The Role of Carotenoids in Resistance of Fungi to Cercosporin

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ABSTRACT

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Cercospora spp. produce large quantities of the singlet-oxygen-generating toxin cercosporin and are not harmed by it. We investigated carotenoid production by these fungi to determine if carotenoids (which quench singlet oxygen) play a role in resistance. *Cercospora* spp. produce high concentrations of β-carotene at early stages of the growth cycle, up to 12 μg/g dry weight. Three cercosporin-sensitive fungi differed in carotenoid production. *Phytophthora cinnamomi* and *Phytophthora parasitica* produced virtually no carotenoids, whereas *Neurospora crassa* produced carotenoids in amounts comparable to those found in *C. nicotianae*. Carotenoid-minus mutants of *N. crassa* and *Phycomyces blakesleeanus* were considerably more sensitive to cercosporin than carotenoid-producing strains. However, there was not a linear relationship between the amount of carotenoids produced by these strains and sensitivity to cercosporin. Three carotenoid inhibitors, norflurazon, mevinolin, and β-ionone, did not inhibit synthesis of carotenoids of *C. nicotianae*, and only low levels of inhibition were obtained with diphenylamine, whereas diphenylamine, norflurazon, and mevinolin effectively inhibited carotenoid synthesis in *N. crassa*. Equivalent amounts of carotenoids were isolated from protoplasts of *N. crassa* and *C. nicotianae*, suggesting that, in *C. nicotianae*, as in *N. crassa*, carotenoids are localized within the protoplast. Our data support the hypothesis that carotenoids play a role in resistance of fungi to cercosporin, but that they are not the only or even major mechanism of cercosporin resistance. This result was unexpected based on previous studies of photosensitizer resistance mechanisms in living organisms.

Additional keyword: carotenoid-synthesis inhibitors.

Cercosporin is a photosensitizing toxin, produced by members of the genus *Cercospora*, which is hypothesized to be involved in the ability of these fungi to parasitize plants (7). Cercosporin generates singlet oxygen and superoxide when irradiated by light (11,13). These oxygen species in turn cause peroxidation of plant membrane lipids leading to changes in membrane fluidity and structure and death of the cell (8,10). Singlet oxygen and superoxide are very generalized toxic agents, causing the oxidation of many cellular components including proteins, carbohydrates, and nucleic acids as well as lipids (31). As would be expected, cercosporin shows generalized toxicity to many organisms. All plants that have been tested are sensitive to cercosporin as are mice and bacteria (2,14,23,38). *Cercospora* species, however, produce high concentrations of cercosporin in the light and apparently are unaffected by it. We recently have found that a number of other fungal species also are resistant even though there is no evidence that they produce similar toxins (9). The resistance of a number of fungi to cercosporin is surprising, considering the generalized toxicity of singlet oxygen and superoxide.

We have been attempting to determine the mechanisms by which *Cercospora* species resist damage by cercosporin. In a previous study, resistant and sensitive fungi were shown not to differ in levels of superoxide dismutase, and no clear correlation could be made between resistance and levels of other oxidative enzymes or of antioxidants (9). The purpose of this study was to investigate the possible role of carotenoids in resistance. Unsaturated, colored carotenoids are potent quenchers of singlet oxygen and of triplet states of photosensitizers (the activated state that transfers energy or electrons to oxygen), and have been shown to be involved in resistance of many organisms to chlorophyll or other photosensitizers (15,16,22). Many fungi produce high levels of carotenoids (17,28,35), but there have been no reports of carotenoid production by *Cercospora* species. In this study we investigated carotenoid production by *Cercospora* species, possible correlations between carotenoid production and resistance and sensitivity of fungal isolates to cercosporin, and the effects of carotenoid inhibitors on resistance of *Cercospora* species to cercosporin. A preliminary report has been published (12).

MATERIALS AND METHODS

Cercosporin. Cercosporin was isolated and purified from cultures of *Cercospora beticola* Sacc. (ATCC #24080) as previously described (7). Stock solutions were prepared in acetone and stored at −20 C in the dark. Final acetone concentrations in all experiments did not exceed 0.5%, a concentration that does not affect fungal growth.

Fungal isolates. The fungal isolates used and their sources are as follows: *Cercospora nicotianae* Ell. & Ev. (ATCC #18366); *Phycomyces blakesleeanus* Burgeff 'Super Producer' and 'Albino' (R. Sutter, West Virginia University, Morgantown); *Neurospora crassa* Shear & Dodge ORS-6a (wild type), 3622 (al-1), 914 (al-2), and 4073 (al-3) (Fungal Genetics Stock Center, University of Kansas, Kansas City); *Phytophthora cinnamomi* Rands; and *Phytophthora parasitica* Dast. (H. D. Shew, North Carolina State University, Raleigh).

Fungal growth. For growth and carotenoid studies, cultures were grown in liquid malt medium (1.5% Difco malt extract, 0.3% Difco peptone, 3% glucose) on a shaker at 150 rpm under continuous light (3 $\mu E \cdot m^{-2} sec^{-1}$). All fungal isolates produced mycelium under these conditions but did not sporulate. The isolate of *C. nicotianae* used in these studies also did not produce cercosporin under these conditions but still expressed normal levels of resistance. Cultures were started by placing five plugs from the edge of a mycelial colony growing on malt agar (malt medium + 1.5% agar) into a 125-ml flask containing 50 ml of medium. Cultures were harvested by filtering them through filter paper in a Büchner funnel. For determination of growth, four separate cultures were filtered individually onto preweighed filter paper, dried in an oven at 65 C for 3 days, and weighed. Dry-weight values reported are the mean of four cultures.

Carotenoid extraction and identification. Carotenoids were extracted by the methods of Tyczkowski and Hamilton (33). This

method is simple, rapid, and sensitive, and allows for analysis of large numbers of samples and small amounts of tissue. The fungus was filtered, and 1 g fresh weight of mycelium was ground for 20 sec in a Sorvall Omnimixer in 30 ml of hexane:acetone:toluene:absolute ethanol (10:7:7:6, v/v/v/v). The fungus-solvent mixture was allowed to incubate overnight at room temperature in the dark. The mixture was saponified by adding 1 ml of 40% KOH in methanol and incubating for 1 hr in the dark. An additional 30 ml of hexane was then added, and the solvent mixture was partitioned against 10% Na_2SO_4 (30 ml). The hexane layer (containing the carotenoids) was removed and evaporated to dryness under N_2. The crude carotenoid extract then was dissolved in 500 μl of iso-octane:methanol:ethyl acetate (96:2:2, v/v/v) and stored frozen until analyzed.

The carotenoids were separated and quantified by high-performance liquid chromatography (HPLC). Carotenoid extracts from *Cercospora* and *Phycomyces* were separated by a modification of the methods of Tyczkowski and Hamilton (33). The extracts were chromatographed on a Waters 5μ Radial-Pak silica gel column with a mobile phase consisting of iso-octane:isopropanol:methanol (80:15:5, v/v/v) at a flow rate of 1.5 ml/min. The multiple carotenoids of *Neurospora* could not be resolved by this procedure and thus were separated by a modification of the procedure of Will and Ruddat (37). Carotenoids were chromatographed on a Waters Radial-Pak C_{18} column with a mobile phase consisting of 2-propanol and acetonitrile:water (9:1, v/v) delivered in a gradient from 30:70 to 75:25 at a flow rate of 2.5 ml/min. Extracts from *Cercospora* also were separated by this method, and the results were identical to those obtained with the more rapid Tyczkowski and Hamilton method.

Carotenoids were detected at 280 and 436 nm and were identified both by co-chromatography with authentic standards and by the absorption spectrum of collected peaks. Carotenoid concentration was computed on the basis of mycelial dry weight. When harvesting cultures for carotenoid extraction, equal amounts of additional cultures were harvested and used for a dry-weight determination.

Carotenoid mutants. Two mutants of *P. blakesleeanus*, an albino mutant and a carotenoid overproducer (Super Producer), and a wild type isolate and three albino mutants of *N. crassa* were tested for differences in sensitivity to cercosporin. The three albino mutants of *N. crassa* were blocked at three different loci: al-1, al-2, and al-3 (25). An agar plug from the margin of a mycelial culture was plated mycelial side down on a plate of malt agar or malt agar containing 1 μM or 10 μM cercosporin. Plates were incubated in the light (80 $\mu E \cdot m^{-2} sec^{-1}$), and radial growth of the fungal colony from the edge of the agar plug was measured in millimeters with a ruler. Percent inhibition was calculated from the ratio of growth on cercosporin-containing medium as compared to growth on medium lacking cercosporin. All isolates also were tested for growth inhibition by cercosporin in the dark; none were inhibited by cercosporin in the dark.

Carotenoid inhibition. Four inhibitors were used to block carotenoid production: diphenylamine (DPA) and β-ionone (Sigma Chemical Co., St. Louis, MO), the herbicide norflurazon (kindly provided by M. Christianson, Zoecon Industries, Inc., Dallas, TX), and the 3-hydroxy-3- methylglutaryl -CoA (HMG-CoA) reductase inhibitor mevinolin (kindly provided by S. B. Tove, North Carolina State University, Raleigh). Mevinolin was obtained in the lactone form and was converted to the sodium salt by heating in 0.1 N NaOH (0.48 mg/ml) at 50 C for 2 hr (6). The solution was then neutralized with HCl and stored frozen in aliquots until used. DPA, β-ionone, and norflurazon were all dissolved in ethanol before being added to the medium. Final ethanol concentration in all experiments equaled 0.03%; this concentration did not affect fungal growth. DPA and mevinolin were used at concentrations of 100 μM; norflurazon and β-ionone were used at concentrations of 200 μM. Concentrations above these levels caused severe growth reduction of the fungal cultures. Because mevinolin also inhibits sterol synthesis, cholesterol (5 μg/ml) (27) was added to medium along with mevinolin. The cholesterol was dissolved in acetone; final acetone concentration equaled 0.2%.

DPA-cercosporin interactions. *C. nicotianae* was inoculated into flasks of malt medium or malt medium containing either 100 or 200 μM DPA. At various times (0 time, 1, 2, and 3 days), 10 μM cercosporin was added to cultures of all three treatments, and the cultures were incubated under lights (80 $\mu E \cdot m^{-2} sec^{-1}$) for 24 hr. Control cultures were treated with acetone and also incubated in the light. After the 24-hr light treatment, the cultures were incubated in the dark for an additional 24 (no DPA treatment) or 48 hr (100 and 200 μM DPA treatments). The cultures then were harvested and the mycelial dry weight was measured.

To test the effect of cercosporin on DPA, cercosporin (10 μM) and DPA (100 μM) were mixed with water and either were incubated in light (80 $\mu E \cdot m^{-1} sec^{-1}$) for 24 hr, incubated in the dark for 24 hr, or stored at −20 C. Solutions of DPA (100 μM) without cercosporin also were incubated in the light for 24 hr or stored frozen. The amount of DPA present in each sample was determined with HPLC with the conditions used for separating carotenoids of *Cercospora* and monitoring with a 280-nm filter.

Carotenoid extraction from protoplasts. Conidia were harvested from 7-day-old cultures of *N. crassa* grown on the basic medium of Sorger and Giles (30) (NMS). Conidia were incubated with shaking in liquid NMS (10^8 conidia/50 ml) for 16 hr. Protoplasts were isolated by the methods of Quigley et al (26), except that 10 μl/ml of β-glucuronidase was added to the enzyme mixture. Mycelium of *C. nicotianae* was grown in potato-dextrose broth as described (19). Mycelium was digested (2 g/40 ml) in an enzyme mixture containing 5 mg/ml Novozyme and 10 μl/ml β-glucuronidase in 50 mM sodium phosphate buffer (pH 5.8) with 10 mM $CaCl_2$ and 0.7 M NaCl. With both *N. crassa* and *C. nicotianae*, part of the mycelium was extracted for carotenoids (total mycelium) and the remainder was used for protoplast isolation. After protoplast isolation, the protoplast enzyme solution was filtered through cheesecloth and glass wool. The residue remaining in the filter was extracted for carotenoids (undigested mycelium). Protoplasts were separated from the enzyme solution by centrifuging at 2,500 rpm for 2 min. Total numbers of protoplasts were counted using a hemacytometer before extracting the protoplasts for carotenoids. The enzyme solution was dried in a rotary evaporator before carotenoid extraction. Enzymes used in protoplast isolation also were extracted for carotenoids as a control.

RESULTS

Carotenoid production. HPLC separation of extracts from *C. nicotianae* resulted in one major carotenoid peak which was identified as β-carotene based on co-chromatography with an authentic standard and the absorption spectrum of the collected peak. β-Carotene production was highest at an early stage of growth and then declined (Table 1). The wild type isolate of *N. crassa*, which is sensitive to cercosporin, produced comparable amounts of carotenoids—approximately 12 μg of carotenoids/g dry weight (24-hr culture). HPLC analysis of carotenoids of *N. crassa* resulted in four major and one minor peak. The minor peak was identified as β-carotene; three of the four major peaks were tentatively identified as neurosporene, γ-carotene, and ζ-carotene. By contrast, two other cercosporin-sensitive fungi, *P. parasitica* and *P. cinnamomi*, produced almost no carotenoids: 23 and 21 ng carotenoids/g mycelium dry weight, respectively. Isolates of two other species of *Cercospora*, *C. beticola* and *C. zeae-maydis*, also

TABLE 1. β-carotene production by *Cercospora nicotianae*

Age of culture (days)	Microgram β-carotene per gram dry weight
2	11.9 ± 1.3[a]
3	12.0 ± 3.5
4	9.9 ± 2.4
5	6.0 ± 2.9

[a] Standard error of the mean.

were shown to produce β-carotene.

Carotenoid mutants. Carotenoid-minus mutants are not available in *Cercospora* spp. *Cercospora* spp. do not accumulate carotenoids in their spores. Thus mutant isolation cannot be done visually and would require extraction and analysis of each mutant. We thus investigated differences in cercosporin resistance among mutants of *N. crassa* and *P. blakesleeanus*. Large differences in carotenoid content were found between carotenoid-producing and albino isolates of *N. crassa* and *P. blakesleeanus* (Table 2). Albino mutants of both fungi were significantly more sensitive to cercosporin than were carotenoid-producing isolates (Table 2).

Inhibition of carotenoids. We were unsuccessful in blocking carotenoid synthesis in *C. nicotianae* with any of the four carotenoid synthesis inhibitors (Table 3). DPA was the only inhibitor that reproducibly reduced carotenoid levels; maximum inhibition by DPA was in the range of 50–60%. Norflurazon consistently stimulated carotenoid production, whereas mevinolin and β-ionone had no consistent effects. By contrast, we had no difficulty inhibiting carotenoid synthesis of *N. crassa* with three of the same inhibitors (Table 3). We obtained more than 90% inhibition with DPA and norflurazon and more than 65% inhibition with mevinolin. The differences in effectiveness of the inhibitors do not appear to be due to lack of uptake of the inhibitors by *C. nicotianae*. All four inhibitors had substantial growth inhibition effects on cultures of both fungi (Fig. 1). The patterns of growth inhibition differed slightly; DPA was more inhibitory to *C. nicotianae* than to *N. crassa*, whereas β-ionone was more inhibitory to *N. crassa* (at least initially). However, by the termination of the experiment (after 2 days' growth for *N. crassa* and 5 days' growth for *C. nicotianae*), the final percent inhibition of growth caused by each of the inhibitors was similar for the two fungi. The only exception was with mevinolin which caused greater inhibition of *N. crassa*.

DPA-cercosporin interactions. Because treatment with 100 μM DPA caused a 50–60% inhibition of carotenoid production by *C. nicotianae*, we attempted to determine what effect this level of inhibition would have on resistance of *C. nicotianae* to cercosporin. Cultures without DPA and with 100 and 200 μM DPA were treated with cercosporin in the light at various times in the growth cycle, and then the final dry weights of these cultures were compared with those of cultures not treated with cercosporin. Results are shown in Figure 2. In cultures without DPA, growth was slightly inhibited overall when cercosporin was added (85–100% of growth of the non-cercosporin-treated control). These data are typical for cultures of *Cercospora* spp., where we routinely see a 5–10% reduction in growth due to the addition of 10 μM cercosporin under the conditions used here. If carotenoid inhibition increased the sensitivity of *C. nicotianae* to cercosporin, we would expect to see an increased inhibition of growth in cercosporin-treated cultures as compared with non-cercosporin-treated cultures in the presence of DPA. The exact opposite occurred. There was a consistent stimulation of fungal growth following cercosporin treatment in cultures containing DPA. This stimulation was most evident with 200 μM DPA.

TABLE 2. Reaction of carotenoid mutants of *Neurospora crassa* and *Phycomyces blakesleeanus* to cercosporin

Isolate	Carotenoid content[a] (μg/g dry wt.)	Growth (% of control)[b] 1 μM[c]	10 μM[c]
N. crassa			
Wild type	11.73	38 ± 3[d]	22 ± 4
al-1	0.39	26 ± 2	10 ± 1
al-2	0.02	19 ± 5	7 ± 3
al-3	0.29	17 ± 5	7 ± 2
P. blakesleeanus			
Super Producer	349.2	86 ± 12	42 ± 10
Albino	0.0314	38 ± 2	10 ± 4

[a] Carotenoid content of *N. crassa* = sum of all carotenoid peaks detected at 436 nm. Carotenoid content of *P. blakesleeanus* = β-carotene, the major carotenoid present. Values reported are from 24-hr cultures of isolates of *N. crassa* and 3-day-old cultures of isolates of *P. blakesleeanus*.
[b] Colony radius at 16 hr for *N. crassa* and 24 hr for *P. blakesleeanus*.
[c] Cercosporin concentration.
[d] Standard error of the mean.

TABLE 3. Inhibition of carotenoid production of *Cercospora nicotianae* and *Neurospora crassa* by inhibitors of carotenoid synthesis

	Percent inhibition			
	Diphenylamine (100 μM)	Mevinolin (100 μM)	Norflurazon (200 μM)	β-ionone (200 μM)
C. nicotianae				
Day 3	60	(−7)[a]	(−94)	4
Day 4	53	34	(−69)	(−16)
Day 5	55	17	(−36)	31
N. crassa				
Day 1	60	43	0	No growth
Day 2	92	66	93	(−14)

[a] (−) denotes stimulation.

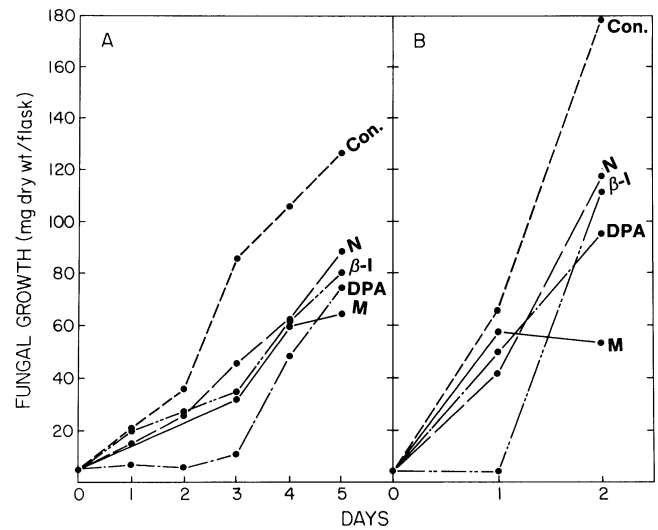

Fig. 1. Growth of **A,** *Cercospora nicotianae* and **B,** *Neurospora crassa* in the presence of carotenoid synthesis inhibitors norflurazon (N), mevinolin (M), β-ionone (β-I), diphenylamine (DPA), control (no inhibitor) (Con.). DPA and mevinolin were added at 100 μM; norflurazon and β-ionone were added at 200 μM.

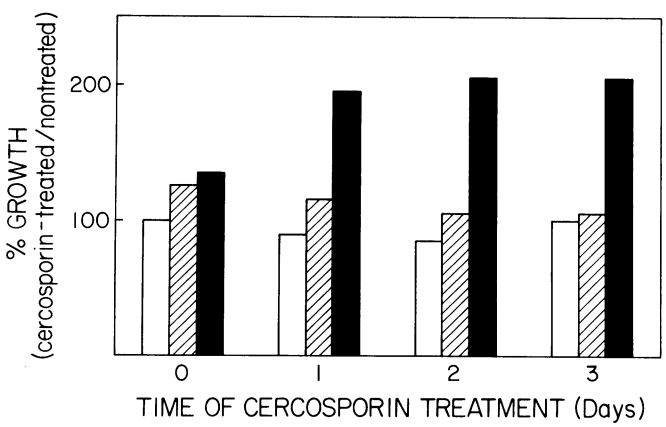

Fig. 2. Percent growth of cercosporin-treated cultures as compared with untreated cultures of *Cercospora nicotianae* in the presence or absence of diphenylamine (DPA). Cultures were treated in the light (80 $\mu E \cdot m^{-2} sec^{-1}$) with 10 μM cercosporin immediately (0 time) or after 1, 2, and 3 days of growth. Percent growth was determined by the dry weight of cultures 2 days (no DPA) or 3 days (with DPA) after cercosporin treatment. Cultures grown with no DPA (open bars) or with 100 μM (shaded bars) or 200 μM (solid bars) DPA.

To try to understand the basis for growth stimulation in the presence of DPA following cercosporin treatment, we looked at the effects of cercosporin on DPA itself. Exposure of a 100 μM solution of DPA to light for 24 hr or incubation with cercosporin in the dark for 24 hr did not cause any reduction in the levels of DPA as determined by HPLC analysis. Incubation of DPA with 10 μM cercosporin in the light for 24 hr, however, reduced the amount of DPA in the solution by 41%, thus indicating that DPA is very effectively degraded by cercosporin in the light. Because DPA has a marked growth inhibition effect on *C. nicotianae* (see Fig. 1), degradation of DPA by cercosporin presumably led to a reversal of this growth inhibition, resulting in the stimulation in growth seen in the previous experiment.

Carotenoid localization. In a previous study, we observed that protoplasts of *C. nicotianae* and *N. crassa* show an identical, sensitive response to cercosporin, and that differential resistance to cercosporin is only expressed as the protoplasts regenerate cell walls (19). Thus, it was important to determine if carotenoids of *C. nicotianae* are localized within the protoplast as is true for *N. crassa* (28), or if some or all of the carotenoids of *Cercospora* could be found in the cell wall. Protoplasts of *N. crassa* were isolated from germinating conidia (16 hr). Protoplasts of *C. nicotianae* were isolated from 24-hr-old mycelium started from mycelial fragments obtained by grinding mycelium in a blender. In both cases, part of the cultures was extracted for carotenoids (total mycelium) and the rest was used for protoplast isolation. Results are shown in Table 4. Because cultures were grown under light, nutrient, and inoculum conditions that favored protoplast release, carotenoid content of total mycelium is not comparable to earlier studies and values from the two fungi are not necessarily comparable to each other, although values were quite similar. Under the conditions used, cultures of both fungi produced high amounts of carotenoids. Similar numbers of protoplasts were released per gram of tissue by the two fungi, and the same proportion of the total carotenoids was present in the protoplasts and in the undigested mycelium. If computed on the basis of total mycelium dry weight, we get values of 8.63×10^{-8} and 8.70×10^{-8} μg of carotenoids per protoplast for *N. crassa* and *C. nicotianae*, respectively. Thus, we conclude that, in *C. nicotianae*, as in *N. crassa*, carotenoids are localized within the protoplast. Small amounts of β-carotene were detected in the enzyme solution (where cell wall components are solubilized) following protoplast release with *C. nicotianae*. No carotenoids were detected with *N. crassa*. Considerable debris was present in the enzyme solution following digestion of mycelium of *C. nicotianae*. This did not occur with *N. crassa*. If the enzyme solution was filtered through a 4.5-μm filter before carotenoid extraction, no carotenoids were detected. Thus, we conclude that the small amount of carotenoids detected in the enzyme solution of *C. nicotianae* was due to mycelial debris.

DISCUSSION

Data presented in this paper support the hypothesis that carotenoids play a role in resistance of fungi to cercosporin. Mycelial cultures of *C. nicotianae* produce β-carotene at a level of approximately 12 μg/g dry weight (under the growth conditions used in this study) which is a value well within the range of the 0–30 μg of carotenoids/g dry weight generally reported for fungi (35). Two oomycetes, *P. parasitica* and *P. cinnamomi*, are very sensitive to cercosporin (100% growth inhibition by 10 μM cercosporin [9]) and produce little if any carotenoids. Further, albino mutants of *N. crassa* and *P. blakesleeanus* are significantly more sensitive to cercosporin than are carotenoid-producing isolates. These last data support results of previous studies with conidia of *N. crassa* and vegetative cultures of *Ustilago violaceae*, which demonstrated that mutants lacking unsaturated, colored carotenoids are more sensitive to light in the presence of the singlet-oxygen-generating sensitizers methylene blue and toluidene blue (32,36). Carotenoids are thus far the only endogenous compound identified that can protect organisms against cercosporin.

Our data, however, do not support the hypothesis that carotenoids are the only or even the major mechanism of cercosporin resistance. Mycelial cultures of *C. nicotianae* and *N. crassa* produce almost identical amounts of carotenoids, yet *N. crassa* is quite sensitive to cercosporin (70–80% growth inhibition by 10 μM cercosporin). The Super Producer mutant of *P. blakesleeanus* produced 30 times the amount of carotenoids as *C. nicotianae* but still was more sensitive to cercosporin. Further, there is not a linear relationship between the amount of carotenoids present in albino and carotenoid-producing isolates and their response to cercosporin. For example, the Super Producer isolate of *P. blakesleeanus* produced 10,000 times the amount of carotenoids as the albino isolate but showed only a two- to fourfold increase in resistance. These results suggest that carotenoids are important but are not the only factor involved in resistance.

We attempted to obtain definitive evidence for a role of carotenoids in resistance of *Cercospora* species to cercosporin by blocking carotenoid synthesis with several potent carotenoid inhibitors. DPA is probably the best studied inhibitor of carotenogenesis in fungi (17,28). In most systems it blocks the desaturation reactions leading from the colorless, saturated carotenoid phytoene (which cannot protect against photosensitizers) to the unsaturated colored carotenoids. Norflurazon is an herbicide that blocks the carotenoid desaturation reactions in plants (3) and also has been shown to inhibit phytoene synthetase in *P. blakesleeanus* (29). β-Ionone has variable results on different fungi, causing everything from a general decline in all carotenoids, to a stimulation of carotenoid synthesis (17,28). To our knowledge mevinolin has never been tested with fungi, but it is a potent inhibitor of HMG-CoA reductase (1,6), which is an important enzyme in pathways eventually leading to carotenoid synthesis. In general, we were unsuccessful in inhibiting carotenoid synthesis in *C. nicotianae* with any of these inhibitors. DPA did cause a 50–60% reduction in the amount of β-carotene produced per gram of mycelium, but this level of inhibition was significantly less than the 92% inhibition we obtained with *N. crassa* or the 90–99.5% inhibition reported with other fungi using the same amount of DPA (24,34). We cannot explain at this time why we were able to inhibit carotenoid synthesis in *N. crassa* but not in *C. nicotianae*. The lack of inhibition does not appear to be due to lack of uptake of the inhibitors, because the inhibitors reduced growth of the two fungi by comparable amounts (and, with DPA, by amounts comparable to those reported for *P. blakesleeanus* [24]). Our inability to inhibit carotenoids of *C. nicotianae* is consistent with a critical role of carotenoids in survival of the fungus. It is important

TABLE 4. Proportion of total mycelial carotenoids present in protoplasts and in undigested mycelium and enzyme solution following protoplast isolation

Fungus	Protoplast yield (no./g fresh wt.)[a]	Total mycelial carotenoids (μg/g dry wt.)	Percent total carotenoids		
			Protoplasts	Undigested mycelium	Enzyme solution
Cercospora nicotianae	$6.4\ (\pm 1.8)^{b}\times 10^{8}$	23.2 ± 0.4	24.2 ± 2.4	59.3 ± 4.1	5.6 ± 2.2
Neurospora crassa	$5.1\ (\pm 1.1)\times 10^{8}$	22.4 ± 1.8	19.8 ± 3.7	61.3 ± 4.8	...[c]

[a] g fresh weight = 193 mg dry weight (*C. nicotianae*) and 203 mg dry weight (*N. crassa*).
[b] ± standard deviation.
[c] None detected.

to emphasize that, because our isolate of *C. nicotianae* does not produce cercosporin under the growth conditions used here, the endogenous presence of cercosporin is not a complicating factor in these studies.

Carotenoids could be more important in cercosporin resistance in *Cercospora* than our data indicate if the cellular localization of carotenoids differed in *Cercospora* as compared with the other fungi studied. Localization of the carotenoids is a critically important consideration. Thomas and co-workers (32) reported that carotenoids protected *N. crassa* conidia from photosensitization by methylene blue and toluidine blue, two sensitizers that act on membranes from outside the cell (20). However, carotenoids had no protective effect against acridine orange, which penetrates into the nucleus and thus acts at a site where carotenoids are not present. The inability of carotenoids to protect plants against cercosporin and other membrane sensitizers also is presumably due to the localization of carotenoids in chloroplasts and other plastids (5). Kjeldstad et al (21) reported that the destruction of protoplasts of *Vicia faba* by a hematoporphyrin derivative was unaffected by the presence or absence of chloroplasts; they concluded that carotenoids or other quenchers in chloroplasts could not protect the plasma membrane against damage.

We recently have shown that protoplasts of *C. nicotianae* and *N. crassa* show an identical, sensitive response to cercosporin. As the protoplasts regenerate cell walls, *C. nicotianae* regains high levels of resistance, whereas *N. crassa* remains at the same response level expressed by the protoplast (19). Thus, if carotenoids were localized in the cell wall of *Cercospora,* they could be critical in the differential resistance expressed by these two fungi. Our studies suggest that this is not the case. Carotenoids of *N. crassa* are localized in lipid bodies in the cytoplasm (28) and thus would be localized within the protoplast. Based on comparative amounts of carotenoids in mycelium and protoplasts of *N. crassa* and *C. nicotianae*, we conclude that carotenoids of *C. nicotianae* also are localized within the protoplast. Carotenoids are thus not the cell wall factor that differentiates *Cercospora* from *Neurospora*. This conclusion is supported by recent work in this laboratory with transformation of *N. crassa* with a *Cercospora* genomic library. We have identified a clone from *C. nicotianae* that when transformed into *N. crassa* results in the overproduction of normal carotenoids of *N. crassa* (Cooperman and Daub, *unpublished*). These transformants show no increased resistance to cercosporin.

Protoplasts of both *C. nicotianae* and *N. crassa*, although sensitive as compared with mycelium of *C. nicotianae* , do express a significant level of resistance to cercosporin. Under the light conditions used in our studies, 50% of the protoplasts of both species survive following exposure to 1 μM cercosporin for 8 hr. One-half-hour exposure to 1 μM cercosporin is sufficient to kill all cells in a plant cell suspension culture. Thus, fungal protoplasts are significantly more resistant to cercosporin than are plant cells, and we believe that carotenoids play a role at the protoplast level. Carotenoids are not the factor, however, that differentiates *Cercospora* from *Neurospora* and that is presumably the major mechanism of cercosporin resistance. This finding is important because, in the majority of studies of resistance to photosensitizers, carotenoids are the only mechanism of resistance identified (4,15,22). Identifying and characterizing the cell wall factor responsible for resistance will make an important contribution to our understanding of photosensitizers and their interactions with cells.

LITERATURE CITED

1. Alberts, A. W., Chen, J., Kuron, G., Hunt, V., Huff, J., Hoffman, C., Rothrock, J., Lopez, M., Joshua, H., Harris, E., Patchett, A., Monaghan, R., Currie, S., Stapley, E., Albers-Schonberg, G., Hensens, O., Hirshfield, J., Hoogsteen, K., Liesch, J., and Springer, J. 1980. Mevinolin: A highly potent competitive inhibitor of hydroxymethylglutaryl-coenzyme A reductase and a cholesterol-lowering agent. Proc. Natl. Acad. Sci. USA 77:3957-3961.
2. Balis, C., and Payne, M. G. 1971. Triglycerides and cercosporin from *Cercospora beticola*: Fungal growth and cercosporin production. Phytopathology 61:1477-1484.
3. Bartels, P. G., and Watson, C. W. 1978. Inhibition of carotenoid synthesis by fluridone and norflurazon. Weed Sci. 26:198-203.
4. Bellus, D. 1979. Physical quenchers of singlet molecular oxygen. Adv. Photochem. 11:105-205.
5. Britton, G. 1982. Carotenoid biosynthesis in higher plants. Physiol. Veg. 20:735-755.
6. Brown, M. S., Faust, J. R., and Goldstein, J. L. 1978. Induction of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity in human fibroblasts incubated with compactin (ML-236B), a competitive inhibitor of the reductase. J. Biol. Chem. 253:1121-1128.
7. Daub, M. E. 1982. Cercosporin, a photosensitizing toxin from *Cercospora* species. Phytopathology 72:370-374.
8. Daub, M. E. 1982. Peroxidation of tobacco membrane lipids by the photosensitizing toxin, cercosporin. Plant Physiol. 69:1361-1364.
9. Daub, M. E. 1987. Resistance of fungi to the photosensitizing toxin, cercosporin. Phytopathology 77:1515-1520.
10. Daub, M. E., and Briggs, S. P. 1983. Changes in tobacco cell membrane composition and structure caused by cercosporin. Plant Physiol. 71:763-766.
11. Daub, M. E., and Hangarter, R. P. 1983. Light-induced production of singlet oxygen and superoxide by the fungal toxin, cercosporin. Plant Physiol. 73:855-857.
12. Daub, M. E., and Payne, G. A. 1985. The role of carotenoids in resistance of fungi to cercosporin. (Abstr.) Phytopathology 75:1298.
13. Dobrowolski, D. C., and Foote, C. S. 1983. Cercosporin, ein erzeuger von singulett-sauerstoff. Angew. Chem. 95:729-730.
14. Fajola, A. O. 1978. Cercosporin, a phytotoxin from *Cercospora* spp. Physiol. Plant Pathol. 13:157-164.
15. Foote, C. S. 1976. Photosensitized oxidation and singlet oxygen: Consequences in biological systems. Pages 85-133 in: Free Radicals in Biology. Vol. II. W. A. Pryor, ed. Academic Press, New York.
16. Foote, C. S., Denny, R. W., Weaver, L., Chang, Y., and Peters, J. 1970. Quenching of singlet oxygen. Ann. N.Y. Acad. Sci. 171:139-148.
17. Goodwin, T W. 1980. The Biochemistry of the Carotenoids. Vol. I. Chapman and Hall, New York.
18. Gwinn, K. D., and Daub, M. E. 1986. Cell wall regeneration does not affect the sensitivity of *Neurospora* protoplasts to cercosporin. (Abstr.) Phytopathology 76:1080-1081.
19. Gwinn, K. D., and Daub, M. E. 1988. Regenerating protoplasts of *Cercospora* and *Neurospora* differ in their response to cercosporin. Phytopathology 78:414-418.
20. Ito, T. 1978. Cellular and subcellular mechanisms of photodynamic action: The $^1O_2$ hypothesis as a driving force in recent research. Photochem. Photobiol. 28:493-508.
21. Kjeldstad, B., Olsten, M. L., Christensen, T., and Johnsson, A. 1986. Photodynamic damage to protoplasts with a hematoporphyrin derivative. Plant Sci. 44:139-143.
22. Krinsky, N. I. 1979. Carotenoid protection against oxidation. Pure Appl. Chem. 51:649-660.
23. Macri, F., and Vianello, A. 1979. Photodynamic activity of cercosporin on plant tissues. Plant Cell Environ. 2:267-271.
24. Olson, J. A., and Knizley, H., Jr. 1962. The effect of diphenylamine on carotenoid, sterol, and fatty acid synthesis in *Phycomyces blakesleeanus*. Arch. Biochem. Biophys. 97:138-145.
25. Perkins, D. D., Radford, A., Newmeyer, D., and Bjorkman, M. 1982. Chromosomal loci of *Neurospora crassa*. Microbiol. Rev. 46:426-570.
26. Quigley, D. R., Taft, C. S., Stark, T., and Selitrennikoff, C. P. 1987. Optimal conditions for the release of protoplasts of *Neurospora* using Novozyme 234. Exp. Mycol. 11:236-240.
27. Rodriguez, R. J., Taylor, F. R., and Parks, L. W. 1982. A requirement for ergosterol to permit growth of yeast sterol auxotrophs on cholestanol. Biochem. Biophys. Res. Commun. 106:435-441.
28. Ruddat, M., and Garber, E. D. 1983. Biochemistry, physiology, and genetics of carotenogenesis in fungi. Pages 95-151 in: Secondary Metabolism and Differentiation in Fungi. J. W. Bennett and A. Ciegler, eds. Marcel Dekker, New York.
29. Sandmann, G., Bramley, P. M., and Boger, P. 1980. The inhibitory mode of action of the pyridazinone herbicide norflurazon on a cell-free carotenogenic enzyme system. Pestic. Biochem. Physiol. 14:185-191.
30. Sorger, G. J., and Giles, N. H. 1965. Genetic control of nitrate reductase in *Neurospora crassa*. Genetics 52:777-788.
31. Spikes, J. D. 1977. Photosensitization. Pages 87-112 in: The Science of Photobiology. K. D. Smith, ed. Plenum Press, New York.
32. Thomas, S. A., Sargent, M. L., and Tuvesen, R. W. 1981. Inactivation of normal and mutant *Neurospora crassa* conidia by visible light and near-UV: Role of $^1O_2$, carotenoid composition and sensitizer location. Photochem. Photobiol. 33:349-354.
33. Tyczkowski, J., and Hamilton, P. B. 1984. HPLC method for analysis of carotenoids in poultry feed and tissues. (Abstr.) Page 42 in: Annu. Meet. Assoc. Off. Anal. Chem., 98th.

34. Valadon, L. R. G., and Mummery, R. S. 1973. Effect of certain inhibitors of carotenogenesis in *Verticillium agaricinum*. Microbios 7:173-180.
35. Weete, J. D. 1980. Lipid Biochemistry of Fungi and Other Organisms. Plenum Press, New York.
36. Will, O. H., III, Newland, N. A., and Reppe, C. R. 1984. Photosensitivity of pigmented and nonpigmented strains of *Ustilago violaceae*. Curr. Microbiol. 10:295-302.
37. Will, O. H., III, and Ruddat, M. 1984. C18 reversed-phase HPLC analysis of carotenes. Liq. Chromatogr. 2:610-612.
38. Yamazaki, S., Okubo, A., Akiyama, Y., and Fuwa, K. 1975. Cercosporin, a novel photodynamic pigment isolated from *Cercospora kikuchii*. Agric. Biol. Chem. 39:287-288.