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Regulation of Cercosporin Accumulation in Culture by Medium and Temperature Manipulation

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ABSTRACT

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The ability to manipulate cercosporin accumulation in specific isolates of *Cercospora* in culture is a necessary prerequisite for studying the regulation of toxin accumulation at a molecular level. This study defined medium, temperature, and light conditions for maximum and minimum cercosporin accumulation in isolates of *C. asparagi, C. beticola, C. kikuchii, C. nicotianae*, and *C. zeae-maydis*. A simple method was developed for the extraction and measurement of cercosporin in cultures of *Cercospora* spp. grown on solid medium. Of six growth media, malt and potato-dextrose agar were generally favorable for cercosporin accumulation, but the effects of medium and isolate on cercosporin accumulation interacted significantly. The ratio of carbon to nitrogen in a defined medium affected cercosporin accumulation in four of the eight isolates tested but not in any consistent manner. Cercosporin accumulation also was regulated by temperature in four of the eight isolates, higher levels accumulating at 20 C than at 30 C. Two isolates of *C. kikuchii* accumulated more cercosporin when grown in light than when grown in darkness, but the effect of light interacted with those of medium and isolate. Patterns of regulation of cercosporin accumulation differed markedly among species and even isolates of the same species of *Cercospora*, making generalizations about the regulation of cercosporin production by environmental factors of limited use. However, the present study did identify certain isolates for future investigation of cercosporin regulation. Our data also show that screening isolates of *Cercospora* for cercosporin production under a single set of cultural conditions is unreliable and question the reliability of correlating toxin production in vitro to the virulence of a *Cercospora* isolate.

The genus *Cercospora* includes species that cause many plant diseases, some of which are economically important, such as Cercospora leaf spot on beet (20) caused by *C. beticola* Sacc., Sigotoka disease of banana caused by *Mycosphaerella fijiensis* Morelet var. *difformis* Mulder and Stover (18), early leaf spot of peanut caused by *C. arachidicola* Hori (13), gray leaf spot of corn, caused by *C. zeae-maydis* Tehon & Daniels (15), and purple-seed stain of soybean, caused by *C. kikuchii* Matsu & Tomoyasu (16).

The nonspecific toxin, cercosporin, has been isolated from at least 34 *Cercospora* species in vitro (1,3,7,14,19), but attempts to isolate the toxin from another 51 species have been unsuccessful (1,3,4,7). The levels of production in vitro varied widely among cercosporin-producing isolates grown under the same conditions (1,3,7). We observed similar variations among the isolates in our collection and also noted variations over time in the level of toxin production by individual isolates. The ability to regulate cercosporin production in vitro is vital for the investigation of the genetic control of toxin production in this fungus. Also, large numbers of isolates of *Cercospora* are often screened for toxin-producing ability on a medium, under growth conditions that favor toxin detection in one good producer with no consideration of variation among isolates (1,3,7). To avoid a premature conclusion about the ability of an isolate to produce toxin, it is important to learn the effect of media and environmental conditions on cercosporin accumulation by different isolates. This is particularly important because toxin production has been used as a criterion for predicting pathogenicity (10) and has been proposed as a taxonomic criterion (3).

Some of the previous studies on effects of light, temperature, and medium on cercosporin accumulation in culture have aimed to optimize in vitro conditions for cercosporin production. Fajola found that light, temperature, and the six media tested affected cercosporin accumulation in *C. ricinella* (3). Kuyama and Tamura (5) found that the five media they tested affected cercosporin accumulation in *C. kikuchii*, and Assante et al (1) found that the effect of amendments to the medium on the accumulation of cercosporin and other secondary metabolites differed among the 23 species of *Cercospora* tested and even between isolates of *C. kikuchii*. Other workers have studied regulation of cercosporin accumulation to shed light on the biochemical pathway of cercosporin production and its regulation. Extensive studies with *C. beticola* isolates have found light (2,8) and growth medium components (1,2,6,8,9) to have marked effects on cercosporin accumulation. The effects on cercosporin accumulation of amino acids, carbon-nitrogen ratio, oxygen, reducing agents, carbon dioxide, trace elements, an uncoupler of oxidative phosphorylation, and an inhibitor of protein synthesis were examined by Lynch and Geoghegan (8). Mumma et al (11) studied the influence of nitrogen source on cercosporin accumulation in one isolate each of *C. hayii* and *C. kikuchii*. The thrust of our study was to define for specific isolates conditions that stimulate or suppress cercosporin accumulation to allow a molecular investigation of the genetic regulation of toxin production in this fungus. Media, light conditions, and temperatures were varied to determine both conditions that would suppress toxin accumulation and conditions that would enhance it. Isolates of several species of *Cercospora* were included in all experiments to assess the generality of the results.

MATERIALS AND METHODS

Isolates of *Cercospora*. *C. asparagi* Sacc. was isolated from asparagus in North Carolina in 1983 and provided by C. Cooperman (North Carolina State University, Raleigh). Isolates IL, IN, and PR of *C. kikuchii* were isolated from soybeans in Illinois, Indiana, and Puerto Rico, respectively, and were provided by J. B. Sinclair (University of Illinois, Urbana), who also provided isolate ATCC 86864 of *C. kikuchii*. F. M. Latterell, USDA, provided the *C. zeae-maydis* Troy (Type A). The other isolates used were *C. beticola* (ATCC 24080) and *C. nicotianae* (ATCC 18366).

Media. Malt medium contained 15 g of malt extract, 3 g of peptone, 30 g of glucose, and 15 g of agar per liter. *Cercospora* sporulation medium (CSM) was an adaptation of that reported by Stavely and Nimmo (17) to induce sporulation in *C. nicotianae* and contained the major and minor salts of Murashige and Skoog (12), 1.6 g of leucine, 5 g of sucrose, 3.6 g of yeast extract, 0.5 mg of thiamine, 1 g of nicotinic acid, 10 mg of biotin, and 18 g of agar per liter. Potato-dextrose agar (PDA) contained 24 g of Bacto dehydrated potato dextrose broth (Difco Laboratories, Detroit, MI) and 15 g of agar per liter. Minimal medium (MM), complete medium (CM), minimal medium with soybean leaves (SBL), sucrose sorbose medium (SS), and sucrose medium (S), all contained 1 g of $Ca(NO_3)_2 \cdot 4H_2O$; 10 ml of a solution containing 2 g of KH_2PO_4, 2.5 g of $MgSO_4 \cdot 7H_2O$, and 1.5 g of NaCl in 100 ml of H_2O, adjusted to pH 5.3 with NaOH; and 15 g of agar per liter. Additional ingredients per liter were: for MM, 10 g of glucose; for CM, 10 g of glucose, 1 g of yeast extract, and 1 g of casein hydrolysate; for SBL, 10 g of glucose and 2 g of dried soybean leaves ground in a blender; for SS, 10 g of sucrose and 1.7 g of sorbose; and for S, 10 g of sucrose. V-8 juice agar with soybean leaves (V8 + SBL) contained 300 ml of V-8 juice (clarified by centrifuging with 4.5 g of $CaCO_3$ for 10 min at 850 *g*) and 15 g of agar per liter. The medium for the carbon:nitrogen (C:N) ratio experiments was the basal medium of Lynch and Geoghegan (8), containing 20 g per liter of sucrose as sole carbon source and varying quantities of $NaNO_3$ as nitrogen source.

Cercosporin assay. Plugs of solid media (6-mm diameter) with fungal colonization were removed with the wide ends of sterile Pasteur pipets and soaked in 5 N KOH in the dark for 4 hr, after which absorbance of the soaking solution was measured at 480 nm (the visible absorbance maximum of cercosporin in base) in a Beckman model 25 spectrophotometer. To compare the amounts of cercosporin detected by this method and a more exhaustive extraction procedure, four plugs (6-mm diameter) were taken from 10-day-old cultures on malt agar of six of the eight isolates of *Cercospora* used in the other experiments. These were either soaked in 8 ml of 5 N KOH, as described, or ground with sand in 8 ml of acetone, filtered, and the absorbance read at 473 nm (the visible absorbance maximum of cercosporin in acetone). To determine if any interfering compounds were present in the extract, 100 μl each of the extracts in 5 N KOH and in acetone were subjected to thin-layer chromatography on silica gel 60 plates (Merck), pretreated in 2% H_3PO_4, and dried overnight at 60 C, using hexane:isopropanol (8:2) as the solvent. Spots with the same R_f as the cercosporin standard were scraped off the plate, resuspended in 2 ml of acetone, and their absorbance read at 473 nm. After correcting for opacity by subtracting the absorbance at 650 nm, absorbances of acetone and KOH extracts were well correlated ($R = 0.99$, $P < 0.0001$, Fig. 1). The acetone extracts also contained compounds that fluoresced blue under long-wavelength ultraviolet light. These were resuspended in acetone and found to have no significant absorbance at 473 nm, indicating that they do

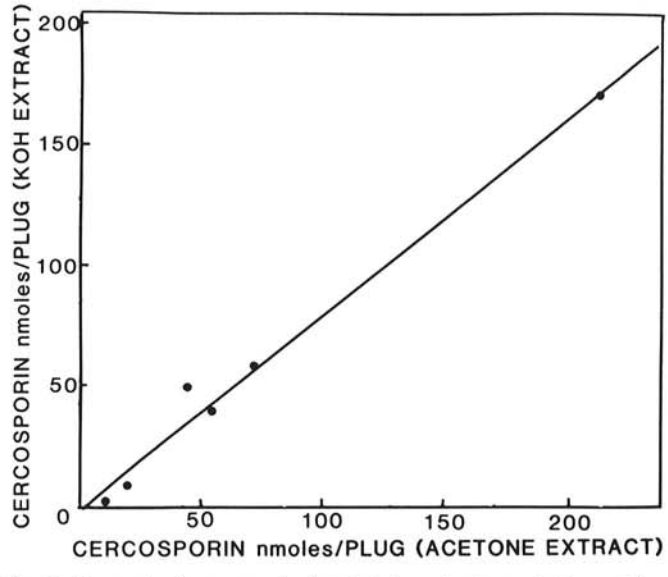


Fig. 1. Amount of cercosporin detected in extracts made by soaking mycelial plugs (6-mm diameter) from 10-day-old cultures of six different isolates of *Cercospora* isolates in 5 N KOH versus amount of cercosporin detected in extracts made by grinding plugs from the same cultures in acetone. The coefficient for correlation between the two methods was 0.99, significant at $P < 0.001$.

not affect the estimation of cercosporin concentration obtained from reading the unpurified extract at 473 nm. No interfering compounds were detected in the KOH extracts.

Isolate/media experiments. Plugs (6-mm diameter) taken from the margin of each isolate of *Cercospora* growing on malt medium were placed in the center of Petri plates containing 25 ml of the test media. Two plates were used for each isolate/medium combination, and the experiment was performed twice. The plates were incubated at 25 C in a chamber with 16 hr of light ($13.5\ \mu E \cdot m^{-2} \cdot sec^{-1}$) and 8 hr of dark. Every 3 days, the diameters of the cultures were measured, and 6-mm-diameter plugs taken from 2 mm behind the margin of growth (one from each plate) were extracted together in 4 ml of 5 N KOH. The correlation between colony diameter and mycelial weight was investigated by inoculating three plates of PDA as described above with each of the eight isolates. After 7 days, colony diameter was measured, and the mycelial mat was cut out and gently scraped to remove the medium. Mycelial mats were placed on weighed filter paper, dried for 72 hr in an oven at 80 C, and then weighed.

To investigate the effect of C:N ratio on cercosporin production, two plates per isolate containing the medium of Lynch and Geoghegan (8) with molar C:N ratios of 10:1, 50:1, 100:1, 150:1, 200:1, or 500:1 were inoculated as described above and incubated at 25 C in a chamber with 16 hr of light ($27\ \mu E \cdot m^{-2} \cdot sec^{-1}$) and 8 hr of dark. After 12 days, the diameters of the cultures were measured, and for each isolate/medium combination, four 6-mm-diameter plugs taken from 2 mm behind the margin of growth on each plate were extracted in 2 ml of 5 N KOH. The experiment was performed three times.

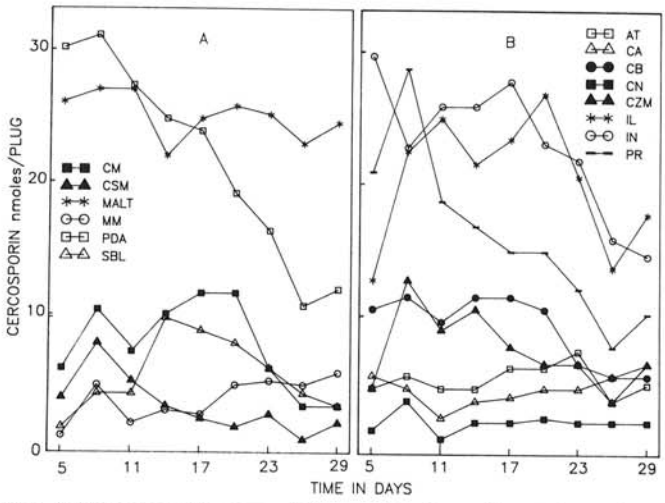


Fig. 2. Mean cercosporin accumulation per plug (6-mm diameter) over time over all eight isolates of *Cercospora* on each medium (A) and over all six media for each isolate (B). Media: malt medium (MALT), potato-dextrose medium (PDA), *Cercospora* sporulation medium (CSM), minimal medium (MM), complete medium (CM), and minimal medium with soybean leaves (SBL). Isolates: *C. asparagi* (CA), *C. beticola* (CB), *C. kikuchii* ATCC 86864 (AT), *C. kikuchii* IL (IL), *C. kikuchii* IN (IN), *C. kikuchii* PR (PR), *C. nicotianae* (CN), and *C. zeae-maydis* (CZM). Means are from two replicates for each isolate/medium combination in each of two trials.

Temperature experiments. Suspensions of aerial mycelium were made by adding sterile deionized water to cultures growing on V8+ SBL and gently scraping the surface with a sterile Pasteur pipet. Suspensions were spread on plates of solid medium, and these were incubated at either 20 or 30 C, with continuous illumination at $1.5\ \mu E \cdot m^{-2} \cdot sec^{-1}$. Plates were shifted from 30 to 20 C after various periods, and four plugs (6-mm diameter) were taken at random from each plate and extracted in 2 ml of 5 N KOH. Eight isolates were included in the experiment, in which plates were shifted from 30 to 20 C after 1, 5, 7, 11, and 14 days and cercosporin was assayed after 2, 6, 8, 12, and 15 days. The experiment was performed three times.

Light experiments. Plugs (6-mm diameter) taken from the margin of isolates of *C. kikuchii* IL and PR growing on malt medium were placed in the center of Petri plates containing 25 ml of PDA or malt medium. Half of the plates were wrapped with aluminum foil to exclude light, and all were placed in chambers at 20 or 25 C with 16 hr of light ($4\ \mu E \cdot m^{-2} \cdot sec^{-1}$) and 8 hr of dark. Three plates were used per isolate/medium/temperature/light combination, and the experiment was performed twice. After 8 days, four 6-mm-diameter plugs from each plate were extracted in 2 ml of 5 N KOH.

RESULTS

Media effects on cercosporin production and growth. In preliminary experiments, several isolates of *Cercospora* produced less red pigment, assumed to be cercosporin, when grown on CSM than when grown on any other medium tested. Omission of individual ingredients of the medium, however, revealed that no one component was responsible for pigment suppression. In *C. beticola* and *C. kikuchii* ATCC 86864, omission of sucrose or thiamine restored the red pigment, but in *C. asparagi* and *C. kikuchii* IL, these components had no effect. Omission of yeast extract partially restored red pigmentation in *C. kikuchii* IL. Because peak time of accumulation of cercosporin varied among both media and isolates (Fig. 2), the best measure of the cercosporin-producing ability of an isolate/medium combination was the highest amount of cercosporin extracted on any harvest date. The numbers in Table 1 are the means of the levels of cercosporin in each trial on the harvest date with the highest level of cercosporin for each isolate/medium combination in that trial.

Comparison of measured amounts of cercosporin produced on

TABLE 1. Maximum production of cercosporin (nmol per plug)[a] by eight isolates of *Cercospora* on six media

	Cercospora isolates								
	C. kikuchii								
Medium	IN	IL	PR	*C. beticola*	*C. zeae-maydis*	*C. asparagi*	*C. kikuchii* ATCC 86864	*C. nicotianae*	Medium mean
Malt[b]	65[c]	120	69	37	7	32	19	8	45 a[d]
PDA	112	74	28	30	64	24	15	11	45 a
CM	50	9	43	20	4	4	6	3	17 b
SBL	47	11	18	5	12	6	10	5	14 b
MM	9	27	24	9	4	4	8	2	11 b
CSM	3	3	43	6	3	6	4	3	9 b
Isolate mean[d]	48 a	41 ab	38 abc	18 bcd	16 bcd	13 cd	10 cd	5 d	

[a] Extracted by soaking 4 hr in 5 N KOH from a 6-mm diameter plug of agar taken from 2 mm behind the margin of growth.

[b] Malt = malt medium; PDA = potato-dextrose medium; CM = complete medium; SBL = minimal medium with soybean leaves; MM = minimal medium; CSM = *Cercospora* sporulation medium.

[c] Mean of the amounts of cercosporin extracted per 6-mm diameter plug on the harvest date with the highest cercosporin level for each isolate/medium combination in each of the two trials.

[d] Means followed by the same letter do not differ significantly $P \leq 0.05$ by Tukey's studentized range test (HSD).

different growth media confirmed that CSM was unfavorable for toxin production in any isolate tested (Table 1, Fig. 2). The medium on which the highest mean level of cercosporin was produced was malt for *C. asparagi, C. beticola, C. nicotianae, C. kikuchii* ATCC 86864, IL, and PR and was PDA for *C. zeae-maydis* and *C. kikuchii* IN (Table 1). Averaged over all isolates, malt and PDA were significantly more favorable for cercosporin accumulation than the other four media tested (Table 1). When averaged over all isolates, peak levels of cercosporin were found after 8 days on PDA, dropping to less than 50% of the maximum by day 29, whereas levels of cercosporin on malt agar remained fairly constant throughout the experiment (Fig. 2).

Overall, *C. kikuchii* IN and IL accumulated the most cercosporin and *C. nicotianae* the least, but differences among isolates were less distinct than among media (Fig. 2, Table 1). Peak times of accumulation averaged over all media types differed among isolates, but cercosporin level declined after 21 days in all isolates. Analysis of variance of maximum cercosporin content showed isolate and medium effects to be significant at $P < 0.0001$ and the isolate × medium interaction to be significant at $P = 0.03$.

The correlation between dry weight of mycelium and colony area as calculated for the eight isolates on PDA was significant ($R = 0.54$, $P = 0.0072$). The most favorable medium for overall growth of the eight isolates of *Cercospora* was PDA and the least favorable SBL (Fig. 3). When averaged over the different media, *C. asparagi* and *C. nicotianae* had the largest areas of mycelium by 20 days and *C. kikuchii* IN the smallest (Fig. 3). Because isolates that accumulated the most cercosporin had grown the least by 20 days, the correlation between maximum cercosporin accumulation and area at 20 days was investigated. The overall correlation was not significant, but when data for each medium were examined separately, area at 20 days and cercosporin accumulation were found to be significantly negatively correlated on CM ($R = -0.73$, $P = 0.0012$) and PDA ($R = -0.79$, $P = 0.002$), two media favorable for cercosporin accumulation. When data for each isolate were examined separately, area of mycelium at 20 days and maximum cercosporin accumulation were found to be significantly correlated for *C. asparagi* ($R = 0.67$, $P = 0.0241$) and *C. beticola* ($R = 0.66$, $P = 0.0198$). Analysis of variance of area at 20 days showed isolate and medium effects to be significant at $P < 0.0001$, but the isolate × medium interaction to be nonsignificant at $P \leq 0.05$.

C:N ratio over the range of 10:1 to 500:1 was found by analysis of variance to have a significant ($P \leq 0.05$) effect on cercosporin accumulation in *C. beticola, C. nicotianae*, and isolates IL and PR of *C. kikuchii*. Cercosporin accumulation in *C. asparagi, C. zeae-maydis*, and isolates AT and IN of *C. kikuchii* was not affected by changes in the C:N ratio in the range tested. Radial growth was not affected by changes in the C:N ratio over this range in any of the isolates. Peak cercosporin accumulation occurred at C:N ratios of 50:1 in *C. beticola*, 10:1 in *C. nicotianae*, 150:1 in *C. kikuchii* IL,

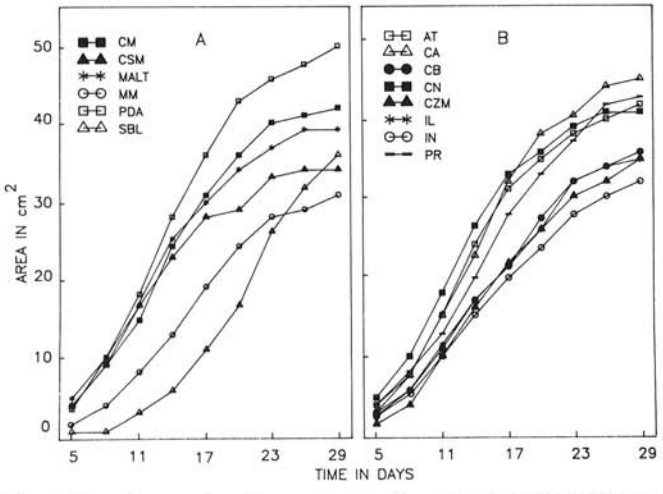


Fig. 3. Mean increase in colony area over time, calculated from diameter (assuming colony to be circular), over all eight isolates of *Cercospora* on each medium (A) and all six media for each isolate (B). Media: malt medium (MALT), potato-dextrose medium (PDA), *Cercospora* sporulation medium (CSM), minimal medium (MM), complete medium (CM), and minimal medium with soybean leaves (SBL). Isolates: *C. asparagi* (CA), *C. beticola* (CB), *C. kikuchii* ATCC 86864 (AT), *C. kikuchii* IL (IL), *C. kikuchii* IN (IN), *C. kikuchii* PR (PR), *C. nicotianae* (CN), and *C. zeae-maydis* (CZM). Means are from two replicates for each isolate/medium combination in each of two trials.

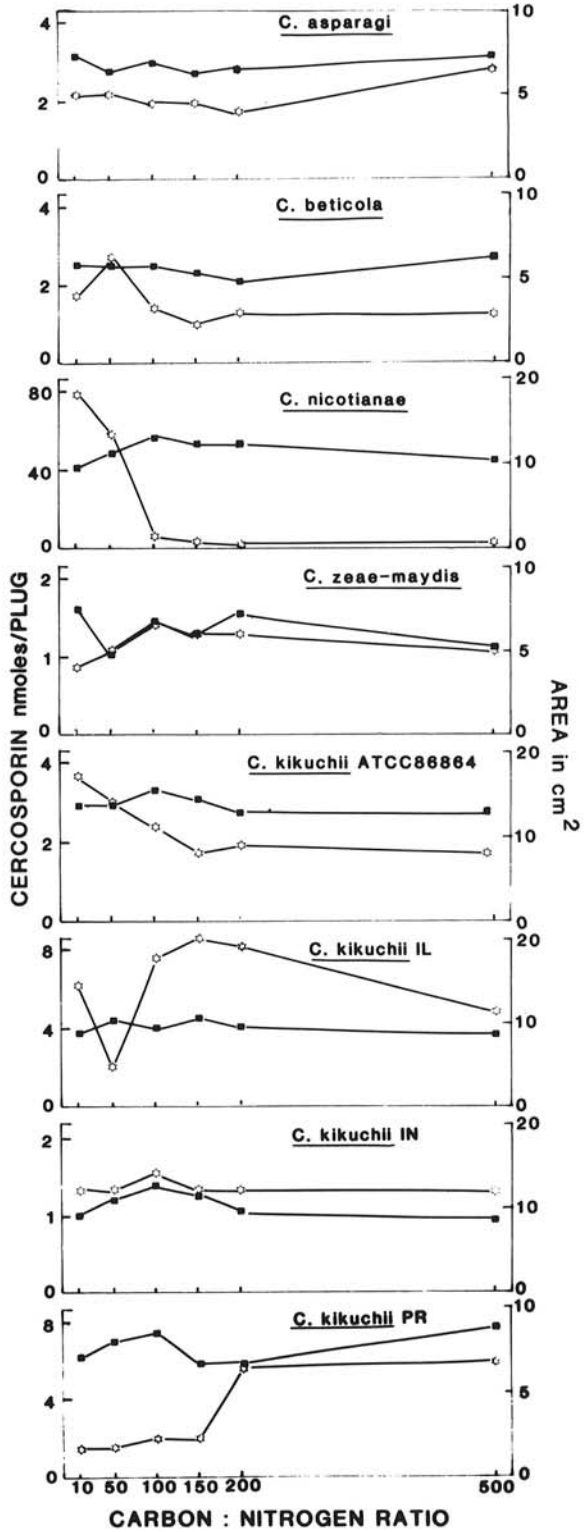
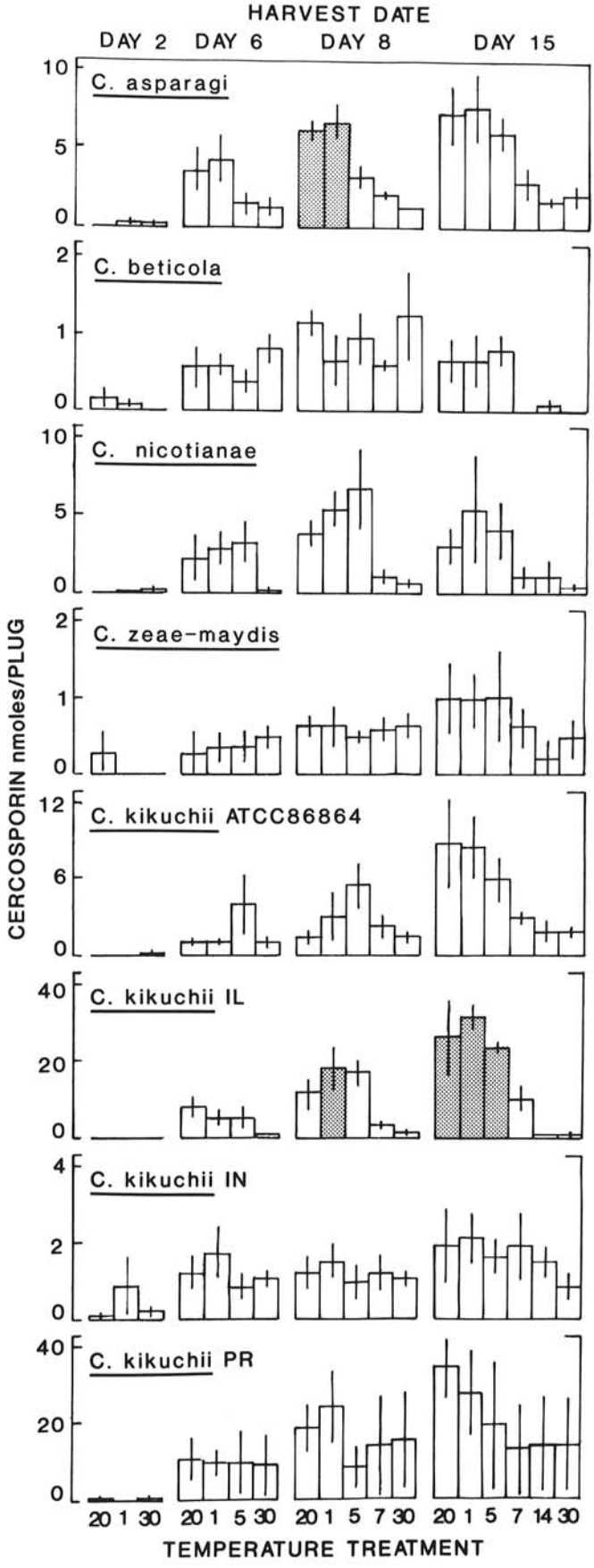


Fig. 4. Mean cercosporin accumulation per plug (6-mm diameter, open stars) and growth (filled squares) by eight isolates of *Cercospora* on media with different C:N ratios. Means are from two replicates in each isolate/C:N ratio combination in each of three trials. Note that ordinate scales are not equivalent.

and 500:1 in *C. kikuchii* PR (Fig. 4).

The most dramatic effects of C:N ratio were on *C. nicotianae* (Fig. 4), which accumulated 36 times as much cercosporin at a C:N ratio of 10:1 as at 500:1. In the other isolates that were significantly affected by C:N ratio, two to four times as much cercosporin accumulated at the most favorable ratio as at the least. In a single trial, media containing no nitrogen or a C:N ratio of 1:1 were included. All isolates of *Cercospora* grew poorly on both these media and all accumulated low or average quantities of cercosporin except *C. beticola*, which accumulated more cercosporin on these media than on those with any other C:N ratio.



Temperature shift experiments. An ultraviolet light-induced mutant of *C. kikuchii* PR, white when grown on SS at 30 C, became red when placed at 4 C overnight. On the assumption that the color change was indicative of rapid toxin accumulation, the effect of temperature on toxin accumulation in eight isolates of *Cercospora* grown on SS was investigated. Temperature conditions during the growth period did not significantly affect levels of cerosporin measured at 2 or 6 days in any isolate or at 8 or 15 days in *C. zeae-maydis* or isolates AT, IN, or PR of *C. kikuchii*. Temperature did have a significant ($P \leq 0.05$) effect on levels of cercosporin measured at 8 days in *C. nicotianae*, at 15 days in *C. beticola*, and at 8 and 15 days in *C. asparagi* and isolate IL of *C. kikuchii*. In *C. asparagi*, significantly more ($P \leq 0.05$) cercosporin accumulated by 8 days in cultures grown at 20 C or switched from 30 to 20 C after 1 day than in cultures grown at 30 C (Fig. 5). In *C. kikuchii* IL, significantly ($P \leq 0.05$) more cercosporin accumulated by 8 days in cultures switched from 30 to 20 C after 1 day, and by 15 days in cultures grown at 20 C or switched from 30 to 20 C after 1 day or 5 days, than in cultures grown at 30 C (Fig. 5). Similar trends were apparent in *C. beticola* and *C. nicotianae*, but differences were not significant.

In a single experiment, in which other media were used to grow cultures at different temperatures, higher levels of cercosporin were found in cultures grown for 5 days at 20 C than at 30 C on PDA (for *C. asparagi, C. beticola*, and isolates ATCC 86864, IL, and PR of *C. kikuchii*), on malt (for *C. asparagi* and *C. kikuchii* IL and PR), and on S medium (for *C. asparagi, C. zeae-maydis*, and *C. kikuchii* IL).

Cultures of *C. kikuchii* IL and PR grown in low light accumulated significantly ($P \leq 0.05$) more cercosporin than those grown in darkness under any set of temperature and medium conditions tested (Fig. 6). There were, however, significant interactions between light and isolate ($P = 0.0022$), light and medium ($P = 0.0001$), and light and temperature ($P = 0.0037$). Darkness did not completely inhibit cercosporin accumulation; for example, as much cercosporin accumulated in dark-grown cultures of PR on PDA as in light-grown cultures of IL on malt medium (Fig. 6).

DISCUSSION

The simple extraction method employed in this study facilitates the measurement of cercosporin accumulation in large factorial experiments. This method is a great improvement on visual estimation of cercosporin content since masking of the red pigment by darker pigments can occur, and all red pigments seen in cultures of *Cercospora* are not cercosporin (4).

Growth media had large effects on the production of cercosporin in agar cultures of the isolates of *Cercospora* tested, and these effects differed among isolates. The best media for cercosporin production in most of the isolates tested were PDA and malt

Fig. 5. Mean cercosporin accumulation per plug (6-mm diameter) in eight isolates of *Cercospora* grown continually at 20 C (20), 30 C (30) or shifted from 30 to 20 C after 1 day (1), 5 days (5), 7 days (7), or 14 days (14) after the start of the experiment. Cercosporin levels were determined at 2, 6, 8, and 15 days after the start of the experiment. Lines on bars represent the standard error of the mean. Shaded bars represent means that differ significantly at $P \leq 0.05$ by Tukey's studentized range test from the mean at 30 C of the same isolate on the same harvest date. Means are from one observation for each isolate/temperature treatment combination in each of three trials.

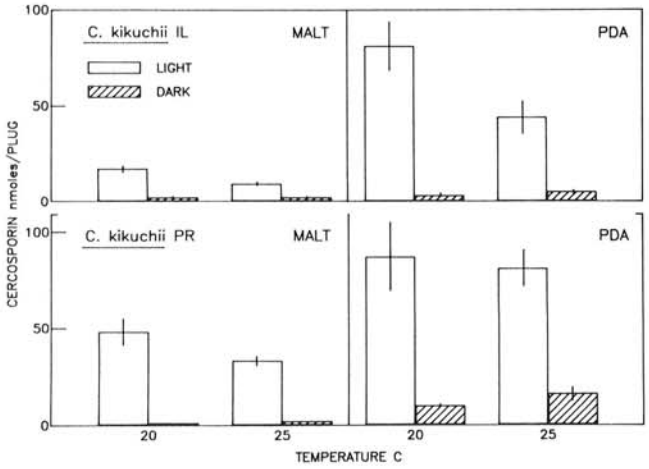


Fig. 6. Mean cercosporin accumulation per plug (6-mm diameter) in two isolates of *C. kikuchii* grown for 8 days at 20 or 25 C on potato-dextrose medium (PDA) or malt medium (MALT), in constant darkness (DARK) or 16 hr of light (4 $\mu E \cdot m^{-2} \cdot sec^{-1}$) and 8 hr of dark (LIGHT). Lines on bars represent standard error of the mean. Means are from three replicates in each of two trials.

medium, but *C. nicotianae* accumulated the most cercosporin on a defined medium containing major and minor salts, sucrose, and $NaNO_3$. CSM did not promote sporulation and suppressed cercosporin accumulation in all the isolates tested. Although the effect of medium on growth was significant at 20 days, the mean area of mycelium of isolates on the least favorable medium for cercosporin production (CSM) was 68% of that on the most favorable (PDA). The cercosporin level on CSM was, however, only 21% of that on PDA, illustrating that the dramatic effects of media on cercosporin production are not necessarily accompanied by similar effects on growth. Dry weight of mycelium is a more accurate measure of growth than area of the colony, since fungal mats may vary in thickness among isolates, temperatures, media, and light conditions. Area and dry weight are, however, significantly correlated. Our results are in agreement with those of Kuyama and Tamura (5), who found malt to be the best of the six media tested for cercosporin production in *C. kikuchii* and Fajola (3), who found PDA the most favorable of the six media tested for cercosporin production in *C. ricinella* Sacc. and Berl.

A thorough study involving C:N ratio effects would involve changing the concentration of C as well as of N. Lynch and Geoghegan (8) varied the concentration of sucrose in media containing a fixed concentration of sodium nitrate as nitrogen source. They found that growth of *C. beticola* was severely reduced at C:N ratios lower than 10:1 but that cercosporin accumulation was minimal at all C:N ratios unless nitrogen was omitted from the medium. Changes in nitrogen content in a medium containing a fixed concentration of sucrose did, however, alter cercosporin accumulation by *C. beticola* very dramatically (8). Because our objective was to find sets of conditions that would drastically alter cercosporin accumulation, we investigated the effects of C:N ratio only in media with constant carbon concentration.

The C:N ratio affected cercosporin accumulation in several of the tested isolates without affecting radial growth. The effect of C:N ratio on cercosporin accumulation differed markedly among isolates; the C:N ratios favoring toxin accumulation were high in *C. nicotianae*, intermediate in *C. beticola* and *C. kikuchii* IL and low in *C. kikuchii* PR, while cercosporin accumulation in the remaining isolates was unaffected by C:N ratio. None of the isolates in this study responded to C:N ratio as did the isolate of *C. beticola* used by Lynch and Geoghegan (8). With their isolate, cercosporin production increased with C:N ratio up to a maximum at 150:1 (8). Thus, while the C:N ratio of the growth medium is quite likely to regulate cercosporin accumulation by any given isolate of *Cercospora*, no generalizations can be made about the nature of such an effect, even with knowledge of the behavior of another isolate of the same species.

Cercosporin accumulation was also regulated by temperature in some isolates. On several media, these isolates showed inhibition of cercosporin accumulation at 30 C and no inhibition at 20 C. Shifting cultures from 30 to 20 C often increased cercosporin content of the cultures, but after a certain age, which differed among isolates, cultures could no longer be induced to increase cercosporin content. Fajola (3) tested growth temperatures between 10 and 35 C and found 22.5 C to be optimum for cercosporin production in *C. ricinella*. Although temperatures between 20 and 25 C do favor cercosporin accumulation in some isolates of *Cercospora*, this was not even true of all eight isolates tested in this study and should not be assumed for untested isolates.

Light is known to be a strong regulator of cercosporin accumulation in *Cercospora* (2,3,8). When all other conditions were held constant, light-grown cultures had much higher levels of cercosporin than those grown in darkness. However, the effect of light interacted significantly with those of isolate, medium, and temperature.

Our examination of the regulation of cercosporin accumulation by medium, temperature, and light has revealed much specific information about the eight isolates of *Cercospora* tested but little information generally applicable to the species. Patterns of regulation differed markedly even among isolates of the same species. Although temperatures from 20 to 25 C seem to favor cercosporin accumulation, this may not be true for every isolate and may also depend on the growth medium. Although malt medium and PDA favor accumulation in general, there are also exceptions. The C:N ratio in a medium is very likely to affect cercosporin accumulation, but how it will do so is unpredictable. Although light strongly affects cercosporin production, its effect is modified by isolate, medium, and temperature. These data cast doubt on failures to detect cercosporin in *Cercospora* species screened by using a single set of cultural conditions and suggest the unreliability of correlating toxin production in vitro to the virulence of an isolate of *Cercospora*, supporting the statements of Yoder (21).

The specific knowledge gained about the regulation of cercosporin production in these eight isolates of *Cercospora* will be used to study the mechanism of the regulation of this toxin in specific isolates. Proteins from cultures grown under sets of conditions that favor or suppress cercosporin accumulation are being subjected to SDS polyacrylamide gel electrophoresis. At 20 C, which favors cercosporin accumulation, *C. kikuchii* IL produces proteins with a banding pattern different from that produced at 30 C, which suppresses cercosporin accumulation. Proteins in cultures grown on media or in light conditions that regulate cercosporin accumulation are under study. Although some of the factors regulating cercosporin production differ among isolates of *Cercospora* isolates, the underlying mechanism of such regulation may prove common to all.

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