hypothesized that this membrane damage provides the fungus, which is limited to growth within plant intercellular spaces, with the nutrients required for growth and sporulation within the host. Cercosporin is toxic to all plants which have been tested as well as to mice, bacteria, and many fungi (9). Efforts to isolate cercosporin-resistant mutants of tobacco and *Neurospora crassa* have been unsuccessful (9a). *Cercospora* spp., by contrast, are highly resistant. The goal of our research is to understand the molecular mechanism of cercosporin resistance in *Cercospora* spp.

Cercosporin is a photosensitizing compound which is converted to an electronically excited state in the presence of light (7). The light-activated triplet state of cercosporin reacts with molecular oxygen to form singlet oxygen ($^1O_2$) and/or superoxide ($O_2^{\cdot -}$) (11, 14, 20). Evidence suggests that $^1O_2$ formation is the major mechanism by which cercosporin exerts its toxicity (7, 11, 14, 20). Mechanisms of resistance to $^1O_2$ and to photosensitizers have not been well characterized. Enzymatic defenses active against many radical oxygen species are not effective against $^1O_2$. There are many compounds which quench $^1O_2$ (4), but only a few of these are found in biological systems, and even fewer have actually been demonstrated to play a role in defense. The best characterized of these are carotenoids, which quench both $^1O_2$ and the triplet state of photosensitizers (15, 22). Carotenoids have been shown to be involved in fungal resistance to cercosporin, but evidence suggests that an additional mechanism is also operating (13).

Cercospora species excrete high concentrations of cercosporin into the medium during growth, forming red crystals which give off a red fluorescence when viewed by fluorescence microscopy. By contrast, the hyphae of these cultures, fluoresce with a bright green color, suggesting that cercosporin may be derivatized when in contact with the fungal cell. We have shown that reduction of the cercosporin molecule by the addition of strong reducing agents results in a fluorescent green compound. Reduced derivatives of cercosporin absorb far less light than the normal oxidized form, are less efficient in $^1O_2$ production, and are less toxic to sensitive fungi (23). These results suggest that maintenance of cercosporin in a reduced form may be a mechanism of cercosporin resistance. The purpose of this study was to investigate the production of reducing power by the fungus and the relationship of that reducing power to cercosporin resistance.

In this study, tetrazolium dyes differing in molecular structure and redox potential were used as indicators of cell surface redox potential. Reduction of the dyes results in the formation of intensely colored formazans which form insoluble precipitates at the point of reduction. In addition to examining cell surface redox potential, we investigated the roles of several reducing agents in protecting cells from cercosporin toxicity and examined sensitive and resistant fungi for endogenous levels of reducing compounds.

MATERIALS AND METHODS

Fungal strains and cultures. The isolates of *Cercospora nicotianae* (ATCC 18366), *Cercospora beticola* (ATCC

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TABLE 1. Tetrazolium dyes used as indicators of fungal redox potential

Dye name[a]	Abbreviation
2-Phenyl-3-(4,5-dimethyl-2-thiazolyl)-5-(4-pyridyl) tetrazolium bromide	PDTPT
3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide	MTT
p-Iodonitrotetrazolium violet	INT
Tetranitro blue tetrazolium	TNBT
Neotetrazolium chloride	NT
Tetrazolium violet	TV
p-Nitro blue tetrazolium	NBT
m-Nitro blue tetrazolium chloride	NBTC
m-Nitroneotetrazolium chloride	NTC
o-Tolyltetrazolium red	*o*-TTR
p-Anisyl blue tetrazolium chloride	ABT
p-Tolyltetrazolium red	*p*-TTR
Thiocarbamyl nitro blue tetrazolium	TCBT
p-Anisyl *p*-nitro blue tetrazolium chloride	ANBT
2,3,5-Triphenyltetrazolium chloride	TTC
Tetrazolium blue chloride	BT
Piperonyl tetrazolium blue	PTB
Veratryl tetrazolium blue	VTB
Cinnamyl nitroblue tetrazolium chloride	DS-NBT
2-(2'Benzothiazolyl)-5-styryl-3(4'phthalhydrazidyl) tetrazolium chloride	BSPT

[a] Dyes are ranked by relative ease of reduction (most easily reduced to most difficult to reduce).

24080), *Penicillium camembertii* (ATCC 6985), and *Penicillium caseicolum* (ATCC 6986) were obtained from the American Type Culture Collection (Rockville, Md.). *Cercospora asparagi* was isolated from asparagus in North Carolina. *Aspergillus flavus* (ATCC 60045), a white-spored strain, was provided by G. A. Payne (North Carolina State University, Raleigh). *Cercospora kikuchii* IN and PR (wild type) and S1 and S2 (toxin-deficient derivatives of PR) were provided by R. G. Upchurch (North Carolina State University). *N. crassa* ORS-6a (4200) and al-1 (3622) were obtained from the Fungal Genetics Stock Center (Kansas City, Kans.). *Alternaria alternata* A5 is a tobacco isolate from North Carolina, provided by H. Spurr (U.S. Department of Agriculture, Oxford, N.C.).

Cultures were routinely maintained on potato dextrose agar (PDA) or potato dextrose broth (PDB) (Difco Laboratories). Media for experiments included PCG (10 g of peptone, 1 g of casein hydrolysate, 0.5 g of glucose, and 17 g of agar per liter); complete medium (CM) (21), a basal salts medium containing glucose, yeast extract, and casein hydrolysate; and minimal medium (MM), consisting of CM minus the yeast extract. Additional media used for the medium interactions study were *Neurospora* medium standard (NMS) (28), a basal salts medium containing tartaric acid and sucrose; malt medium (21); and *Cercospora* sporulation medium (CSM) containing leucine, sucrose, and yeast extract in addition to basal salts (21).

Chemicals. Reduced glutathione (GSH), oxidized glutathione (GSSG), *o*-phthalaldehyde, *N*-ethylmaleimide, 5,5'-dithio-bis(2-nitrobenzoic acid), L-cysteine, sodium ascorbate, and the tetrazolium dyes (for abbreviations, see Table 1) BT, TNBT, INT, TTC, TV, MTT, NT, and NBT were obtained from Sigma Chemical Company (St. Louis, Mo.). The tetrazolium dyes ANBT, ABT, TCBT, *p*-TTR, *o*-TTR, DS-NBT, NTC, BSPT, NBTC, PDTPT, and VTB were obtained from Research Organics (Cleveland, Ohio).

Cercosporin. Cercosporin was extracted and purified from cultures of *C. kikuchii* PR as previously described (7). Cercosporin was stored as an acetone stock at −20°C in the dark. In all assays, acetone was added at an equal concentration (0.5%, vol/vol) to control treatments.

Tetrazolium dye test. Twenty tetrazolium dyes (Table 1) were tested as indicators of cell surface redox potential of nine fungal isolates differing in their resistance to cercosporin. Tetrazolium dye stock solutions (2.5 mg/ml) were prepared in water and incubated overnight before being filter sterilized. Dyes were added to PCG medium at a final concentration of 0.05 mg/ml. Cercosporin-resistant fungi tested were *C. kikuchii* PR and S2 and *C. nicotianae*. Cercosporin-sensitive fungi tested were *P. caseicolum*, *P. camembertii*, *A. flavus*, and a wild-type strain and an albino strain of *N. crassa*. *Alternaria alternata*, which is intermediate in resistance to cercosporin, was also tested. Plates were inoculated in the center with agar plugs cut from the edges of 1-week-old fungal colonies. Plates were wrapped with Parafilm and incubated at 25°C in the light with two replicates for each fungus-dye interaction. Dye reduction was scored by visual observation of formazan crystals in the medium and by microscopic observation of mycelial plugs harvested from the plates.

Medium interactions. Seven *Cercospora* isolates, *C. nicotianae*, *C. beticola*, *C. asparagi*, and *C. kikuchii* PR, S1, S2, and IN, were inoculated onto plates of seven different media containing 10 μM cercosporin. The media used were NMS, malt, CM, CSM, MM, PDA, and PCG. Growth was measured after 7 days and compared with growth of controls without cercosporin to calculate percent growth. The seven isolates were also inoculated onto the same seven media without cercosporin but containing 0.05 mg of TTC or *p*-TTR per ml and grown for 7 days (malt, CM, CSM, MM, PDA, and PCG) or 11 days (NMS). Reduced dyes (formazans) were extracted by placing four 6-mm plugs from each plate into 2 ml of 100% ethanol. Control plates of the fungi without dye were also extracted. The A_{478} of each dye was measured. For both formazan absorbance and cercosporin resistance, mean values for all seven isolates on each medium were determined, and correlation coefficients were calculated for these means for each dye.

Protection assays. The reducing agents cysteine, GSH, and ascorbate were tested for their effects on cercosporin toxicity. Mycelial plugs of *C. kikuchii* PR, *A. flavus*, and *N. crassa* ORS-6a were inoculated into the centers of plates of

PCG medium with and without 10 μM cercosporin and amended with cysteine or GSH (both at 0, 1, 5, 10, and 20 mM) or sodium ascorbate (0, 1, 5, 10, 20, 30, and 40 mM). Plates were incubated under fluorescent lights (60 microeinsteins · m^{-2} · s^{-1}). Radial growth was measured after 16 h (*N. crassa*) or 5 days (other fungi).

Reaction of reducing agents with cercosporin. To determine whether the reducing agents reduced or reacted with cercosporin, spectrophotometric and thin-layer chromatography (TLC) analyses were conducted. A 10 μM cercosporin solution containing 10% acetone was prepared in PDB. Dithionite was added at a concentration of 10 mg/ml. Cysteine, GSH, or ascorbate was added at a concentration of 20 mM. Spectra were recorded from 650 nm to 400 nm on a Beckman model 25 spectrophotometer. Spectra were recorded at zero time (all treatments) and after incubation for 24 h at room temperature in the light (cysteine, ascorbate, and GSH treatments). Solutions of cercosporin incubated with the reducing agents for 24 h were also extracted and analyzed for the presence of cercosporin by TLC. Solutions were mixed with a 1/10 volume of chloroform to extract cercosporin. The chloroform layer was spotted onto silica gel TLC plates (Merck), and the plates were developed with hexane-isopropanol (6:4). Cercosporin was detected at an R_f of 0.56 as a red fluorescent spot under long-wave UV light.

Analysis of endogenous reducing agents. Endogenous reducing agents from mycelial extracts of 4-day-old (*N. crassa* ORS-6a) or 5-day-old (*A. flavus* and *C. kikuchii* PR and S2) cultures grown in PDB were assayed. Mycelial extracts were prepared by filtering mycelium, grinding it in liquid nitrogen, and extracting the mycelial powders with 0.1 M sodium phosphate buffer (pH 8.0) containing 5 mM EDTA. Total thiol content was determined on the basis of a method by Grill et al. (18) using 5,5'-dithio-bis(2-nitrobenzoic acid). Total thiols from both fresh mycelium (soluble thiols) and acetone powders (protein thiols) were measured. Cysteine concentration was measured by the acid ninhydrin technique (16). GSH and GSSG contents were analyzed by the methods published by Barak and Edgington (3). GSH and GSSG contents were determined both from culture supernatants and from mycelial extracts. GSH was measured by reaction with *o*-phthalaldehyde; GSSG was measured the same way after treatment with *N*-ethylmaleimide.

RESULTS

Tetrazolium dye reduction. Results for the reduction of the 20 dyes by the eight fungi are summarized in Fig. 1. Reduction was determined both by visual assessment of colored formazans in culture and by microscopic visualization of hyphae to ensure that fungal pigments did not interfere with evaluation. Reduction was scored as positive when pigment formation occurred in the cultures in the area where hyphae were growing and when formazan crystals were microscopically observed outside intact hyphae. In some cultures, formazan pigments were visible only in the centers of colonies and microscopic examination revealed crystals inside hyphae which appeared empty or senescent. These reactions were interpreted as negative for dye reduction. Dyes which were easily reduced by all of the fungi included TNBT, INT, MTT, and PDTPT. Dyes BSPT and DS-NBT were not visibly reduced by any of the fungi. The remaining dyes (BT, TTC, ANBT, TCBT, ABT, *p*-TTR, *o*-TTR, NTC, NBTC, NBT, TV, and NT) were differentially reduced. These dyes were consistently reduced by resistant fungi and not reduced or variably reduced by sensitive fungi.

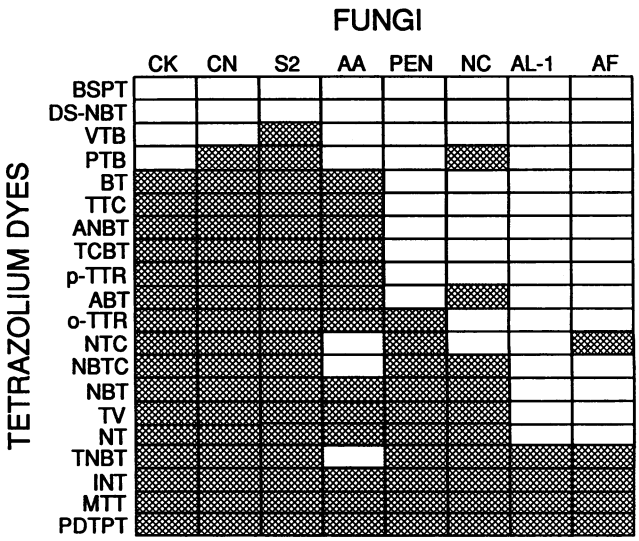

FIG. 1. Tetrazolium dye reduction by fungi. Resistant fungi *C. kikuchii* (CK), *C. nicotianae* (CN), cercosporin-negative mutant of *C. kikuchii* (S2), and *Alternaria alternata* (AA) and sensitive fungi *P. caseicolum* and *P. camembertii* (PEN), *N. crassa* (NC), *N. crassa* albino mutant (AL-1), and *A. flavus* (AF) were grown on PCG medium amended with 20 different tetrazolium dyes (see Table 1 for abbreviations). Hatched boxes indicate dye reduction by the fungus; open boxes indicate no reduction. Dyes are ranked by relative ease of reduction as observed in this study (most easily reduced to most difficult to reduce).

Alternaria alternata, which is intermediate in cercosporin resistance, was found to be intermediate in the number of dyes which it reduced compared with more resistant or more sensitive fungi.

Medium effects. In preliminary experiments, medium composition was found to affect dye reduction. Cercosporin resistance of *Cercospora* spp. is also altered by different growth media. Thus, in order to further correlate resistance with dye reduction, seven *Cercospora* isolates were tested for both cercosporin resistance and reduction of TTC and *p*-TTR on seven different media. As shown in Fig. 2, there was a significant correlation ($r = 0.85$ and $P = 0.0143$ for *p*-TTR; $r = 0.74$ and $P = 0.0591$ for TTC) between the ability of the fungi to reduce dyes on a particular medium and their resistance to cercosporin on that medium. The correlation was strongest for *p*-TTR.

Protection assays. The presence of reducing agents in media protected *N. crassa* and *A. flavus* from cercosporin toxicity to varying degrees but did not have any effect on the resistance of *Cercospora* spp. (Fig. 3). Both cysteine and GSH were highly effective in protecting *A. flavus* and *N. crassa*, with increased growth occurring at levels as low as 5 mM. Ascorbate also protected *A. flavus* against cercosporin but had only a small effect on *N. crassa*. None of these agents affected the resistance of *C. kikuchii* to cercosporin. The presence of these agents also did not affect growth of control cultures, with the exception of GSH, which was inhibitory to growth of *C. kikuchii* and *N. crassa* at high concentrations (20 mM).

Spectrophotometric analysis. To determine whether the reducing agents reduced or reacted with cercosporin, spectrophotometric and TLC analyses were conducted. Reduction of cercosporin by the addition of the reducing agent dithionite caused a color change from red to green and a

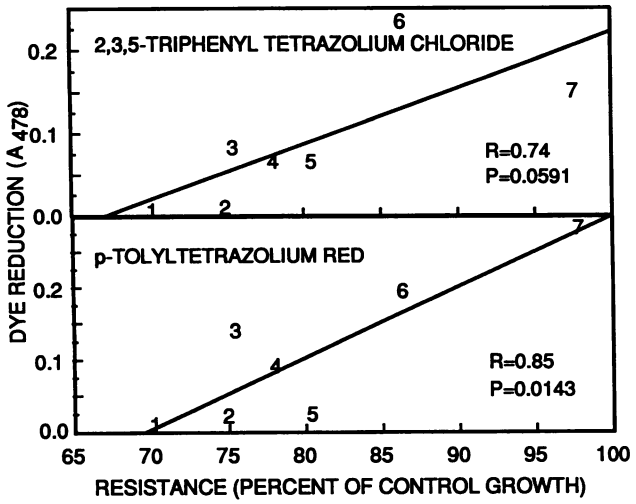

FIG. 2. Correlation between dye reduction and cercosporin resistance for *Cercospora* isolates on seven media. Seven *Cercospora* isolates were tested for cercosporin resistance and ability to reduce TTC (2,3,5-triphenyl tetrazolium chloride) and *p*-TTR (*p*-tolyltetrazolium red) on seven different media. Resistance was determined by comparing growth on media with and without 10 μM cercosporin. Dye reduction was determined by extraction and quantitation of the formazan product. Numbers on the graph represent the mean values for all seven isolates on each of the different media: 1, NMS; 2, malt; 3, CM; 4, CSM; 5, MM; 6, PDA; and 7, PCG. A significant correlation between resistance of the *Cercospora* fungi on a medium and the amount of dye reduction on that medium was found.

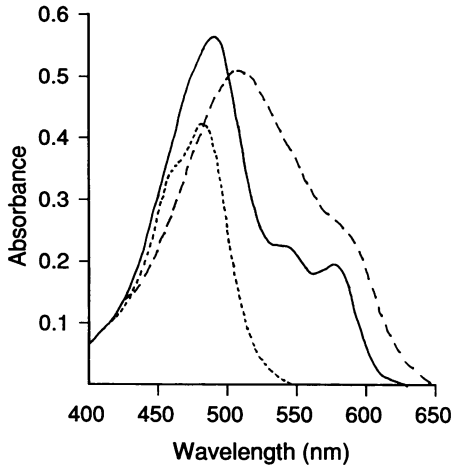

FIG. 4. Absorbance spectra of cercosporin (——), dithionite-reduced cercosporin (– – – –), and cercosporin treated with GSH for 24 h (— — —). A 10 μM cercosporin solution containing 10% acetone was prepared in PDB. Dithionite was added at a concentration of 10 mg/ml; GSH was added at a concentration of 20 mM and incubated for 24 h at room temperature in the light. The GSH-cercosporin spectrum at zero time showed no change from the untreated cercosporin spectrum.

marked difference in the absorption spectrum (Fig. 4). The reduced form was highly unstable and reverted back to the oxidized form when the solution was aerated and when the cercosporin was extracted from the dithionite solution with chloroform. Glutathione, cysteine, and ascorbate at a concentration which protects sensitive fungi (20 mM) did not cause a color shift or a change in the cercosporin absorption spectrum when assayed immediately after addition. After a 24-h incubation at room temperature in the light, the spectra of the solutions containing cysteine and ascorbate were still unchanged, although cercosporin appeared to be less stable in the presence of ascorbate; total peak area of cercosporin in the presence of ascorbate decreased by approximately 25%. By contrast, the GSH-containing solution changed color (purple-pink instead of red), and there was a pronounced red shift in the absorption spectrum (Fig. 4). TLC analysis showed that there was no cercosporin present in the GSH-containing solution after 24 h and confirmed that cercosporin was still present in the ascorbate- and cysteine-treated samples, with no new breakdown products observed.

Assays for endogenous reducing substances. There was no

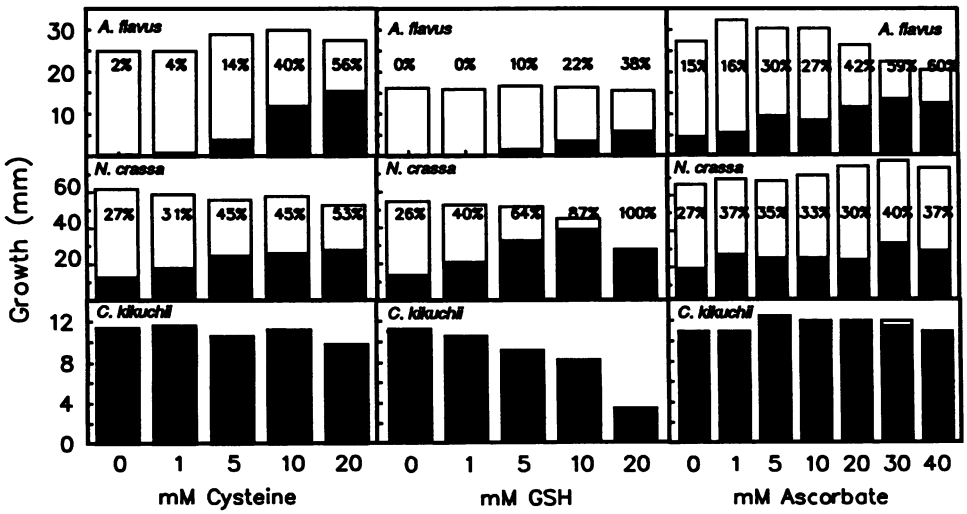

FIG. 3. Protection of fungi by reducing agents. Growth of fungi on media with (dark bars) or without (open bars) 10 μM cercosporin in the presence of the reducing agents cysteine, GSH, and ascorbate. The presence of reducing agents decreased cercosporin toxicity for the cercosporin-sensitive fungi *A. flavus* and *N. crassa* without affecting the growth of *C. kikuchii*. Numbers above the bars indicate percentages of growth in the presence of cercosporin compared with growth of non-cercosporin-containing controls.

evidence that *Cercospora* spp. have higher levels of cysteine, glutathione, or total thiols than the sensitive fungi *N. crassa* and *A. flavus*. Total thiols ranged from 0.5 to 1.0 μmol/g (dry weight) for soluble thiols and from 2.3 to 4.6 μmol/g of acetone powder for protein thiols. No correlation was found between cercosporin resistance and total thiol levels. Cysteine levels (average of 2.3 μmol/g [dry weight]) did not vary between fungi. Low levels of GSH and GSSG were detected in culture filtrates (less than 1 μmol/g [dry weight] of mycelium). Mycelial GSH and GSSG contents of the different fungi (1.2 to 1.5 and 0.1 μmol/g [dry weight] for GSH and GSSG, respectively) fell within the range of published values for fungi (3), and no differences between cercosporin-resistant and cercosporin-sensitive fungi could be found.

DISCUSSION

In this study, we investigated the hypothesis that resistance of fungi to the photosensitizing compound cercosporin was due to the production of reducing power by resistant fungi. Reducing power of fungi was estimated by using 20 tetrazolium dyes of different molecular structures. Tetrazolium dyes are widely exploited as indicators of biological reducing systems (2, 26). Reduction of the dyes results in formation of stable formazans, which are intensely colored compounds and which precipitate at the point of reduction. The molecular structure of each dye determines the ease with which it is reduced. Although the precise redox potentials of most of the dyes have not been determined, studies using a variety of approaches have demonstrated large differences in the ease of reduction between dyes and have provided relative rankings of some of the commonly used dyes (2, 26). Information about seven of the dyes used in our study (MTT, INT, TNBT, NT, TV, NBT, and BT) is available. Our rankings agree very closely with those of Altman (1, 2), who used pairs of dyes incubated with rat tissue and measured quantitatively the amount of each formazan produced.

The ability of fungi to reduce the tetrazolium dyes was strongly correlated with their resistance to cercosporin. Cercosporin-resistant fungi were able to reduce substantially more of the dyes than were the sensitive fungi. Further, when resistance of *Cercospora* species was altered by different growth media, resistance levels were significantly correlated with the ability to reduce dyes on those media, as measured by extraction and quantification of the formazan products. The strong correlation between expression of resistance and ability to reduce dyes both for species within the genus *Cercospora* and for unrelated species provides support for the hypothesis that cell surface reduction is involved in resistance.

If reducing power plays a role in resistance, then exogenously added reducing agents should protect sensitive fungi against cercosporin. We examined changes in the toxicity of cercosporin in the presence of several potent reducing agents. All three reducing agents (cysteine, ascorbate, and GSH) were able to protect sensitive fungi against cercosporin. Protection was seen at levels which did not affect growth of control cultures. Although our data suggest that GSH protection may be due to the formation of a cercosporin derivative rather than to reducing power alone, cysteine and ascorbate protected the fungi without altering the cercosporin molecule.

On the basis of our results, we have constructed a model (Fig. 5) to explain how reducing power may function in the protection of *Cercospora* species against cercosporin. This model proposes that *Cercospora* species generate reducing power at the cell surface (presumably at the plasma membrane), which renders the cercosporin molecule nontoxic. After secreting cercosporin, the fungus is able to keep it reduced because of the presence of reducing power at the cell surface. Reduced cercosporin spontaneously reoxidizes as it diffuses away from the cell surface reducing environment. The oxidized toxin molecule, activated by light, reacts with oxygen to form $^1O_2$, which causes lipid peroxidation of membranes of plants and other sensitive organisms.

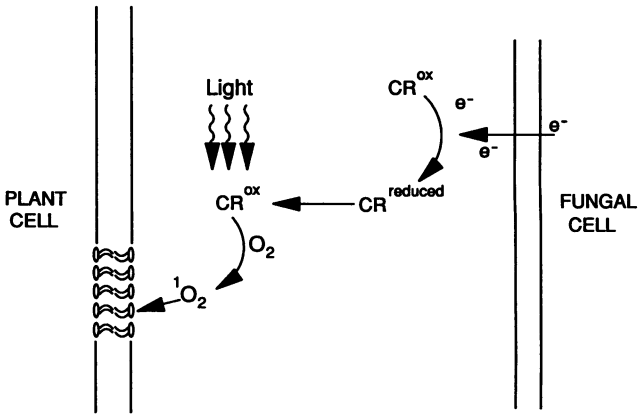

FIG. 5. Proposed model to explain the role of reducing power in cercosporin resistance. After secreting cercosporin, *Cercospora* species are able to keep it reduced because of the presence of reducing power at the cell surface. In the reduced form, cercosporin generates less singlet oxygen and is less toxic. Reduced cercosporin spontaneously reoxidizes as it diffuses away from the cell surface reducing environment. The oxidized toxin molecule, activated by light, reacts with oxygen to form $^1O_2$, which causes lipid peroxidation of cell membranes of plants and other sensitive organisms.

This model is supported by several lines of evidence in addition to those presented in this report. We have recently synthesized stable, reduced derivatives of cercosporin and have shown that they absorb less light, generate far less $^1O_2$, and are less toxic than oxidized cercosporin (23), supporting the argument that reduction detoxifies the cercosporin molecule. Second, fluorescence microscopy studies using bandpass filters, which allow us to differentiate between fluorescence emissions from cercosporin and reduced cercosporin, indicate that hyphae of *Cercospora* species are capable of reducing cercosporin, whereas hyphae of sensitive fungi are not (12). *Cercospora* hyphae killed by heat or chloroform vapor are not capable of reducing cercosporin, demonstrating that reduction requires living cells. We have also observed that crystals of cercosporin accumulate near hyphae in culture, supporting our observation that reduced cercosporin is unstable and spontaneously reoxidizes upon diffusion away from the source of reducing power.

One observation in this study which appears inconsistent with the model described above is that reducing agents do not directly reduce cercosporin at concentrations which protect sensitive fungi. However, by use of fluorescence microscopy, we have recently shown that sensitive fungi can reduce cercosporin when incubated with reducing agents (12), although reduction was dependent on the specific reducing agent and conditions of incubation. These data suggest that reducing agents in combination with reducing power generated by sensitive fungi may allow cercosporin

reduction when neither alone is sufficient. It is also possible that reducing power protects directly against the toxic effects of active oxygen species in addition to reduction and detoxification of the photosensitizer molecule. Thiol compounds have been reported to react directly with $^1O_2$ (27), to act as antioxidants (19), and to protect cells against ionizing radiation and increased oxygen tensions (24). Also, transmembrane dehydrogenases which protect against oxidative damage by reducing lipid hydroperoxides have been identified in human erythrocytes (6, 17).

In order to determine the source of reducing power, we assayed for the presence of endogenous reducing substances. Although compounds such as GSH, cysteine, and ascorbate were able to protect sensitive fungi, observed differences in endogenous levels of GSH, cysteine, and total thiol groups did not correlate with cercosporin resistance. Thus, it appears that the presence of simple reducing agents in the mycelium is not responsible for the reducing power which protects resistant fungi. Another possibility for the source of reducing power is the presence of transmembrane redox systems. Plasma membrane redox enzymes are a general characteristic of plant, animal, bacterial, and fungal cells (5, 17) and function to transport reducing power across the membrane. Tetrazolium dye reduction is a common method used to detect activity of transmembrane redox systems (26); thus, reduction of tetrazolium dyes by *Cercospora* spp. may be indicative of such enzyme systems.

In summary, we have shown that *Cercospora* spp. have a strong reducing capability which correlates with cercosporin resistance. We believe that reducing power results in the direct reduction and detoxification of the cercosporin molecule in contact with the cell surface. Reducing power may also protect against the damaging effects of $^1O_2$ itself. Because no differences between levels of reducing substances for resistant and sensitive fungi were found, we hypothesize that differences between the cell surface redox potentials of sensitive and resistant fungi are due to the presence of a transmembrane redox system(s). If this hypothesis is correct, then cercosporin resistance could result from the presence of a specific cercosporin reductase or could be due to a complex interaction of several enzyme systems in the membrane, together providing the necessary level of reducing power. Present efforts are directed toward the isolation of *Cercospora* mutants which are altered in their ability to reduce cercosporin. Through complementation of these mutants, we hope to identify genes involved in cercosporin reduction.

ACKNOWLEDGMENTS

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