

## Symposium-in-Print

# Dihydrocercosporin Singlet Oxygen Production and Subcellular Localization: A Possible Defense Against Cercosporin Phototoxicity in *Cercospora*

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## ABSTRACT

Fungi in the genus *Cercospora* produce cercosporin, a potent singlet oxygen ( $^1\text{O}_2$ )-generating photosensitizer that plays a critical role in the ability of these fungi to parasitize plants. Although plants, mice, bacteria and many fungi are sensitive to cercosporin, *Cercospora* species are resistant to its toxicity. The cellular resistance of these fungi to cercosporin has been correlated with fungal cell surface reducing ability and the ability to maintain cercosporin in a chemically reduced state. As a model for reduced cercosporin we employed a reduced, acetylated derivative (hexaacetyl-dihydrocercosporin, HAC) that we tested for  $^1\text{O}_2$  production in a range of solvents. We found that as a  $^1\text{O}_2$  photosensitizer, HAC was only moderately effective in organic solvents ( $\phi_{\text{SO}} = 0.14\text{--}0.18$ ) and very poor in water ( $\phi_{\text{SO}} = 0.02\text{--}0.04$ ). By contrast, the  $^1\text{O}_2$  quantum yield of cercosporin itself was unaffected by solvent ( $\phi_{\text{SO}} = 0.84\text{--}0.97$ ). To investigate the localization of reduced cercosporin in fungal cells, we developed a fluorescence assay using laser scanning confocal microscopy. This assay showed a uniform green fluorescence, indicative of reduced cercosporin, in the cytoplasm of hyphal cells treated with cercosporin. We hypothesize that the main protection mechanism against cercosporin phototoxicity in the fungus consists of transformation of cercosporin to a reduced state and localization of this reduced form in the aqueous compartment of the cell, thus decreasing intracellular  $^1\text{O}_2$  production to levels that can be tolerated by the fungus. In addition, we have, for the first time, directly detected  $^1\text{O}_2$  phosphorescence from fungal culture, either stained with the photosensitizer rose bengal or actively synthesizing cercosporin, demonstrating  $^1\text{O}_2$  production *in vivo* and from cercosporin in culture.

## INTRODUCTION

The plant pathogenic fungus, *Cercospora*, produces the perylenequinone photosensitizer cercosporin, a toxin that plays an important role in the ability of this pathogen to parasitize plants (1,2). Upon activation by light, cercosporin generates singlet oxygen ( $^1\text{O}_2$ )<sup>†</sup>, leading to peroxidation of host plant membrane lipids and breakdown of the host plasma membrane (3–5). Nutrients leaking from host cells provide the pathogen, whose colonization is limited to the leaf intercellular spaces, with the nutrients required for growth and sporulation in the host. *Cercospora* species are highly successful plant pathogens, causing disease worldwide on a wide range of plant species. Unlike most plant diseases, naturally occurring resistance to these fungi is rare in cultivated crops. We hypothesize that these pathogens are successful because they have evolved a uniquely effective pathogenesis mechanism. All plants require light for growth, and the generalized toxicity of  $^1\text{O}_2$  provides the fungus with the means to parasitize multiple host species. There is a report that *Cercospora* can also cause skin lesions in humans (6).

Cercosporin is a potent producer of  $^1\text{O}_2$  with a high  $^1\text{O}_2$  quantum yield (0.81) (7). In addition to plants, cercosporin toxicity has been demonstrated against mice, bacteria and many fungi (8,9). There has only been one report of plant resistance to cercosporin (10). In this case, resistance of a wild rice species was attributed to the presence of carotenoids and the lack of uptake of cercosporin by the plant cells. With this single exception, all other cases of cercosporin resistance are in fungi, both *Cercospora* species and fungi in other genera reported to produce similar perylenequinone toxins (9). Whereas plant cells are killed by less than micromolar concentrations of cercosporin (1), *Cercospora* fungi produce millimolar concentrations in culture in the light and tolerate its effects (11). This extreme level of resistance to a potent  $^1\text{O}_2$ -generating photosensitizer is unusual and

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<sup>†</sup>Abbreviations: HAC, hexaacetyl-dihydrocercosporin;  $^1\text{O}_2$ , singlet oxygen; PDB, potato dextrose broth; SDS, sodium dodecyl sulfate.

provides an excellent system for defining cellular resistance to photosensitizers.

Previous work correlated the resistance of *Cercospora* species to cercosporin with cellular reducing ability and the ability of the fungus to maintain cercosporin in a chemically reduced state (12–14). Estimates of fungal cell reducing potential were obtained by assaying reduction of tetrazolium dyes of differing redox potential (14). Resistant fungi had greater ability to reduce dyes than did sensitive fungi. Cyclic voltammetry experiments showed the formal redox potential of cercosporin to be  $-0.16$  V/SHE at pH 7.1 (12), a value well within the reduction capability of living cells. Also, fluorescence microscopy studies using filters specific for fluorescence of cercosporin and reduced cercosporin correlated fungal resistance with the presence of reduced cercosporin within hyphae, although crystals of nonreduced cercosporin are readily visible outside of the cells (13). Based on these studies, a model was proposed (14) that fungi resist cercosporin by maintaining it in a reduced form; cercosporin excreted by the fungus was proposed to oxidize spontaneously to the photoactive form toxic to the host plants.

As part of these studies, the photoactivity and toxicity of reduced derivatives were also investigated (15). Cercosporin is reduced by the addition of dithionite or zinc dust. Cercosporin spontaneously reoxidizes when extracted away from reducing agents, but a stable reduced acetylated derivative (hexaacetyl-dihydrocercosporin, HAC) was synthesized and tested for  $^1\text{O}_2$  yields and phototoxicity. Light absorption by the HAC was found to be less than half of that by cercosporin. In addition, HAC was significantly less efficient in  $^1\text{O}_2$  production. Per quantum of light absorbed, the HAC produced only 17% of the  $^1\text{O}_2$  of cercosporin. Phototoxicity of HAC was assayed both by a lipid peroxidation test and by inhibition of growth of sensitive fungi. The HAC was approximately 60% less active in the lipid peroxidation assay but showed only a 20% reduction in toxicity to sensitive fungi.

The purpose of this report is to investigate further the importance of cercosporin reduction as a possible mediator of cellular resistance. The strong correlation between resistance and cellular reducing activity and the absolute correlation between resistance and the presence of reduced cercosporin in hyphae argue strongly for reduction as an important defense mechanism. However, although significantly reduced  $^1\text{O}_2$  production by the HAC was noted, the HAC still produces  $^1\text{O}_2$  and shows toxicity to sensitive organisms. Here we show that the production of  $^1\text{O}_2$  by HAC is strongly solvent dependent and that cellular localization of reduced cercosporin may be critical in resistance. In addition, we demonstrate spectrally for the first time,  $^1\text{O}_2$  production by cercosporin in fungal culture.

## MATERIALS AND METHODS

**Materials.** Cercosporin was isolated and purified from cultures of *Cercospora kikuchii* (strain PR) as previously described (1). Hexaacetyl-dihydrocercosporin was prepared by the reductive acetylation of cercosporin as described (15). Rose bengal, perinaphthenone, acetonitrile, ethylene carbonate, propylene carbonate, 1,4-dioxane and ethanol were purchased from Aldrich Chemical Co., Milwaukee, WI. Sodium dodecyl sulfate (SDS) was from Schwarz/Mann Biotech, Cleveland, OH. Deuterated water was purchased from Cambridge Isotope Laboratories, Andover, MD.

**Fungal strains and culture conditions.** *Cercospora nicotianae* strain ATCC 18366 was maintained on solid malt medium (16) at 24°C in the dark. Inoculum for both the *in vivo*  $^1\text{O}_2$  emission studies and confocal studies was produced by harvesting 4–5 mm agar plugs from the margins of 2 week-old mycelial cultures and grinding in water (6 plugs/5 mL) with glass beads.

**Fluorescence and absorption spectra of cercosporin and HAC.** Absorption spectra were measured using a Hewlett Packard 8452A diode array spectrophotometer. Fluorescence spectra were recorded on an SLM SPC 823-SMC 220 spectrofluorometer (SLM Instruments, Urbana, IL). The spectra were corrected and normalized for the same number of absorbed photons. Relative fluorescence values were calculated assuming that fluorescence intensity in  $\text{D}_2\text{O}$  was equal to 1.

**Quantum yields of  $^1\text{O}_2$  production for cercosporin and HAC.** Quantum yields of  $^1\text{O}_2$  generation were calculated from phosphorescence spectra obtained using a steady-state  $^1\text{O}_2$ -spectrophotometer (17). Samples were excited using a 200 W mercury lamp, with the light filtered through a 436 nm interference filter. The spectra were normalized to the same number of absorbed photons using the Lambert–Beer law. Quantum yields of  $^1\text{O}_2$  production were calculated relative to the perinaphthenone (phenalene) standard (18), as described (19).

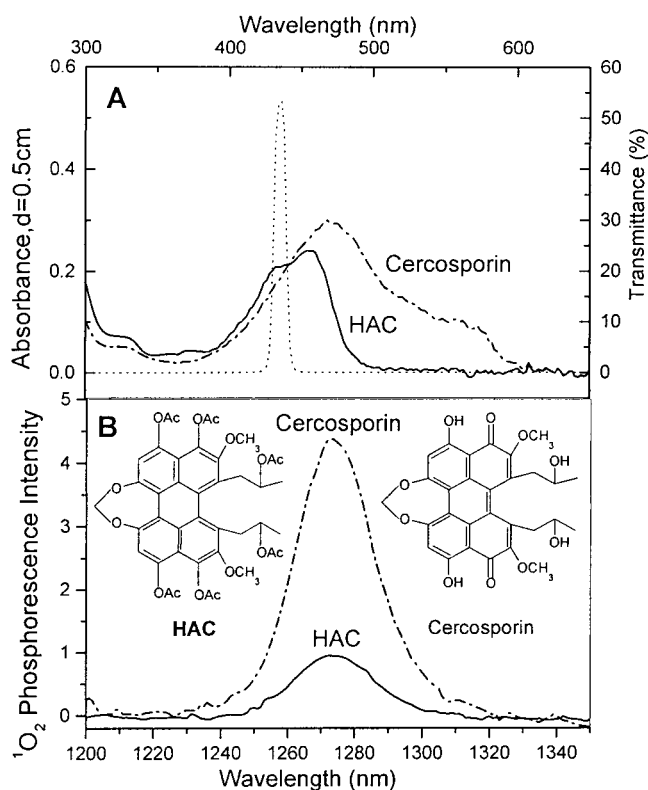
**Confocal microscopy.** For confocal microscopy, mycelial inoculum produced as described above was added to flasks of potato dextrose broth (PDB, Difco) (3 mL/50 mL). Cultures were incubated in the dark at room temperature on a rotary shaker for 36 h. Cercosporin (10  $\mu\text{M}$  final concentration) was then added to the cultures, and the cultures were incubated for an additional 4 h before being viewed on a Zeiss LSM 410 UV laser scanning confocal microscope equipped with a C Apo 40 $\times$  1.25 numeric aperture water immersion lens (20). The sample was excited with the 488 nm line of a krypton–argon mixed gas laser (Omnichrome, Inc.), and fluorescence emission was collected using a 515–565 nm bandpass filter with a pinhole setting of 8 and a resolution of 1  $\mu\text{m}$  for the cross-sectional  $z$ -scan.

***In vivo* emission of  $^1\text{O}_2$  phosphorescence from *Cercospora nicotianae*.** Assay of *in vivo*  $^1\text{O}_2$  production utilized methods developed for  $^1\text{O}_2$  emission from keratinocytes (17). Sterile plastic coverslips (Nalge Nunc International, Naperville, IL) were placed in 60 mm petri dishes to which 4 mL medium (minimal, malt or PDB) and 100  $\mu\text{L}$  of macerated mycelial inoculum was added. Cultures were incubated in a 24°C growth chamber and illuminated with constant fluorescent light (30  $\mu\text{E m}^{-2} \text{s}^{-1}$ ) for 4–5 days, until a confluent mat of hyphae had grown across the surface of the coverslip. Spectra were initially obtained from fungal cultures treated with rose bengal as described (17). Measurement of  $^1\text{O}_2$  in the presence of cercosporin was obtained from cultures endogenously producing cercosporin. The coverslips with fungal mycelium were washed with a 10% dimethylsulfoxide solution to remove external rose bengal or cercosporin. For assay, the coverslips with mycelium were fitted diagonally into a 1 cm quartz cuvette, and assayed with the  $^1\text{O}_2$  steady-state spectrophotometer, using a 418 nm cutoff filter combination (17). For determination of quenching activity of the fungal culture medium, different volumes of culture medium were mixed with aqueous rose bengal solutions (40  $\mu\text{M}$  final rose bengal concentration). Samples were excited using a 475 nm cutoff filter combination, and phosphorescence intensity was recorded with the  $^1\text{O}_2$  steady-state spectrophotometer as a function of medium concentration.

## RESULTS

### Production of $^1\text{O}_2$ by HAC and cercosporin in different solvents

We measured the solvent effect on  $^1\text{O}_2$  production by HAC and cercosporin. Both these compounds absorb at 436 nm, a wavelength transmitted by a selected interference filter, allowing easy and accurate comparison between cercosporin and HAC (Fig. 1A). As expected, cercosporin produced much stronger  $^1\text{O}_2$  phosphorescence than HAC in ethanol (Fig. 1B). The same experiment was performed with the two compounds in different organic and aqueous solutions, and



**Figure 1.** Measurement of <sup>1</sup>O<sub>2</sub> production from cercosporin and HAC. (A) Absorption spectra from cercosporin and HAC in ethanol, and the transmission of the 436 nm interference filter used for excitation (.....). (B) <sup>1</sup>O<sub>2</sub> phosphorescence spectra from cercosporin and HAC in ethanol using conditions as shown in 1A.

the quantum yields of <sup>1</sup>O<sub>2</sub> relative to perinaphthenone were computed (Table 1). Solvent had little effect on <sup>1</sup>O<sub>2</sub> yields from cercosporin, with  $\phi_{SO}$  values ranging from 0.84 in D<sub>2</sub>O to 0.97 in dioxane. By contrast, the presence of water had a pronounced effect on <sup>1</sup>O<sub>2</sub> yields from HAC (Table 1). While the quantum yields in organic solvents ranged from 0.14 to 0.18, they decreased almost 10 times ( $\phi_{SO} = 0.02$ – $0.04$ ) in aqueous solution. These data clearly show that a simple aqueous environment is able to transform the otherwise mild HAC photosensitizer into a very weak <sup>1</sup>O<sub>2</sub> producer.

#### Fluorescence assay for the localization of cercosporin in fungal hyphae

The observation that different chemical environments strongly impact <sup>1</sup>O<sub>2</sub> yields from HAC prompted us to reinvestigate the localization of reduced cercosporin in fungal hyphae. Cercosporin and HAC absorb and fluoresce in different spectral regions that previously allowed their separate identification in fungal culture by fluorescence microscopy (13). These studies demonstrated that cercosporin, which is in its normal form external to hyphal cells, is reduced when localized within the cell. However, subcellular localization of reduced cercosporin was not determined. Because little information was available on HAC fluorescence properties, we first examined how different solvents may affect the position and intensity of fluorescence spectra. Although relative fluorescence yields of HAC increased almost two-fold in non-

**Table 1.** Photochemical properties of cercosporin and HAC: quantum yields\* of singlet oxygen ( $\phi_{SO}$ ) and relative fluorescence yields ( $\phi_F$ )

Solvent‡	HA-cercosporin†		Cercosporin	
	$\phi_{SO}$	$\phi_F$ §	$\phi_{SO}$	$\phi_F$
Acetonitrile	0.18	1.3	0.87	1.2
Ethanol	0.18	1.8	0.91	1.1
1,4-Dioxane	0.14	1.9	0.97	1.1
Carbonates	0.04	ND¶	ND¶	1.0
SDS (10 <sup>-1</sup> M)/D <sub>2</sub> O	0.03	1.2	0.86	1.0
D <sub>2</sub> O	0.02	1.0	0.84	1.0

\*Quantum yields were measured as described in the Materials and Methods and are relative to perinaphthenone.

†Hexaacetyl-dihydrocercosporin.

‡All solutions contained 5% acetonitrile.

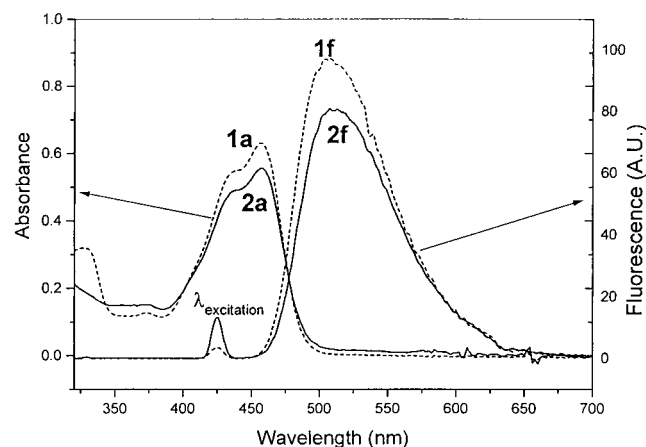
§The  $\phi_F$  values are relative to fluorescence in D<sub>2</sub>O solutions.

||Carbonates contained 95% ethylene carbonate 5% propylene carbonate.

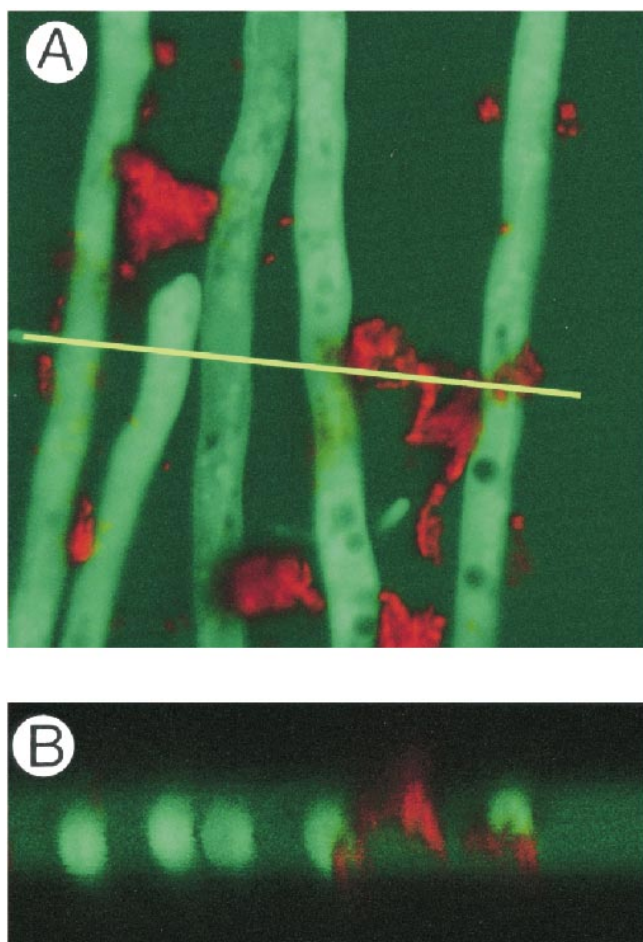
¶Not determined.

aqueous solvents as compared to water (Table 1), solvent had little effect on the fluorescence wavelength (Fig. 2). Thus, localization of reduced cercosporin in aqueous *versus* lipid domains of the cell cannot be determined by the wavelength of fluorescence emission. As with the HAC, solvent also had little effect on fluorescence emission from cercosporin (Table 1).

We thus chose to study localization of reduced cercosporin by laser confocal microscopy. Because the absorption and fluorescence emission spectra from HAC were not affected by solvent (Fig. 2), the same excitation/detection conditions could be used to detect reduced cercosporin in both aqueous and lipid compartments of the cell. Mycelial cultures were grown and incubated in the presence of cercosporin prior to viewing them on the confocal microscope. The samples were excited at 488 nm and emission collected using a 515–565 bandpass filter, allowing for visualization of both cercosporin and reduced cercosporin. In agreement with the previous studies (13), cercosporin localized within hy-



**Figure 2.** Absorbance and fluorescence spectra of HAC in aqueous and organic solvents. (1a) Absorption spectrum from HAC in dioxane. (2a) Absorption spectrum from HAC in 10<sup>-1</sup> M SDS in D<sub>2</sub>O. (1f, 2f) Fluorescence emission spectra from samples 1a and 2a, respectively, excited at 425 nm.

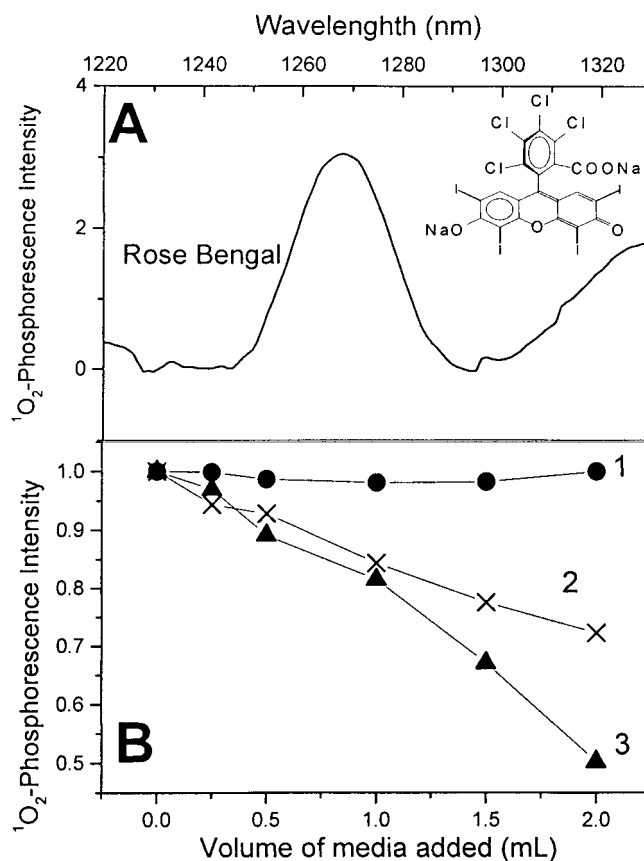


**Figure 3.** Confocal micrograph of hyphae of *C. nicotianae* in the presence of cercosporin. Conditions for microscopy are as described in the Materials and Methods. (A) View shows five hyphae with growing tips oriented to the top of the figure. Green fluorescence within hyphal cells indicates the presence of reduced cercosporin. Small dark (nonfluorescent) structures toward the hyphal tips are nuclei, and larger, distinctly nonfluorescent spots in the older part of the hyphal strands are vacuoles. Fluorescent red crystals external to the hyphae are nonreduced cercosporin. Line indicates the plane of the  $z$ -scan (cross section). (B) The  $z$ -scan (cross section) of the hyphae showing uniform green fluorescence indicative of reduced cercosporin in the cytoplasm of the cells.

phae was in a reduced form, as evidenced by green fluorescence, and cercosporin in crystals external to hyphal cells was not reduced (red fluorescence) (Fig. 3A). The pattern of green fluorescence in the hyphae suggested that the reduced cercosporin was localized in the cytoplasm and excluded from vacuoles and nuclei. The conclusion of hydrophilic localization is supported by the  $z$ -scan (cross-sectional view). The plane of the  $z$ -scan is shown in Fig. 3A (line), and the scan itself is in Fig. 3B. The  $z$ -scan shows a cross section of uniform green fluorescence across the hyphae, indicative of cytoplasmic localization.

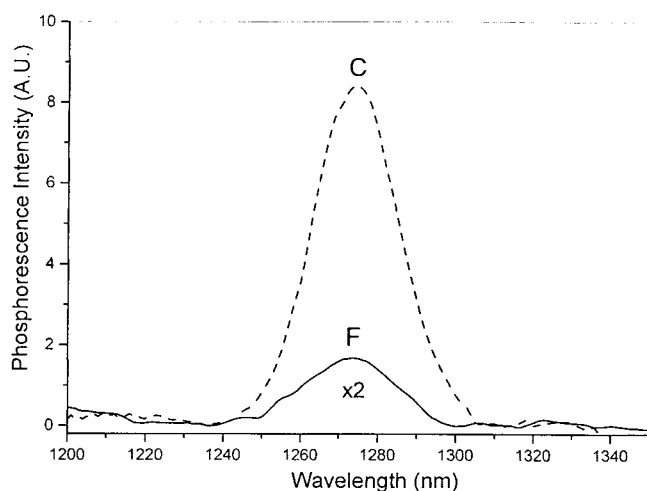
#### *In vivo* emission of $^1\text{O}_2$ from cultures of *C. nicotianae*

Singlet oxygen formation from cercosporin *in vitro* has been demonstrated in several studies (5,7,15,21), but production of  $^1\text{O}_2$  by cercosporin in fungal cells *in vivo* has never been



**Figure 4.** Optimization of the conditions for assay of *in vivo*  $^1\text{O}_2$  production from cercosporin in cells of *C. nicotianae*. (A) The  $^1\text{O}_2$  phosphorescence spectrum from *C. nicotianae* mycelium grown on coverslips in minimal medium and stained with rose bengal followed by washing. The  $^1\text{O}_2$  emission can be visualized from the cultures. (B) Inhibition of  $^1\text{O}_2$  phosphorescence from rose bengal solutions with the addition of fungal growth medium: (1) minimal medium, (2) PDB and (3) malt medium. The  $^1\text{O}_2$  phosphorescence signal was significantly decreased in the presence of PDB and malt medium, the two media that support cercosporin production by the fungus.

confirmed spectrally. Our previous success in detecting  $^1\text{O}_2$  in keratinocytes stained with rose bengal (17) prompted us to develop a similar system for assaying *in vivo*  $^1\text{O}_2$  production in cultures of *C. nicotianae* producing cercosporin. By testing different inoculation and culturing conditions, we developed a culture system that allowed for growth of a thin mycelial mat that adhered to plastic coverslips and could be mounted and viewed in the  $^1\text{O}_2$  spectrophotometer. The system was first tested following staining of the mycelium with rose bengal as described for the keratinocytes (17). Slides were treated with rose bengal and then washed thoroughly to remove extracellular rose bengal. Strong red fluorescence from the rose bengal-stained fungus was observed by fluorescence microscopy (not shown), indicating that the rose bengal was not in an aqueous environment, where it shows little fluorescence. This result is consistent with our previous studies with keratinocytes that indicated rose bengal accumulation in the nucleus and membranes (17), presumably due to formation of highly hydrophobic ion pairs, allowing rose bengal incorporation inside micelles and solubility in hexane (22,23). As shown in Fig. 4A, a strong  $^1\text{O}_2$  phos-



**Figure 5.** Production of  $^1\text{O}_2$  by cercosporin *in vivo* in contact with fungal hyphae. This figure shows the  $^1\text{O}_2$  phosphorescence spectrum from 25 mM cercosporin (C) in  $\text{D}_2\text{O}$  and from cercosporin from *C. nicotianae* mycelium grown in malt medium (F). The *in vivo* signal was multiplied by a factor of two for presentation.

phosphorescence signal could be obtained from the rose bengal-treated mycelial cultures.

For measurement of  $^1\text{O}_2$  production from cercosporin in fungal culture, we first investigated the effect of fungal nutrient medium composition on  $^1\text{O}_2$  phosphorescence *in vitro*, using rose bengal as the photosensitizer, and on production of cercosporin by the fungus. As shown in Fig. 4B,  $^1\text{O}_2$  phosphorescence from rose bengal was strongly inhibited when increasing concentrations of malt medium or PDB medium were added to rose bengal solutions. Minimal medium did not inhibit  $^1\text{O}_2$  phosphorescence. However, minimal medium also did not support production of cercosporin by the fungal cultures (data not shown). Thus, cultures endogenously producing cercosporin grown in malt and PDB were used for the *in vivo*  $^1\text{O}_2$  assays. The  $^1\text{O}_2$  phosphorescence from a cercosporin solution and from a mycelial culture of *C. nicotianae* grown in malt medium are shown in Fig. 5. In spite of the inhibitory effect of malt medium on  $^1\text{O}_2$  phosphorescence detection, a clear signal is detectable from the fungal culture. This is the first direct spectral evidence for  $^1\text{O}_2$  production by this fungus during endogenous cercosporin production.

## DISCUSSION

The studies reported here expand on previous work investigating  $^1\text{O}_2$  yields from cercosporin and also support our previous hypothesis that resistance of the producing fungus to cercosporin is strongly correlated with cellular reduction of cercosporin. Dobrowolski and Foote (7) first reported the quantum yield ( $\phi_{\text{SO}}$ ) of  $^1\text{O}_2$  from cercosporin in deuterated benzene to be 0.81. Here we obtained similar values for cercosporin, ranging from 0.84 to 0.97 in both organic and aqueous environments. These data confirm that cercosporin is a very strong generator of  $^1\text{O}_2$  and also demonstrate that production is independent of chemical environment, in contrast to HAC.

In previous work, we reported that reduced cercosporin was a weaker photosensitizer and was significantly less pho-

totoxic than cercosporin (15). Synthesis and analysis of a stable, reduced, acetylated derivative of cercosporin (HAC) indicated that chemical reduction decreased both light absorption and  $^1\text{O}_2$  production. Previous analysis of relative  $^1\text{O}_2$  yields by HAC in chloroform were 0.16–0.17, indicating that HAC is a moderate photosensitizer, with some toxicity to sensitive organisms. Here we expand on that work and present quantum yields for  $^1\text{O}_2$  production by HAC in several solvents, chosen to emulate different chemical environments in fungal cells. Our  $\phi_{\text{SO}}$  values in organic solvents such as acetonitrile or ethanol are 0.18, giving relative values of 0.20–0.21, similar to the relative values reported previously. However, in aqueous environment  $\phi_{\text{SO}}$  was about 0.02, which makes HAC a very weak photosensitizer, in contrast to cercosporin whose  $^1\text{O}_2$  quantum yield was little affected by solvent.

The demonstration that the HAC is a very poor generator of  $^1\text{O}_2$  in an aqueous environment helps to explain a previous puzzle in our studies of the basis of cercosporin resistance in *Cercospora* species and in other perylenequinone-resistant fungi. In earlier studies we correlated resistance to cercosporin in diverse fungal species to cell surface reducing ability and the ability of a fungus to maintain cercosporin in a reduced state (13,14). Although reduced cercosporin is attenuated in production of  $^1\text{O}_2$ , under the assay conditions used in the previous study (15) it still produced significant  $^1\text{O}_2$  and showed some toxicity to sensitive organisms. The results of our current study suggest that cellular localization may have an additional significant impact on reduced cercosporin efficacy as a photosensitizer, as reduced cercosporin localized in an aqueous cellular environment would be significantly less active than one localized in a lipid environment. Our confocal microscopy studies are consistent with the localization of reduced cercosporin in an aqueous environment, in the cytoplasm of the hyphal cells. Although we cannot exclude the possibility that some of the reduced cercosporin also localizes in membranes within the cell, the reduction of cercosporin and significant cytoplasmic localization would still result in a major decrease in  $^1\text{O}_2$  generation within the cell. Together, these results are consistent with our hypothesis that the ability of the producing fungus to reduce cercosporin is a major mechanism of detoxification and help explain the ability of these fungi to tolerate such high concentrations of this potent photosensitizer.

The importance of photosensitizer localization in the fungus is also supported by previous studies on toxicity of rose bengal to *Cercospora* fungi. Unlike cercosporin and most other photosensitizers that are not toxic to these fungi, rose bengal causes significant growth inhibition (13). We previously attributed this toxicity to differences in reduction potential. Using cyclic voltammetry, we reported the reduction potential of cercosporin and rose bengal to be  $-0.14$  V and  $-0.72$  V, respectively, *versus* the normal hydrogen electrode. Such a negative redox potential for rose bengal suggests that it is unlikely to be reduced by cells, a hypothesis that was supported by the bright red fluorescence emitted by fungal hyphae stained with rose bengal (13). Here we suggest that localization may provide an additional explanation. Despite its anionic form, rose bengal is a very hydrophobic compound (23), and it localizes predominantly in the hydrophobic cell regions such as the nucleus and membranes (17).

We hypothesize that the combined effects of chemical reduction of and localization to aqueous compartments of the cell combine to provide protection against cercosporin toxicity, and that neither operates in response to rose bengal.

Finally we also report spectral evidence of  $^1\text{O}_2$  production in fungal culture. All previous studies on  $^1\text{O}_2$  generation from cercosporin were done *in vitro* in various solvents (5,7,15,21). However, cercosporin is also capable of reacting *via* type I reactions if incubated in the presence of a reducing substrate such as methionine or urate (5,21). Thus, definitive evidence for  $^1\text{O}_2$  production *in vivo* was lacking. By optimizing an assay system previously used to detect  $^1\text{O}_2$  production by rose bengal in keratinocytes, we demonstrated for the first time the production of  $^1\text{O}_2$  by cercosporin produced in fungal culture. Thus, cercosporin does react *via* a type II mechanism when in contact with the fungus. It is not clear at this time whether the  $^1\text{O}_2$  produced is from reduced cercosporin inside fungal cells or from cercosporin external to the cells. Because reduced cercosporin present inside the hyphae is a poor  $^1\text{O}_2$  photosensitizer, it is unlikely that it would produce enough  $^1\text{O}_2$  for spectral detection. However, our experiments with rose bengal showed that  $^1\text{O}_2$  can be detected inside the fungus.

Whether or not the  $^1\text{O}_2$  detected from culture originates from outside or inside the cell, our results demonstrate that the fungal cell is exposed to  $^1\text{O}_2$  when producing cercosporin. Thus, in addition to reduction of internal cercosporin, cellular defenses targeted against  $^1\text{O}_2$  must be present and active in the cells for full expression of resistance by the fungus. Currently, we are investigating mutants that are sensitive to cercosporin and are characterizing genes involved in cercosporin resistance (24–27). These studies have suggested a role for cellular pyridoxine in  $^1\text{O}_2$  quenching and in cercosporin resistance and are helping to define the complex mechanisms used by *Cercospora* fungi for cercosporin resistance.

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