Triglycerides and Cercosporin from Cercospora beticola: Fungal Growth and Cercosporin Production

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ABSTRACT

Compounds which are phytotoxic to sugar beet leaves were isolated from *Cercospora beticola*. The factors affecting their production and chemical composition were studied. They were identified as cercosporin, and mixtures of triglycerides having octadecenoic, octadecadienoic, octadecatrienoic, and hexadecanoic acids. Auto-oxidation products of fatty acids were also detected. Analytical and spectrometric data suggest that the molecular formula of cercosporin is $C_{29}H_{26}O_{10}$. Cercosporin was found to be photosensitive at pH values higher than 7.7. Inhibition of growth of *C. beticola* and enhancement of cercosporin production were induced by light only in colonies growing on media supplied with ascorbic acid. Phytopathology 61: 1477-1484.

Additional key words: characterization, photodecomposition, nuclear magnetic spectrum.

The sudden appearance of necrotic area in the leaves of sugar beet following infection with *Cercospora beticola* Sacc. has been attributed to the action of a toxin. Evidence for this possibility was first reported by Schlösser (13). He isolated from cultures of *Cercospora beticola* a chloroform-soluble, yellow substance which inhibited growth of many species of bacteria, and was phytotoxic to higher plants. Schlösser (14) postulated that this unidentified substance could account for the difference in virulence among the various isolates of *Cercospora beticola*.

However, as early as 1957, Kuyama & Tamura (7) isolated a crystalline red pigment, cercosporin, from the mycelium of *Cercosporina kikuchii*. Further investigations suggested that this pigment was a perylene derivative having an extended quinone system (6, 8). Venkataramani (18) also reported the isolation of cercosporin from *Cercospora personata*, which he found was toxic to groundnut plants

The present work deals with the isolation and characterization of compounds produced by *Cercospora beticola* and conditions favoring their photo-oxidation.

MATERIALS AND METHODS.—*Source and maintenance of isolates.*—In most experiments, a monosporic isolate (C-2) from the NB 7 cultivar of sugar beet growing at the Colorado State University Agronomy Farm was used. Three other isolates were also used to compare their ability to produce cercosporin: C-1, a monosporic isolate obtained from R&G Pioneer growing at the Colorado State University Agronomy Farm; D-4 isolate, kept in culture for at least 3 years; and C-2a, initially obtained from a white segment that appeared in a culture of the C-2 isolate. The fungi were cultured on potato-dextrose agar (PDA), and were stored at 4 C.

Culture media.—Beet leaf dextrose agar.—(4). Fresh sliced sugar beet leaf blades (200 g) were boiled in 1 liter distilled water for 15 min and strained through double-layered cheesecloth. The extract was diluted to 1 liter with distilled water and autoclaved at 1 atm for 20 min in 50-ml lots before storage at room temperature. The beet leaf-dextrose medium contained 100 ml beet leaf extract, 20 g dextrose, and 15 g Bacto-agar/liter.

Synthetic agar medium.—Composition of this medium was as follows: $MgSO_4 \cdot 7H_2O$, 0.5 g; $FeCl_3$, 0.2 mg; $ZnSO_4\ 7H_2O$, 0.2 mg; $MnSO_4 \cdot 4H_2O$, 0.1 mg; KH_2PO_4, 0.908 g; K_2HPO_4, 1.160 g; $NaNO_3$, 1.0 g; dextrose, 20 g; Difco yeast extract, 1.0 g; and Bacto-agar, 15 g/liter.

Liquid medium for pigment production.—This medium contained 900 ml synthetic medium and 100 ml beet leaf extract/liter.

Growth conditions.—The effect of various treatments on growth and cercosporin production were studied by culturing the fungus on 10 ml nutrient agar in 125-ml conical flasks. Various amounts of ascorbic acid or other growth stimulants were added in 1 ml water to 10 ml melted synthetic agar medium. All flasks were autoclaved at 1 atm for 20 min and, when cool, each was inoculated at the center with a 4-mm agar disc cut from the margin of a growing colony on PDA. Three out of six replicate flasks for each treatment were wrapped with aluminum foil, and the other three were left uncovered. The cultures were incubated in a constant-temperature cabinet at

25 ± 1 C. The light source consisted of two cool-white fluorescent lamps (F20T12/CW) arranged along the ceiling of the cabinet. Both dark-grown and light-grown cultures were kept in the same cabinet, and were arranged at random 25 cm from the lamps.

For large-scale production of colored compounds, 500 ml of liquid culture solution in conical flasks (2,800-ml capacity) were inoculated with 1.0 ml of *C. beticola* spore suspension. Eleven flasks were wrapped with aluminum foil; the other 11 were left uncovered. All flasks were kept at 25 C, and ca. 30 cm from two (cool-white F20T12/CW) fluorescent lamps. During the 22-day incubation period, the cultures were shaken once a day.

Extraction of colored compounds.—Colonies on nutrient-agar, or the freeze dried mycelium from liquid cultures, were repeatedly extracted with ethyl acetate until the filtrate was colorless. The filtrates were washed several times with distilled water; then the ethyl acetate fraction was evaporated in a vacuum rotary evaporator. The colored compounds in the culture filtrates were isolated by lowering the pH to about 2.0 with hydrochloric acid, then extracting several times with $CHCl_3$.

Thin-layer chromatography (TLC).—Precoated TLC plates (20 x 20 cm) of silica gel without a fluorescence indicator were used to separate the pigments. The plates were pretreated by dipping in 2% H_3PO_4 and drying in an oven at 100 C. The solvent system was ethyl acetate:benzene (2:3).

Isolation of colored compounds.—The compounds were separated on "dry columns" (1 x 60 cm) of silica gel. In preparation, 200 g silica gel were mixed with 500 ml of an aqueous solution containing 10 g H_3PO_4 and 20 g $Ca(H_2PO_4)_2 \cdot H_2O$. The resulting slurry was dried in an oven at 100 C for 24 hr and, when cool, homogenized in a glass mortar. The crude extract from the *C. beticola* mycelium was dissolved in $CHCl_3$; silica gel (3 g) was then added and mixed thoroughly when the solution was adsorbed, and the silica gel was dried and placed at the top of the "dry column". Ethyl acetate:benzene (2:3) was percolated slowly into the column, and the separate colored fractions were collected as they were eluted. The first bright yellow fraction (YF) was collected and evaporated in vacuo to a thick oil. A small red band was rejected, but the next band, which was a deep red (RF), was also collected and evaporated in vacuo to dryness. The YF was further purified by column chromatography on untreated silica gel using benzene:methanol (95:5) for elution. The RF dry residue was dissolved in methanol and mixed with an equal volume of 2% HCl in water. The resulting clouded solution was allowed to stand at 0 C, and needle crystals with a metallic reflection were separated after 3-4 days. These were purified still further by recrystallization.

During the process of purification, the pigments were checked for homogeneity by TLC. Care was also taken to avoid high temperatures (< 24 C), and the pigments were stored at −10 C.

Quantitative determination of cercosporin.—Preliminary tests showed that solutions of ethanol prepared by eluting a part of the TLC plate which had been irrigated by the solvent system but not spotted with crude extract had the same absorbance as the pure ethanol at the wavelength 480 mμ. From these tests, it was evident that H_3PO_4 and possible contaminants in the silica gel eluted with ethanol could have little, if any, interference during the quantitative estimation of cercosporin. However, the determinations were carried out using a blank ethanol solution prepared by eluting a part of the TLC plate of a similar size to the spots given by cercosporin.

The R_f values of cercosporin on silica gel plates, treated with 2% H_3PO_4 in four different solvent systems, were used as a criteria for purity. The solvent systems were ethyl acetate:benzene (2:3), R_f 0.110; cyclohexane:benzene (10:2), R_f 0.0; benzene:methanol (8:2), R_f 0.485; *n*-butanol:pyridine:saturated solution of NaCl (1:1:2) (organic phase), R_f 0.851. Molar extinction coefficient at 480 mμ is $\epsilon = 2.27 \times 10^{-4}$.

Beet leaves were extracted with ethyl acetate, and the extract was mixed with a known amount of cercosporin. The TLC of this mixture showed that cercosporin was separated from other substances. The cercosporin was recovered from the plates quantitatively, providing evidence that the method was specific for cercosporin estimation.

The crude extract from *C. beticola* cultures on nutrient-agar was separated quantitatively by the TLC method described. The red-stained area corresponding to the position of cercosporin was scraped out, then eluted with ethanol. The silica gel scrapings were removed by filtration through a fritted glass Büchner funnel (pore diam 10 to 15 μ). All quantitative estimations were carried out spectrophotometrically by the measurement of the absorbance of the ethanol filtrate completed to a known volume, at 480 mμ (λ max). Values of absorbance were transformed to mg of cercosporin through an experimentally standardized curve.

Phytotoxicity tests.—The following methods were used to assess the ability of the various fractions to cause necrosis of sugar beet leaves. (i) Four mg of the compound under test were dissolved in 0.4 ml ethanol; then 0.1% aqueous $NaHCO_3$ added to give a final volume of 4 ml. Each leaf was punctured at various locations with a fine needle, and two to three droplets (ca. 0.1 ml) of the solution were applied on each injury on the upper surface of half the leaf. The other half of the leaf was treated similarly with a solution of the same composition as above except that the test compound was omitted. (ii) Droplets of an ethanolic solution of the test compound (1 mg/ml) were applied on half of each beet leaf, but in this test the leaf was not injured mechanically. As in the previous test, the other half of the leaf acted as a control and was treated with pure ethanol.

Leaves of five different cultivars of sugar beet growing in pots in a greenhouse were used; after treatment, the plants were covered with polyethylene bags and kept in the greenhouse. The second test was repeated on detached leaves in the laboratory. In this case, the leaves were put in a glass cabinet and were

kept 30 cm from two cool-white fluorescent lamps (F20T12/CW) arranged above the cabinet.

RESULTS.—*Isolation of pigments.*—Table 1 shows that RF in both culture filtrate and mycelial extract was produced in larger quantities in the light than in darkness, yet the mycelial growth was favored by darkness. The YF from mycelium extract was produced about the same in the light and darkness. In subsequent experiments, the effect of light on fungal and RF production was examined more closely.

Characterization of YF.—The infrared absorption spectrum (KBr window) gave bands at 2,920 cm^{-1} and 2,890 cm^{-1} for methyl and methylene groups, 1,737 cm^{-1} for ester carbonyl groups, 1,185 cm^{-1} for ester -C-O-, and 723 cm^{-1} for long-chained methylene groups which are characteristic of esters of polyalcohols and long-chain fatty acids. Furthermore, a peak at 3,000 cm^{-1} indicated the presence of double bond(s) in the fatty acid chains, whereas absence of O-H stretching region at about 3,340 cm^{-1} and C-O stretching at about 1,040 cm^{-1} is characteristic of triglycerides (11).

The nuclear magnetic spectrum (NMR) ($CDCL_3$·60 MH_Z) showed signals at Σ 0.88 assigned to the terminal methyl protons, at Σ 1.29 of methylene groups in carbon chains, at Σ 2.00 of CH_2 protons adjacent to -CH= in a chain, at Σ 2.30 assigned for CH_2 in -O-C-CH_2-, a weak signal at Σ 2.80 assigned to 1,4-diene (=$CHCH_2$CH=), at Σ 4.10 for CH_2 in -CH_2-O-C-, and at Σ 5.40 assigned to ethylenic double bonds. All these signals and the absence of signals at Σ 3.60 for CH_2 in -CH_2OH and Σ 3.80 for CH in -CHOH suggest the presence of triglycerides.

The mass spectrum revealed peaks at m/e 882, 880, 878, 876, 872, 856, 854, 852, 832, 830, and 828 which are consistent with molecular ions for triglycerides of octadecenoic (oleic), octadecadienoic (linoleic), octadecatrienoic (linolenic), and hexadecanoic (palmitic) acids.

In addition, a number of peaks at m/e 265, 263, 261, and 239 must be ascribed to the acyl ions of octadecenoic, octadecadienoic, octadecatrienoic, and hexadecanoic acids, respectively.

TABLE 1. Growth of *Cercospora beticola* and colored compound production in presence/absence of light after 22 days' incubation at 25 C in 5.5 liters liquid culture medium[a]

	Light	Darkness
Mycelial dry wt (g)	20.0	27.1
Red fraction (mg) from		
Culture filtrate	7.0	0.0
Mycelial extract	82.5	0.8
Yellow fraction (mg) from		
Mycelial extract	250.0	248.4

[a] Liquid culture medium contained 900 ml synthetic medium and 100 ml beet extract/liter. Synthetic medium and beet leaf extract described under MATERIALS AND METHODS.

Methanolysis.—The methyl esters of fatty acids of the triglyceride mixture were prepared according to the method of H. Kurz described by Markley (9) and Kato et al. (5).

The infrared absorption spectrum of the methyl esters gave stretching vibrations very similar to those reported by Kato et al. (5) for methyl esters obtained by methanolysis of monoglycerides from *Sepedonium ampullosporum*. It is noticeable that all the bands were characteristic of methyl esters of long-chained fatty acids with double bonds in the chains.

The NMR spectrum showed a strong signal at Σ 3.67 assigned to methyoxy groups, whereas the signal at Σ 4.10 for CH_2 in -CH_2-O-C- in the spectrum of the untreated YF disappeared. This suggests that the glycerol was removed from the triglyceride molecule during methanolysis, and that the fatty acids were esterified with methanol. This was further confirmed by the detection of glycerol in the aqueous phase after methanolysis and the mass spectrum of the methyl esters. The fact that the infrared spectrum of a product obtained from the aqueous phase on methanolysis was identical with the spectrum shown by authentic glycerol leaves no doubt of the identity of this product as glycerol.

The mass spectrum showed prominent peaks at m/e 296, 294, 292, and 270 corresponding to the molecular ions of methyl esters of octadecenoic, octadecadienoic, octadecatrienoic, and hexadecanoic acids, respectively. Also, the acyl ions were accordingly shown at m/e 265, 263, 261, and 239.

Thus, all the above information leads to the conclusion that the YF isolated from the cultures of *C. beticola* is mainly a mixture of triglycerides of the previously mentioned acids.

Detection of auto-oxidation products.—Since methylene-interrupted polyunsaturated systems are rapidly oxidized by atmospheric oxygen (17), it is reasonable to expect that a certain degree of auto-oxidation had occurred during the process of isolation and purification of the triglyceride mixture.

Oxidative decomposition of YF was first suspected by the following observation. One dimension TLC of the YF fraction revealed, besides the principal spot (R_f = 0.60), several other minor spots visible only under ultraviolet light. Furthermore, second dimension chromatography of plates previously kept for a few hours in the air showed that the YF spot was again separated, giving the spots shown by the first dimension chromatography. However, this second fractionation of the YF spot was eliminated by keeping and chromatographing the plates in dark and in an inert atmosphere of nitrogen.

This observation was considered as evidence that the unidentified YF at that time was an unstable, easily oxidized substance. The presence of auto-oxidation products of polyunsaturated fatty acids was further confirmed by thiobarbituric acid tests (19) and the production of an orange-red color, or the so-called "alkali color", when the YF substance was dissolved in alcoholic sodium hydroxide (17).

Characterization of the RF substance.—Samples of the crystalline preparation of RF (See MATERIALS

AND METHODS) were used for all analytical and spectrometric studies.

The crystals were found to contain no nitrogen, chlorine, sulfur, or phosphorus, and melted at 238 C (Thomas Hoover, capillary melting apparatus, Unimel) without sublimation or decomposition. They were insoluble in water and sodium hydrogen carbonate, partially soluble in sodium carbonate (giving a pale greenish solution), and very soluble in dilute sodium hydroxide (giving a clear solution with an intense green color). They were insoluble in petroleum ether and benzene, partially soluble in carbon tetrachloride, and readily soluble in pyridine, dioxane, chloroform, ethyl acetate, alcohol, and acetone. They were soluble in concentrated sulfuric acid, giving a purple color without fluorescence, and a blue-purple precipitate was formed when water was added. The alcoholic solution turned brown-red with ferric chloride and green with magnesium acetate. Reduction with zinc dust gave, in glacial acetic acid, a yellow solution with green fluorescence and, in alkali, an orange solution with yellow fluorescence. Oxidation of the reduced solution by air gave the original color.

All the above properties are identical to those reported by Kuyama & Tamura (7) and Venkataramani (18) for cercosporin.

The identity of the RF compound with cercosporin is further supported by the following spectrometric data. The ultraviolet spectra, Fig. 1, have absorption maxima (λ max) similar to those given for cercosporin (7, 18), whereas the infrared absorption spectrum (KBr window), Fig. 2, is identical. Major bands occurred at 3,400, 2,940, 1,619, 1,585, 1,554, 1,455, 1,428, 1,395, 1,348, 1,315, 1,268, 1,223, 1,170, 1,145, 1,113, 1,075, 1,055, 1,017, 978, 938, 921, and 850 cm^{-1}.

The NMR spectrum ($CDCl_3$, 90 MH_Z) shows a singlet at Σ 14.82 (1 H), suggesting the presence of a hydrogen-bonded phenol or enol; a singlet at Σ 5.63 (1 H) assigned to a quinoid carbonyl group; and a singlet at Σ 4.14 (5 H), probably due to two methoxys. The high resolution mass spectrum (HRMS), Fig. 3, revealed a molecular ion at m/e 534.1542 corresponding to an elemental composition of $C_{29}H_{26}O_{10}$.

Although the empirical formula, $C_{30}H_{28}O_{10}$, has been proposed for cercosporin (7, 18), the formula $C_{29}H_{26}O_{10}$ is in very good agreement with the analytical data given for this compound. Calculated for $C_{29}H_{26}O_{10}$: C, 65.17; H, 4.90. Found by analysis of cercosporin: C, 65.50, 65.67; H, 4.83, 4.85 (7); C, 65.54; H, 4.82 (18). Furthermore, a molecular weight of 540 reported by Venkataramani (18) is closer to the parent peak m/e 534 than to 584 as given for cercosporin.

In view of the chemical and physical properties and the spectrometric data of the RF compound, it is reasonable to conclude that this substance is the same as cercosporin isolated from *Cercosporina kikuchii* (7) and *Cercospora personata* (18).

Analysis of the crystals grown in diluted aqueous ethanolic hydrochloric acid (see MATERIALS AND METHODS) showed the presence of one molecule of water of crystallization for each molecule of cercosporin. Calculated for $C_{29}H_{26}O_{10}\,H_2O$: C, 63.04; H, 5.11; O, 31.85. Found: C, 63.27; H, 5.53; O, 31.16.

Effect of light and pH on decomposition of cercosporin.—Although cercosporin in ethanol, or other common organic solvents, is stable on exposure to light, it was photosensitive in dilute alkali solution. A solution of cercosporin in 0.1 *N* sodium hydroxide has a clear green color, and its ultraviolet spectrum is characterized by absorption maxima (λ max) at 223, 254, 299, 479, 595, and 640 mμ (Fig. 1). Exposure of this solution to a 20 w daylight lamp for 36 hr changed the green to a yellow color, and all absorption bands at long wavelength disappeared (Fig. 1).

In two further experiments, the effect of pH on the photodecomposition of cercosporin was studied. Aliquots of 0.05 ml of a methanol solution of cercosporin were made up to 20 ml with the following buffer solutions in pyrex glass tubes: 0.1 *M* KH_2PO_4-0.1 *M* NaOH; 0.02 *M* borax-0.1 *M* HCl; 0.025 *M* borax-0.1 *M* NaOH; 0.05 *M* Na_2HPO_4-0.1 *M* NaOH; and 0.2 *M* KCl-0.2 *M* NaOH. The final concentration of cercosporin was 0.1 mg/10 ml. The tubes of one of the two pH series were wrapped with aluminum foil, whereas those of the second series were left uncovered. Controls without cercosporin were similarly prepared. All tubes were arranged at a distance of ca. 30 cm from a 20 w daylight lamp. For the first experiment the absorbance, at λ = 480 mμ, was measured immediately after preparation of the solutions and after 20-hr exposure to light, whereas in the second experiment, the decomposition of cercosporin was assessed at various intervals up to 20 hr after exposure to light.

It is evident from Fig. 4 that, in the presence of light, though not in darkness, the amount of cercosporin decomposed increased rapidly with increase in pH above 7.7. As might have been anticipated, the rate of photodecomposition of cercosporin was enhanced similarly in solutions adjusted at a pH higher than 7.7 (Fig. 6). It is of interest to note that solutions with pH 8.4 or higher were green, whereas those with pH 7.7 or lower were red. Thus, in this range, cercosporin behaves as an indicator. At low pH values, cercosporin was separated from its solution, giving a red precipitate.

Phytotoxicity tests.—Both cercosporin and the mixture of triglycerides from *C. beticola* were

TABLE 2. Production of cercosporin by four *Cercospora beticola* isolates grown on beet leaf-dextrose agar for 8 days at 25 C and diurnal exposure to daylight

Isolates	Diam of colonies (mm)	Cercosporin (mg/culture)
C-1	25.3	0.090
C-2	36.0	0.191
C-2, 1	36.5	Traces
D-4	37.3	0.025

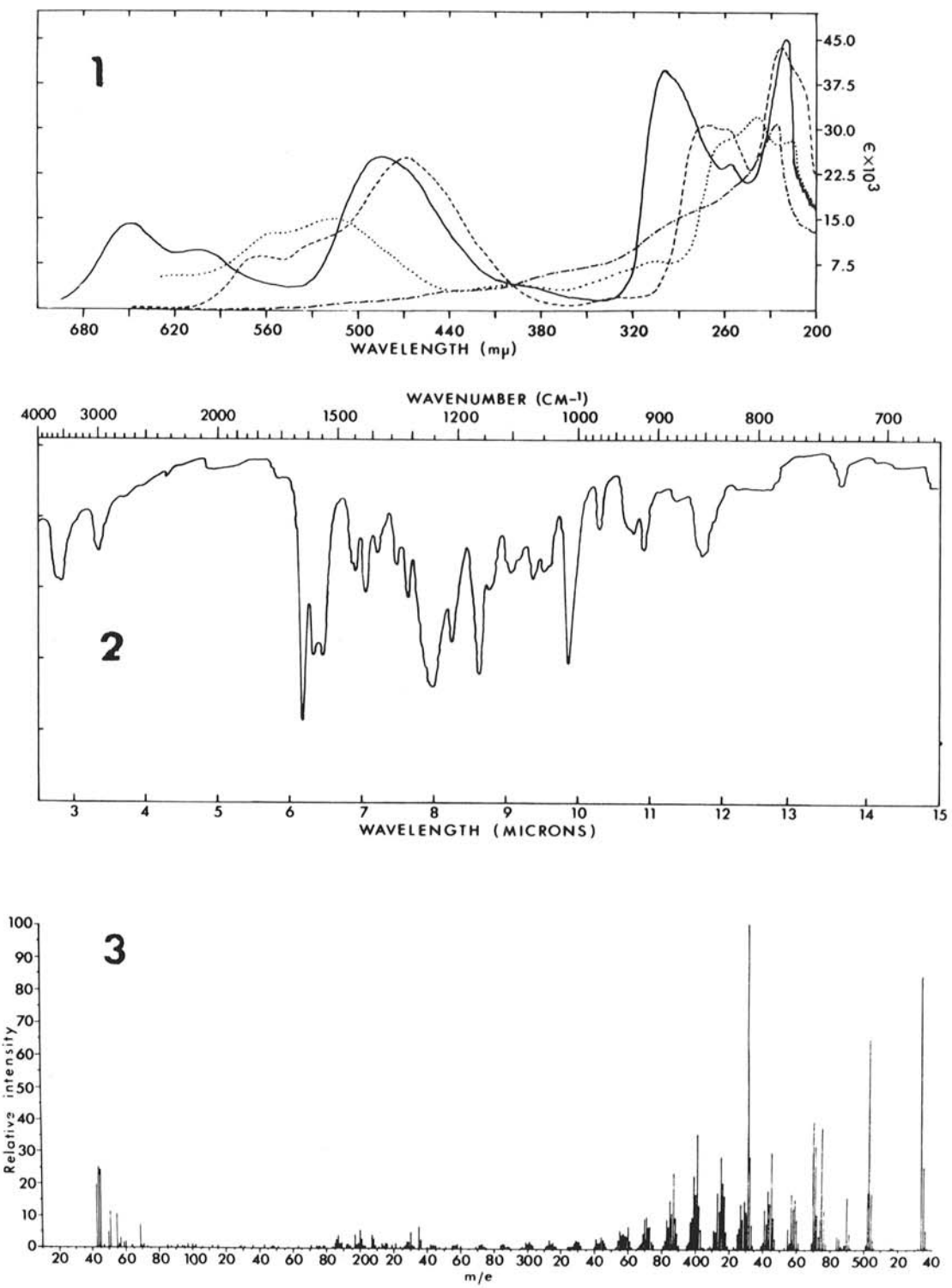
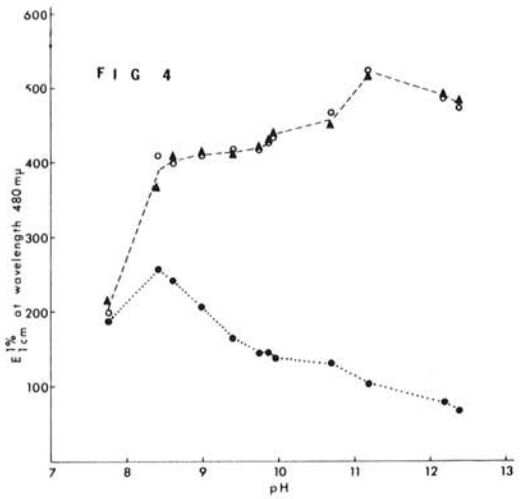
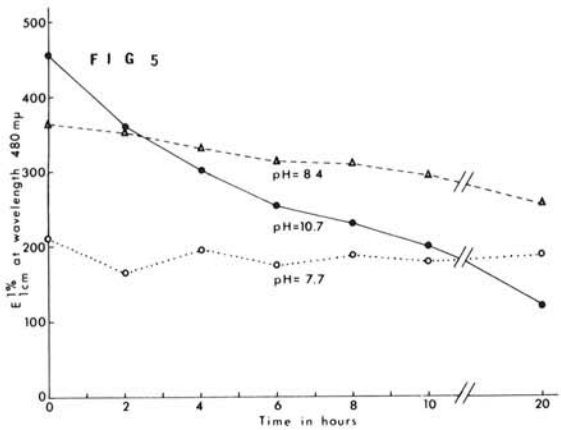


Fig. 1-3. 1) Ultraviolet spectrum of cercosporin: . . . . in concentrated H_2SO_4; – – – – in methyl alcohol; _____ in 0.1 *N* NaOH; —·—·— in 0.1 *N* NaOH after exposure to 20-w daylight lamp for 36 hr. 2) Infrared spectrum of cercosporin (KBr pellet). 3) Mass spectrum of cercosporin.





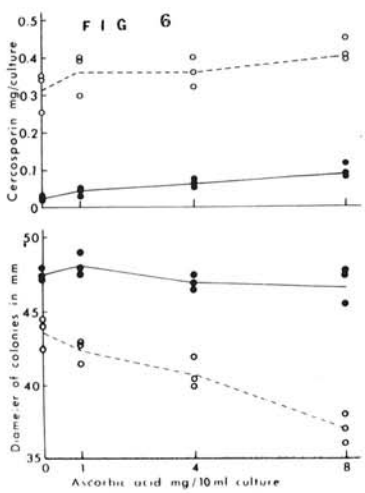


Fig. 4, 5, 6. 4) Effect of pH on photodecomposition of cercosporin: ○ fresh solution; ▲ After 20 hr in darkness; ● after exposure to 20-w daylight lamp for 20 hr. **5)** Effect of pH on photodecomposition rate of cercosporin; **6)** Effect of ascorbic acid on growth of *Cercospora beticola* and cercosporin production in presence/absence of light. ● Cultures kept in darkness; ○ cultures kept under light. Incubation for 12 days at 25 ± 1 C.

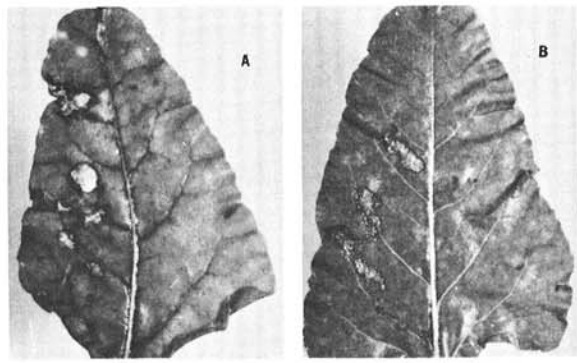


Fig. 7. Lesions on sugar beet leaf surface caused by 0.1 mg of cercosporin (A) and 0.1 mg of triglycerides (B) from *Cercospora beticola*. On left part of leaf, compound dissolved in ethanol; on right side, ethanol alone.

phytotoxic to sugar beet leaves (Fig. 7). However, results were variable with the YF, and cercosporin appeared to be more toxic.

In bioassays of cercosporin, where the leaves were not injured mechanically, necrotic spots appeared within 3 to 4 days with a gray-brown halo in most of the tested cultivars, or a red one in naturally red or reddish varieties of sugar beet. Mechanical injury at the time of application of the test solution hastened the development of the spots, but these spots were similar to those described above. In a number of tests, young and old leaves showed similar sensitivity to cercosporin.

When leaves treated with cercosporin were kept for longer periods of time (up to 6 or more days), a mycelium covered the necrotic areas. Microscopic examination and isolation techniques showed that nearly all spots had been infested by a fungus identified as *Alternaria tenuis*. No tests were made to detect the presence of bacterial contaminants, but we do not want to exclude the possibility. The development of *Alternaria* should be mentioned.

Bioassays with five cultivars of sugar beet in the greenhouse showed a positive relationship between resistance to leaf spot disease and sensitivity of the tested cultivars to cercosporin. However, subsequent tests, on detached leaves from five more cultivars of plants growing in the field, showed that the previously observed relationship was not real.

Production of cercosporin by four C. beticola isolates.—Cercosporin production was assessed on triplicate 8-day-old cultures of four different isolates of *C. beticola* grown on beet leaf-dextrose agar at 25 C with diurnal exposure to daylight. Table 2 shows that various isolates of *C. beticola* differ greatly in ability to produce cercosporin. It is interesting to note that the most active cercosporin producers were the isolates C-2 and C-1, which had been isolated recently from plants growing in the field.

Effect of light and antioxidants on growth of C. beticola and production of cercosporin.—Observations of an increased colored-substance production in cultures of *C. beticola* kept under light for sporulation led us to suspect that light and

TABLE 3. Effect of various antioxidants on growth of *Cercospora beticola* and cercosporin production[a]

Compound added 0.01% synthetic agar medium	No ascorbic acid		Ascorbic acid (8 mg/10 ml culture)	
	Light	Darkness	Light	Darkness
	Diam of colonies in mm			
Control	31.0	35.0	25.0	33.5
Quercetin	33.0	34.7	24.5	32.5
Quercitrin	32.0	33.5	25.7	32.5
Myricitin	31.7	34.5	22.3	32.0
3-Hydroxytyramine-HCl	30.0	33.7	24.2	35.2
	Production of cercosporin mg/culture			
Control	0.188	Traces	0.480	0.076
Quercetin	0.310	Traces	0.328	Traces
Quercitrin	0.308	Traces	0.560	0.168
Myricitin	0.190	Traces	0.480	0.072
3-Hydroxytramine-HCl	0.328	Traces	0.640	0.020

[a] The fungus was grown on synthetic agar medium for 8 days at 25 C in presence/absence of light. Traces represent amounts of cercosporin detectable by TLC, but giving absorbance near zero.

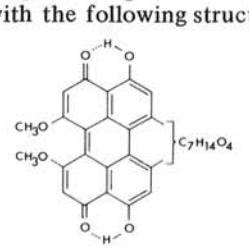
perhaps oxidation conditions might be affecting the pigmentation of *C. beticola*. To ascertain whether either of these factors affect the production of cercosporin, *C. beticola* was grown for 12 days on synthetic agar media containing 0, 1, 4, and 8 mg ascorbic acid (not neutralized) per 10 ml culture medium, under conditions detailed in MATERIALS AND METHODS.

The data on fungal growth and cercosporin production given in Fig. 6 show that whereas light strongly promoted the production of cercosporin to more than 3 times that in the darkness, the growth of *C. beticola* was inhibited. These diverse effects of light are enhanced progressively with an increase in the concentration of ascorbic acid in the culture medium. However, other antioxidants such as quercetin, quercitrin or myricitin were not as active as ascorbic acid in this respect (Table 3). It is evident from the data given in Table 3 that growth of *C. beticola* was inhibited in the presence of light only on the media which had been supplied with ascorbic acid, regardless of the presence or absence of other antioxidants. Cercosporin production, on the other hand, was enhanced by light, particularly in the presence of ascorbic acid. In cultures kept in darkness, the production of cercosporin was also increased by ascorbic acid, but not by the other antioxidants.

DISCUSSION.—It is apparent from the foregoing data that *C. beticola* has the ability to produce at least two phytotoxic substances. These results do not essentially conflict with Schlösser's (13) observations; but suggest that the material which he isolated was the mixture of triglycerides (YF) and cercosporin. In fact, the paper chromatographic techniques employed by Schlösser were inadequate for separation of the triglycerides from cercosporin. With solvent systems such as benzene:methanol (8:2) or *n*-butanol:pyridine:saturated solution of sodium chloride (1:1:2) (organic phase), both cercosporin and triglyceride mixture were moved together with the front of the solvent system, giving one spot only (R_f=1.00). With cyclohexane:benzene (10:2), cercosporin, with its yellow photocomposition product, remained at the starting point, and the YF formed a band which was extended from the starting point to the front. Furthermore, TLC of a precipitate obtained by the method described by Schlösser showed the presence of triglycerides (YF), cercosporin, and traces of iso-cercosporin.

Regarding the toxic properties shown by the YF, the oxidation products of the polyunsaturated fatty constituents are more likely to be responsible for the detected toxic effect on sugar beet leaves than were the triglycerides themselves. Bernheim et al. (1) showed, for example, that oxidized fatty acids are potent inhibitors of certain enzymes, including succinoxidase and cytochrome oxidase of rat liver. The effect of the YF from *C. beticola* and the oxidation products of the triglycerides on the plant tissues need further study. Since cercosporin is insoluble in water, its presence in the culture filtrate must be due to its solubility in fats. Actually, droplets of the YF were detected in the culture solutions along with cercosporin.

From the work of Kuyama (6), it has been established that the parent hydrocarbon of cercosporin is a benzo (ghi) perylene. Furthermore, the groups shown by the NMR spectrum; i.e., quinoid carbonyl, two phenolic hydroxyl groups in position *peri* to the quinone carbonyls and two methoxys, are in agreement with those reported by Kuyama & Tamura (8). The spectroscopic data already presented are consistent with the following structure:



The mass spectral fragmentation appears to be dominated by losses of portions of the $C_7H_{14}O_4$ part of the molecule, but unfortunately, neither mass spectrum nor NMR provided conclusive evidence as to the structure of the $C_7H_{14}O_4$ part of the molecule.

Effects of light similar to those observed on growth and pigmentation of *C. beticola* have often been reported for other fungi (2, 10, 12, 15). However, the observed synergistic effect of ascorbic acid was quite unexpected. Inhibition of the growth of *C. beticola* by light in absence of ascorbic acid was scarcely detectable, and this is in agreement with early observations (3) on the effect of light on sporulation and zonation of this fungus. Furthermore, whereas ascorbic acid increased slightly the production of cercosporin, in the darkness, it did not affect fungal growth. It is, therefore, the combined effect of light and ascorbic acid which caused the observed inhibition of growth and enhancement of cercosporin production. Although these results were obtained by culturing *C. beticola* on artificial media, it is possible that these two factors may affect the fungus similarly in its host plants. This hypothesis is not a mere speculation. It has been shown, for example, that light results in an accumulation of ascorbic acid in various plants (16). If a similar accumulation of ascorbic acid occurs with light in sugarbeet leaves, then the plant, through ascorbic acid and perhaps the synergistic effect of other antioxidants, may possess a mechanism of resistance which merits further exploration.

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