APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1991, p. 2940–2945
0099-2240/91/102940-06$02.00/0

Vol. 57, No. 10

Mutants of *Cercospora kikuchii* Altered in Cercosporin Synthesis and Pathogenicity

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Received 2 May 1991/Accepted 18 July 1991

We have obtained spontaneous and UV-induced stable mutants, altered in the synthesis of cercosporin, of the fungal soybean pathogen *Cercospora kikuchii*. The mutants were isolated on the basis of colony color on minimal medium. The UV-induced mutants accumulated, at most, 2% of wild-type cercosporin levels on all media tested. In contrast, cercosporin accumulation by the spontaneous mutants was strongly medium regulated, occurring only on potato dextrose medium but at concentrations comparable to those produced by the wild-type strain. UV-induced mutants unable to synthesize cercosporin on any medium were unable to incite lesions when inoculated onto the soybean host. Cercosporin was reproducibly isolated from all inoculated leaves showing lesions. Although cercosporin involvement in disease has been indirectly suggested by many previous studies, this is the first report in which mutants blocked in cercosporin synthesis have been used to demonstrate that cercosporin is a crucial pathogenicity factor for this fungal genus.

Cercospora kikuchii (Matsumoto & Tomoyasu) Gardner, a fungal soybean pathogen capable of attacking all aerial plant parts (25), is the causative agent of purple seed stain, leaf spot, and pod blight diseases (22). Purple seed stain is the most prevalent and widely distributed fungal disease of soybean seed, and no immune or highly resistant commercial soybean cultivars are available (24). Although the economic losses due to *C. kikuchii* infections depend on weather conditions, reduction in seed germination (28) and seed quality (30) have been regularly documented and are of concern to growers. *Cercospora* species do not penetrate the cell walls of their host plants, but instead grow throughout the intercellular spaces. It is postulated that production of the necrosis-inducing toxin cercosporin by *Cercospora* species allows the fungus to obtain nutrients required for growth and sporulation within the host.

Cercosporin, a non-host-specific phytotoxin, was first isolated from *C. kikuchii* in 1957 (15) and has since been isolated from many other *Cercospora* species (1, 12, 18). The structure of this red perylene quinone, 1,12-bis(2-hydroxypropyl)-2,11-dimethoxy-6,7-methylenedioxy-4,9-dihydroxyperylene-3,10-quinone, molecular weight 534 (Fig. 1), was elucidated independently by Lousberg et al. (17) and Yamazaki and Ogawa (29). Three lines of evidence suggest that cercosporin plays an important role in diseases caused by *Cercospora* species. First, cercosporin is a light-activated compound, and light has been shown to be important for the development of disease symptoms in several *Cercospora* diseases (6, 9, 20). Light induces the production of singlet oxygen and superoxide by cercosporin, and these active oxygen species are capable of the peroxidation of plant membrane lipids, leading to increased membrane rigidity, electrolyte leakage, and cell death (4, 8). Second, treatment of plant tissue with pure cercosporin reproduces the ultrastructural changes that are both consistent with the known mode of action of cercosporin and similar to the disease symptoms caused by the pathogen itself (2). Third, cercosporin has been isolated from the necrotic lesions of several infected plant hosts (12, 27). There are no known examples of plant resistance to cercosporin.

Present experimental approaches for studying the chemistry of cercosporin synthesis are arduous, and progress has been slow. Nuclear magnetic resonance and mass-spectrometric analysis of the reaction products of *Cercospora* mycelia fed ^{13}C-labeled acetate and formate demonstrated a polyketide route of synthesis and confirmed the structure of the end product cercosporin, but suggested only one polyketomethylene intermediate (23). To date, no enzymes or chemical intermediates have been isolated for the cercosporin biosynthetic pathway. To study several aspects of the biosynthesis of cercosporin and to more directly assess the role of cercosporin in the pathogenicity of *Cercospora* species, it would be useful to have mutants blocked or altered in the biosynthesis of this polyketide metabolite.

In this study we report the isolation and preliminary characterization of *C. kikuchii* mutants altered in the biosynthesis and regulation of cercosporin and altered in pathogenicity on the soybean host. Of the five stable mutants isolated, two were spontaneous mutants, which were altered in the regulation of cercosporin biosynthesis and were pathogenic, and three were UV induced, which were essentially cercosporin negative on all media tested and were nonpathogenic.

MATERIALS AND METHODS

Strains, media, and culture conditions. *C. kikuchii* PR was isolated from soybeans in Puerto Rico and was kindly provided by J. B. Sinclair, University of Illinois, Urbana. Fungi were grown on the following media in liquid and agar-solidified forms: minimal medium (MM) (16); MM modified by the addition of 5 g of D-sorbose per liter for mutant selection; complete medium (CM) (16); potato dextrose broth (PDB; Difco Laboratories, Detroit, Mich.); and the *C. kikuchii* sporulation medium of El-Gholl et al. (11) modified by the addition of 2 g of dried, senescent soybean leaf powder per liter. Fungi cultured in liquid medium were

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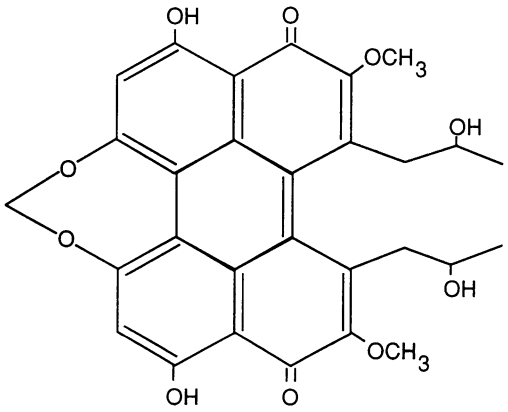

FIG. 1. Structural formula of cercosporin.

grown in 50-ml volumes in 250-ml Erlenmeyer flasks for 7 days on a rotatory shaker (180 to 200 rpm) at 20°C under continuous white fluorescent light (approximately 15 microeinsteins · m^{-2} · s^{-1}) or continuous dark (achieved by wrapping flasks in foil and dark cloth). *Cercospora* strains were maintained in pure culture by hyphal-tip transfer.

Growth measurements. Radial growth was measured by taking the average of the diameters (measured at right angles to each other) of each colony grown for 10 days on agar medium in a 10-cm petri dish that had been inoculated with a 5-mm-diameter mycelial plug taken from the periphery of a 10-day-old culture on MM.

Induction of sporulation. A single 5-mm fungal plug taken from the periphery of a *C. kikuchii* colony grown for 10 days on potato dextrose agar (PDA) was vortexed in a sterile tube containing 3 ml of sterile water and 2 g of 4-mm glass beads until the plug was completely disrupted. Aliquots of 0.1 ml of this mycelial suspension were spread on sporulation agar. Plates were incubated for 4 to 7 days at approximately 24°C with a 12-h light and 12-h dark cycle. Conidia were harvested by flooding the plates with sterile water and gently scraping fungal colonies with a rubber-tipped glass rod. Conidial suspensions were passed through four plies of sterile cheesecloth and concentrated by centrifugation at 6,000 × *g*. Conidial suspensions were kept on ice prior to use.

Mutant isolation. *C. kikuchii* PR conidia were quantified by using a hemacytometer, and the suspension was adjusted to approximately 2.0×10^6 spores per ml in sterile water. Aliquots of 300 μl were irradiated for various times with UV light (500 μW · cm^{-2}), and serial dilutions of the mutagenized conidia were plated in low room light on MM-sorbose and incubated in the dark for 3 days in the growth chamber. Ten days after plating, white and tan colonies were selected from the background of red colonies for further study. Rarely, white colonies of putative spontaneous mutants were observed and recovered from control plates.

Cercosporin assay. Aliquots (10 ml) of the liquid cultures (mycelia plus medium) were blended in a Waring blender and treated with 1 vol of 5 N KOH (14) and clarified by centrifugation. Cercosporin concentrations were calculated from the A_{480} and the molar extinction coefficient of 23,300 (29) for cercosporin in base. Dry weights of mycelial samples were determined by weighing samples that had been lyophilyzed.

Fungal sensitivity to cercosporin. The sensitivity of *Cercospora* isolates to cercosporin was tested by inoculating 5-mm fungal plugs onto divided petri plates of CM and PDA medium, with the agar medium of one-half of each plate amended by the addition of a cercosporin stock solution to give a final concentration of 10 μM as outlined by Daub (5). The plates were inoculated at 25°C under continuous fluorescent light (80 microeinsteins · m^{-2} · s^{-1}). Radial growth was measured at 24-h periods.

Soybean inoculation studies. The leaves of glasshouse-grown, 6-week-old soybean plants, cultivars Lee-68 and Centennial, were inoculated with *C. kikuchii* PR and cercosporin mutants by two methods. In method 1, 5-mm fungal plugs, taken from the periphery of dark-grown CM cultures, were attached to the leaf underside with surgical tape (3MM, St. Paul, Minn.). The upper leaf surface was observed at 24-h intervals for lesion formation. This experiment was done twice, with three replications per experiment. Inoculum for the second method was prepared by blending approximately 0.5 g (fresh weight) of washed, dark-grown (CM) mycelia of each strain in 20 ml of sterile water in a sterile Waring blender. Mycelial suspensions were atomized onto the soybean leaves until the leaves dripped. Plants were covered with plastic bags for 24 h. Lesion formation was monitored over a 10-day period. Disease rating stages (25) that relate increasing severity to increasing area of the leaf covered by lesions were used to rate disease severity. The experiment was done once for each cultivar, and the leaf data from each experiment was combined from three replications with four plants per replication.

Extraction of cercosporin from plant tissue. Soybean leaf tissue was ground to powder in liquid nitrogen in a chilled mortar. Approximately 500 mg of leaf powder was extracted with 3 ml of ethyl acetate for 12 h at 4°C. The tissue suspension was then centrifuged at top speed in a microcentrifuge for 10 min to pellet the tissue. The supernatant was taken to dryness under a stream of nitrogen gas and then resuspended in a minimal volume of ethyl acetate. Extract components of uninfected, uninfected plus pure cercosporin standard in ethyl acetate, and infected leaf tissue were resolved by thin-layer chromatography as described by Fore et al. (13).

RESULTS

Production and isolation of mutants. Approximately 8 min of UV light exposure (500 μW · cm^{-2}) was required to kill 95 to 99% of the *C. kikuchii* PR conidia. Mutagenized conidia were plated on MM to exclude the selection of auxotrophic mutants. In five separate mutagenesis experiments, a total of 4,820 survivors were isolated. Of these survivors, a total of 18 white-tan, putative cercosporin-deficient mutants, representing 0.37% of the survivors, were identified. Repeated subculturing of these 18 isolates on various media yielded three stable mutants, U1, U2, and U4, with white-tan phenotypes on all media tested, representing 0.06% of the survivors. While these experiments were in progress, two spontaneous white mutants, S1 and S2, were recovered from control plates. The two spontaneous mutants had stable white-tan phenotypes on MM and CM medium, but were red-brown on PDA. Mutants were examined morphologically and were similar in appearance to wild-type PR except for coloration. Typical fungal colonies of wild-type PR (which is intensely red-brown) and the five tan-white mutants, U1, U2, U4, S1, and S2, grown on CM medium are shown in Fig. 2.

Growth and sporulation analysis. On agar medium, cercosporin-blocked mutants U1, U2, and U4 grew more rapidly than cercosporin-producing wild-type PR and mutants S1

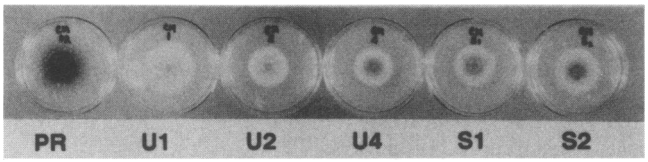

FIG. 2. Appearance of *C. kikuchii* PR (wild type) and five mutant strains altered in cercosporin production after growth on CM medium for 10 days in continuous light at 20°C. Strains U1, U2, and U4 were UV induced, and strains S1 and S2 are spontaneous mutants. Colonies were photographed through the plate from the bottom.

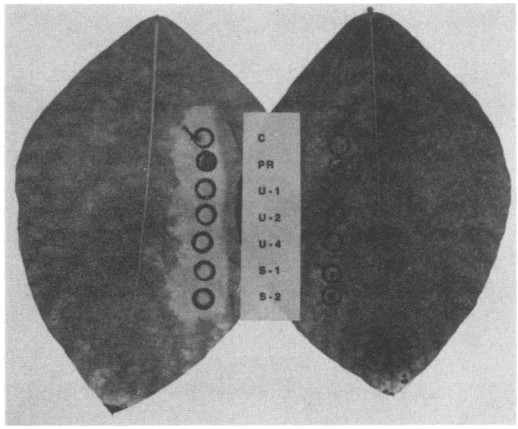

FIG. 3. Soybean leaves of cultivar Lee-68 7 days after inoculation with fungal plugs of *C. kikuchii* PR and cercosporin mutants U1, U2, U4, S1, and S2. On the left is the leaf undersurface showing the inoculation tape; on the right, the leaf surface is shown.

and S2 (producing cercosporin on PD medium). Radial growth of strain PR on solid media was 68 to 88% of that of mutants on MM and 57 to 67% of that of mutants on CM. Strain PR and mutants S1 and S2 were all intensely red-brown on PDA, but their growth was only 70 to 85% of that of the other mutants on this medium. In liquid culture, on the other hand, whether cercosporin producing or not, wild-type PR and the mutants all achieved similar final dry weight accumulation (approximately 2 g/50 ml of culture) after 7 days. Mutants U1, U2, and U4 produced, at most, about 1% of the amount of spores routinely recovered from PR (10^6/ml). On the other hand, sporulation of S1 and S2 was similar to that of PR (data not shown).

Analysis of cercosporin synthesis. Spectrophotometric analysis of cercosporin accumulation (Table 1) revealed that UV-induced mutants U1, U2, and U4 were essentially cercosporin negative on all three media, producing no more than 2% of the wild-type level of cercosporin in liquid culture in the light. In contrast, spontaneous mutants S1 and S2 were strongly medium regulated, producing less than 0.5% of wild-type cercosporin levels on MM and CM but wild-type quantities or greater when grown on PDA in the light. In agreement with previous findings (2, 14, 19), our data indicated that cercosporin synthesis required exposure to light for its induction. Cercosporin accumulation for PR, S1, and S2 was, respectively, 50- to 90-fold, 136-fold, and 50-fold greater in the light. In 50 ml of liquid culture, no consistent difference in growth yield (2 g [dry weight]) between PR and the mutants was detected after 7 days of growth.

Sensitivity to cercosporin. The growth of *C. kikuchii* PR and the spontaneous and UV-induced mutants was not inhibited by the presence of 10 μM cercosporin in either CM or PDA medium in the light (data not shown).

Analysis of pathogenicity. Since UV-induced mutants U1, U2, and U4 had sharply reduced sporulation, we used fungal-plug and mycelial inoculation techniques to evaluate pathogenicity on soybean leaves. Necrotic lesions were observed on the upper surface of soybean leaves of both cultivars 5 to 7 days after inoculation with mycelial plugs of PR and mutants S1 and S2, but not after inoculation with mutants U1, U2, and U4 (cultivar Lee-68 [Fig. 3]). Irregularly shaped, 1- to 5-mm lesions with necrotic centers were observed on leaves 10 days after inoculation with mycelial suspensions of PR and mutants S1 and S2, but not with mutants U1, U2, and U4. Pinpoint flecks were observed infrequently on plants inoculated with U1, U2, or U4. Such flecks may indicate low virulence for these mutants. Numbers of leaves with lesions were used to calculate the percent incidence, and the severity class was determined visually by using a lesion stage rating scale based on the area of the lesion. The incidence and severity of disease caused by wild-type PR and medium-regulated mutants S1 and S2 indicate that they are pathogens of soybean under glasshouse conditions (Table 2). Lesions typically observed on cultivar Centennial 10 days after inoculation are shown in Fig. 4. Strains morphologically identical to wild-type PR and mutants S1 and S2 were isolated from plants inoculated with these *C. kikuchii* strains. Attempts to isolate mutants U1, U2, and U4 from their respective inoculated plants were unsuccessful.

Infected-tissue extraction. Thin-layer chromatography of

TABLE 1. Accumulation of cercosporin in three media inoculated with *C. kikuchii* PR and five mutants grown for 7 days in liquid culture at 20°C in continuous light or continuous dark

Strain	Concn of cercosporin (nmol/mg [dry wt]) in[a]:					
	Light			Dark		
	MM	CM	PD	MM	CM	PD
PR	5.9 ± 0.8	93.1 ± 6.0	112.8 ± 2.5	–[b]	1.0 ± 0.3	2.1 ± 0.4
U1	–	1.8 ± 0.3	1.8 ± 0.2	–	–	–
U2	–	1.2 ± 0.3	0.6 ± 0.2	–	–	–
U4	–	2.0 ± 0.3	2.3 ± 0.2	–	–	–
S1	–	0.5 ± 0.1	136.3 ± 4.5	–	–	1.0 ± 0.3
S2	–	0.5 ± 0.1	124.4 ± 3.8	–	–	2.2 ± 0.3

[a] The mean and standard deviation for three replications are shown.
[b] –, cercosporin was not detected in the sample.

TABLE 2. Disease incidence and severity in 6-week-old soybean plants (cultivar Centennial) 10 days after incubation with mycelial suspensions of *C. kikuchii* PR and five mutants

Strain	No. of leaves evaluated	% Incidence[a,b]	Severity class[a,c]
Control	240	1.0	0.01
PR	300	88.0	3.20
U1	264	2.0	0.01
U2	240	0.0	0.00
U4	312	3.0	0.01
S1	240	77.0	2.20
S2	252	72.0	2.20

[a] Values are the means of three replications with four plants per replication.
[b] Percentage of leaves with lesions.
[c] Average disease rating 0 to 9, where 0 is no disease and 9 is 90% of leaf area covered by lesions.

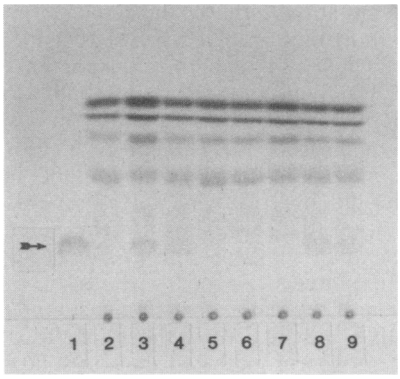

FIG. 5. Thin-layer chromatogram of partially purified extracts of infected leaves of the soybean cultivar Centennial. Lanes: 1, cercosporin standard (10 μl of 10 μM solution); 2, uninoculated leaf; 3, uninoculated leaf with the addition of cercosporin standard; 4, leaf inoculated with *C. kikuchii* PR; 5, leaf inoculated with U1; 6, leaf inoculated with U2; 7, leaf inoculated with U4; 8, leaf inoculated with S1; and 9, leaf inoculated with S2. The arrow at lane 1 indicates the migration point of authentic cercosporin.

extracts from lesions of plants inoculated with PR and mutants S1 and S2 showed a compound that migrated at the same R_f of 0.23 as authentic cercosporin (Fig. 5). Although lesions were not observed on soybean leaves inoculated with mutant U1, U2, or U4, these tissues were also extracted and extracts were chromatographed. No cercosporin was detected.

DISCUSSION

Genetic manipulation of the pathogen is the ideal for a rigorous analysis of the involvement of factors, such as toxins, in disease initiation and development. *C. kikuchii* and many other important fungal plant pathogens, however, are not manipulable by classical genetics because no teleomorphic stage is known and no mode of parasexual recombination has been described. Therefore, as an initial step in a molecular genetic approach to study cercosporin synthesis and its role in pathogenicity, we have isolated cercosporin mutants of the soybean fungal pathogen *C. kikuchii*. Strain PR was chosen for this study because it produced cercosporin on all laboratory media tested, could be readily induced to sporulate, and, in glasshouse soybean susceptibility tests, was pathogenic on 22 of 22 cultivars tested (26).

Prior to this study, one other cercosporin mutant of *C. kikuchii*, mutant "alba," had been isolated, but characterization of its pathogenicity was not reported (21). The isolation of mutants that are affected in the production of secondary metabolites often proves difficult (3), and our work with *C. kikuchii* illustrates this. It was not possible to employ the strategies commonly used for the isolation of conditionally lethal mutants, because secondary metabolites, unlike primary metabolites, are not essential for growth (3). In spite of a simple mutant selection scheme based on colony color, only three stable UV-induced mutants were recovered from the initial pool of survivors. The multiseptate and multinucleate structure of *Cercospora* conidia may partially explain the apparent reversion to wild-type color of most putative cercosporin mutants and also the duration of UV exposure required for 95 to 99% conidial kill. Owing to the method of mutant isolation on minimal medium, auxotrophic phenotypes were excluded. This was done because it has been shown for other fungi that such mutants have a high probability for instability and pleiotrophy to secondary metabolite production (3).

In this study, two classes of cercosporin mutants were isolated: class I (spontaneous, toxin accumulation regulated by medium, sporulation proficient, and pathogenic), and class II (UV induced, toxin negative on all media, sporulation deficient, and nonpathogenic). On the basis of these observations, it appears that UV-induced mutants U1, U2,

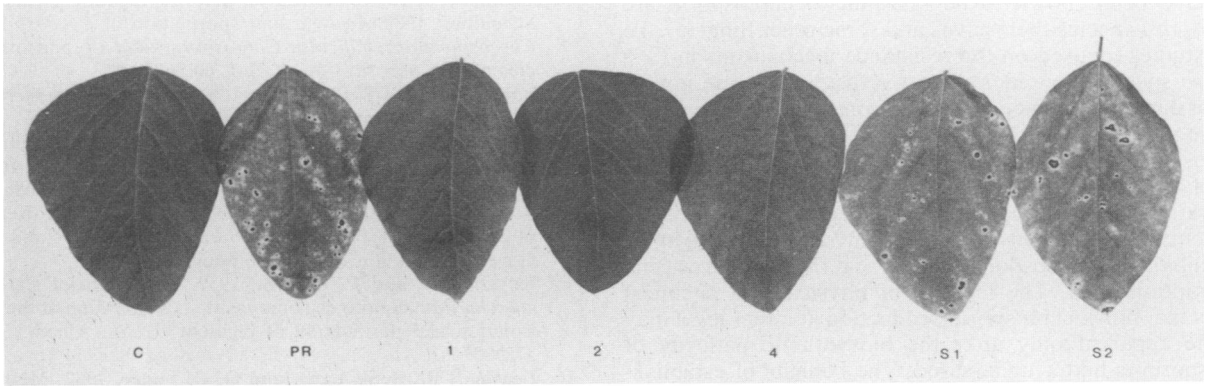

FIG. 4. Appearance of lesions on leaves of soybean cultivar Centennial 10 days after inoculation with mycelial suspensions of *C. kikuchii* PR and cercosporin mutants U1, U2, U4, S1, and S2.

and U4 have mutations either in the cercosporin biosynthetic genes or in the genes involved in the reception and transmission of the light cue, or both. The spontaneous mutants, S1 and S2, are wild type in their ability to synthesize cercosporin, but appear to have a mutation(s) whose phenotype is the acquisition of medium-regulated cercosporin synthesis. Cocultivation of all of the mutants in all possible combinations did not result in any observed biochemical complementation (data not shown). The potential connection between toxin synthesis and proficient sporulation remains to be explored.

Comparison of the radial growth measurements of cercosporin-negative mutants with those of PR, S1, and S2 producing cercosporin indicated that on solid medium cercosporin synthesis was correlated with reduced growth. This finding is supported by the results of Jenns et al. (14), who found a significant negative correlation between cercosporin accumulation on agar medium and colony area when data for each medium were examined separately. In contrast to growth on solid medium, however, results from liquid culture growth experiments indicated that there was no significant difference between the growth (dry weight accumulation) of cercosporin-producing cultures of PR and the nonproducing mutants (26). Growth, as defined by liquid culture, is thus apparently not a significant factor in the lack of pathogenicity of mutants U1, U2, and U4.

In choosing mycelial plant inoculation, we have been able to show that cercosporin-negative mutants U1, U2, and U4 are nonpathogenic. The lack of sporulation for these mutants, while undesirable, is not likely to be a confounding factor in the interpretation of our short-term glasshouse infection tests. The lack of sporulation is likely to be important in the field for the ability of the pathogen to initiate repeating cycles of infection and for biological fitness and survival. We have isolated *Cercospora* strains identical to PR, S1, and S2 from soybean leaf lesions. Thus, cercosporin synthesis appears to be a crucial pathogenicity factor for this fungus. It was not unexpected that mutants S1 and S2, which produce cercosporin on PD medium, would also produce cercosporin in planta since both substrates provide plant-derived nutrients.

The mechanism of photosensitization of cercosporin in the presence of light and oxygen is well established (6). The photosensitization mechanism and the apparent complexity of factors required for plant resistance partially explain why no plants resistant to the toxin have been found and why attempts to select plant cells for resistance in the presence of cercosporin have been unsuccessful (5). The only organisms that have been shown to be resistant to cercosporin are *Cercospora* species themselves and some other fungi (5). To date, studies focused on the resistance mechanisms in *Cercospora* species have identified two factors that are important for partial resistance, namely carotenoid production (10) and the production of reducing power at the cell surface (7). The resistance to *Cercospora* diseases that exists in some crop plants, however, is thought to be directed against fungal penetration of the host and not against cercosporin itself (5). A potentially important mechanism of resistance might be the inhibition of cercosporin synthesis at the enzyme or gene transcription level. The identity of physical and chemical factors that block cercosporin production at either level may become apparent only after the biosynthetic pathway of cercosporin is better understood. The benefits of establishing disease control at these levels make the elucidation of the cercosporin biosynthetic pathway and the understanding of its genetics and regulation a major research goal of our laboratory.

The ability to obtain mutants altered in the synthesis of cercosporin allows the application of biochemical and molecular genetic approaches to the study of cercosporin biosynthesis and regulation. This is the first study in which blocked and regulatory cercosporin mutants were used that shows that cercosporin synthesis is necessary for lesion production on soybean in the glasshouse. Biochemical and molecular genetic studies involving these mutants are presently being conducted; they should contribute to a better understanding of both the cercosporin biosynthetic pathway and its regulatory controls. The mutants described in this study are especially well suited to these studies because they have proven to be stable over a 2- to 3-year period. Finally, such mutants should be useful for the isolation of genes necessary for cercosporin biosynthesis.

ACKNOWLEDGMENTS

We thank G. A. Payne for helpful discussions and critical evaluation of the manuscript and Marvin Williams for photographing the specimens.

REFERENCES

1. **Assante, G., R. Locci, L. Camarda, L. Merlini, and G. Nasini.** 1977. Screening of the genus *Cercospora* for secondary metabolites. Phytochemistry **16**:243–347.
2. **Balis, C., and M. G. Payne.** 1971. Triglycerides and cercosporin from *Cercospora beticola*: fungal growth and cercosporin production. Phytopathology **61**:1477–1484.
3. **Beremand, M. N.** 1987. Isolation and characterization of mutants blocked in T-2 toxin biosynthesis. Appl. Environ. Microbiol. **53**:1855–1859.
4. **Daub, M. E.** 1982. Peroxidation of tobacco membrane lipids by the photosensitizing toxin, cercosporin. Plant Physiol. **69**:1361–1364.
5. **Daub, M. E.** 1987. Resistance of fungi to the photosensitizing toxin, cercosporin. Phytopathology **77**:1515–1520.
6. **Daub, M. E.** 1987. The fungal photosensitizer cercosporin and its role in plant disease, p. 271–280. *In* R. Heitz and K. R. Downum (ed.), Light activated pesticides. American Chemical Society, Washington, D.C.
7. **Daub, M. E.** Unpublished data.
8. **Daub, M. E., and S. P. Briggs.** 1983. Changes in tobacco cell membrane composition and structure caused by cercosporin. Plant Physiol. **71**:763–766.
9. **Daub, M. E., and R. P. Hangarter.** 1983. Light-induced production of singlet oxygen and superoxide by the fungal toxin, cercosporin. Plant Physiol. **73**:855–857.
10. **Daub, M. E., and G. A. Payne.** 1989. The role of carotenoids in resistance of fungi to cercosporin. Phytopathology **79**:180–185.
11. **El-Gholl, N. E., S. A. Alfieri, Jr., W. H. Ridings, and C. L. Schoulties.** 1982. Growth and sporulation of *Cercospora apii*, *Cercospora arachidicola*, *Cercospora kikuchii*, and other species of *Cercospora*. Can. J. Bot. **60**:862–868.
12. **Fajola, A. O.** 1978. Cercosporin, a phytotoxin from *Cercospora* spp. Physiol. Plant Pathol. **13**:157–164.
13. **Fore, S. A., M. E. Daub, and M. K. Beute.** 1977. Phytotoxic substances produced by some isolates of *Cercospora arachidicola* are not cercosporin. Phytopathology **78**:1082–1086.
14. **Jenns, A. E., M. E. Daub, and R. G. Upchurch.** 1989. Regulation of cercosporin accumulation in culture by medium and temperature manipulation. Phytopathology **79**:213–219.
15. **Kuyama, S., and T. Tamura.** 1957. A pigment of *Cercospora kikuchii* (Matsumoto et Tomoyasu). 1. Cultivation of the fungus, isolation and purification of pigment. J. Am. Chem. Soc. **79**: 5725–5726.
16. **Leach, J. B., B. R. Lang, and O. C. Yoder.** 1982. Methods for selection of mutants and *in vitro* culture of *Cochliobolus heterostrophus*. J. Gen. Microbiol. **128**:1719–1729.

17. **Lousberg, R. J. J. C., U. Weiss, C. A. Salemink, A. Merlini, and G. Nasini.** 1971. The structure of cercosporin, a naturally occurring quinone. J. Chem. Soc. Chem. Commun. **1971**:1463–1464.
18. **Lynch, F. J., and M. J. Geoghegan.** 1977. Production of cercosporin by Cercospora species. Trans. Br. Mycol. Soc. **69**:496–498.
19. **Lynch, F. J., and M. J. Geoghegan.** 1979. Regulation of growth and cercosporin photo-induction in *Cercospora beticola*. Trans. Br. Mycol. Soc. **73**:311–327.
20. **Macri, F., and A. Vianello.** 1979. Photodynamic activity of cercosporin on plant tissue. Plant Cell Environ. **2**:267–271.
21. **Matsueda, S., M. Nagaki, Y. Susuta, K. Tanaka, K. Takagaki, M. Shimoyama, T. Imaizumi, and K. Tsubaki.** 1983. *Cercospora kikuchii* (Matsumoto et Tomoyasu) Gardner mut. alba, a novel mutant of the pathogen of the soybean purple speck disease. Sci. Rep. Hirosaki Univ. **30**:42–46.
22. **Murakishi, H. H.** 1950. Purple seed stain of soybean. Phytopathology **41**:305–318.
23. **Okubo, A., S. Yamazaki, and K. Fuwa.** 1975. Biosynthesis of cercosporin. Agric. Biol. Chem. **39**:1173–1175.
24. **Roy, K. W., and T. S. Abney.** 1976. Purple seed stain of soybeans. Phytopathology **66**:1045–1049.
25. **Sinclair, J. B. (ed.).** 1982. Compendium of soybean diseases, 2nd ed., p. 2 and 14. American Phytopathological Society, St. Paul, Minn.
26. **Upchurch, R. G.** Unpublished data.
27. **Venkataramani, K.** 1967. Isolation of cercosporin from *Cercospora personata*. Phytopathol. Z. **58**:379–382.
28. **Wilcox, J. R., and T. S. Abney.** 1973. Effects of *Cercospora kikuchii* on soybeans. Phytopathology **63**:796–797.
29. **Yamazaki, S., and T. Ogawa.** 1972. The chemistry and stereochemistry of cercosporin. Agric. Biol. Chem. **36**:1707–1718.
30. **Yeh, C. C., and J. B. Sinclair.** 1981. Effect of *Cercospora kikuchii* on soybean seed germination and its interaction with *Phomopsis* sp. Phytopathol. Z. **105**:265–270.