

Ethylene Production by Cultures of *Cylindrocladium floridanum* and *C. scoparium*

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ABSTRACT

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Ethylene was produced by *Cylindrocladium floridanum* and *C. scoparium* in culture. Production was methionine-dependent and occurred during the active growth of the fungus, beginning shortly after spore germination. Ethylene was produced by *Cylindrocladium* both from actively growing mycelium (direct) and nonenzymically from culture filtrates (indirect). Both direct and indirect systems were light mediated, and appeared to involve a fungal metabolite, a flavinlike compound (FLC),

Additional key words: azalea blight, hormone, leaf abscission.

either contained in the mycelium or secreted into the medium. The FLC in culture filtrates exhibited light-absorbing properties similar to flavin mononucleotide, a known cofactor in at least one reported ethylene-generating pathway. Another possible system (either direct or indirect) of ethylene production by *Cylindrocladium* occurred in the dark, exhibited a production lag, and produced less ethylene than the light-mediated system.

Ethylene is an endogenous plant growth regulator that is produced by healthy higher plants, as well as by many microorganisms (8,9). It is frequently produced in increased amounts by diseased plants (4,10,27). Linderman (14) reported that ethylene was a product of the interaction between the fungal pathogen *Cylindrocladium* and azalea (*Rhododendron obtusum* (Lindl.) Planch.) tissue, and was most likely involved in the leaf abscission that occurs with the disease. He further showed that abscised infected leaves play a key role in the epidemiology of the root rot and wilt phase of the disease (13). In any host-parasite interaction in which ethylene is a product, as with the *Cylindrocladium* disease, the question arises as to whether the ethylene evolved came from the host, the pathogen, or both.

Methionine is a precursor for ethylene biosynthesis in higher plants (11) and also enhances ethylene production by some microorganisms (6,16,21,24,26). The role methionine plays in the biosynthesis of ethylene by microorganisms, however, is not clearly understood. The report by Billington et al (1), however, suggests that some microorganisms produce the intermediate 4-methylthio-2-oxobutanoate during ethylene biosynthesis from methionine.

The first objective of this study was to determine whether *Cylindrocladium* spp. were capable of producing ethylene in the absence of a plant host and the second was to learn whether methionine or specific environmental conditions were required for ethylene biosynthesis. The overall objective was to relate that information to possible known or proposed biosynthetic pathways and to the pathogenesis of *Cylindrocladium* on azaleas.

MATERIALS AND METHODS

Cultural conditions and inoculation procedure. Cultures of *Cylindrocladium floridanum* Sobers & Seymour (isolated from *Cercis* sp. by G. Holcomb) and *C. scoparium* Morgan (isolated from azalea by R. G. Linderman) were grown on half-strength malt extract, yeast extract, malt yeast dextrose agar (MYDA) (12) at

25 ± 1 C with a 12-hr photoperiod under fluorescent lights. Single-spore stock cultures were renewed every 3-4 wk. Conidial inoculum, in sterile distilled water, was prepared from 1-wk-old cultures mass-transferred from stock cultures. The spore concentration was adjusted to 10,000-20,000 spores per milliliter with a hemacytometer and checked by dilution plating. One milliliter of spore inoculum was aseptically pipetted into each of a number of volume-calibrated 125-ml Erlenmeyer flasks containing 40 ml sterile modified Pratt's medium (23) supplemented with 10 mM DL-methionine (Sigma Chemical Co., St. Louis, MO 63178). Control flasks were inoculated with 1.0 ml of spore inoculum filtrate obtained by passage through a 0.22 µm Millipore filter. Flasks were covered with a foam plug and aluminum foil, and incubated in a growth chamber at 25 ± 1 C under a 14-hr photoperiod by using fluorescent and incandescent lights (mean light intensity in the growth chamber was 8,694 lux with a standard error of the mean, 228 lux).

Ethylene analysis. Before ethylene analysis, flasks were flushed with 150-200 cm³ of filtered ambient air and held for 15 min under a laminar flow hood before being sealed with ethanol-sterilized serum caps. Sealed flasks were incubated in the growth chamber for 1.0 hr in the light before gas analysis. A 1.0-cm³ gas sample was withdrawn from the flasks with a hypodermic syringe and injected into the gas chromatograph. A Perkin-Elmer 3920 gas chromatograph (Perkin-Elmer Corp., Norwalk, CT 06852) equipped with a flame-ionization detector and a 2.4-m Poropak N (177-149 µm, 80-100-mesh) column was used to determine ethylene concentration in the head space of flasks. Ethylene was identified by cochromatography with an ethylene-in-air standard.

After ethylene analysis, mycelium was collected on Whatman #1 predried and preweighed filter paper. The mycelium was rinsed thoroughly with distilled water in a Büchner funnel and oven-dried for 24 hr at 70 C before weighing.

Statistical analysis. Treatments were replicated four to six times depending upon the experiment. Experiments were repeated twice and replicated the same each time. All experiments were unpaired, and when applicable, a completely randomized design or unpaired Student's *t*-test was used to determine significant differences. Experiments dealing with a percent decrease in ethylene production were analyzed with a statistical equation (17) that gave

an estimate of the standard deviation of the ratio of the treatment contribution/total ethylene and that took into account the variance of all sample flasks in both treatments.

Variations in experimental procedure. In several experiments we varied the standard cultural conditions to test specific treatments on ethylene production. For example, we supplemented modified Pratt's medium with 0, 2, 4, 6, 8, 10 mM DL-methionine to determine the effect of methionine concentration on ethylene production. The methionine concentration of *Cylindrocladium*-inoculated modified Pratt's medium, supplemented with 10 mM methionine, decreased an average of 0.55 mM per day (amino acid analysis was done daily for 96 hr of incubation by the Biochemistry and Biophysics Department, Oregon State University). The assay medium was supplemented with 10 mM methionine for all further experiments to ensure that methionine was not a limiting factor in ethylene biosynthesis. This concentration of methionine only slightly decreased the growth rate of *Cylindrocladium*.

In another experiment, ethylene production was monitored after germination of conidia. Experiments were conducted in volume-calibrated 60-ml vials containing 20 ml of modified Pratt's medium.

Two milliliters of spore inoculum were added and ethylene was sampled 0, 3, 6, 9, 12, and 24 hr after inoculation of vials.

Ethylene production from culture filtrates and heat-killed mycelium also was determined. Filtrates were collected by passing the culture first through cheesecloth and then through a 0.22 or 0.45 μ m Millipore filter. Filtrate was pipetted into an empty sterile 125-ml flask, incubated 1.0 hr, and ethylene-analyzed. The mycelium was heat-killed in a water bath at 75 C for 1.0 hr to determine whether viable mycelium was needed for continued ethylene production. We renewed the original culture medium by centrifuging heat-killed mycelium at 10,000 rpm for 15 min, decanting off the medium, adding 20 ml of sterile distilled water, recentrifuging, decanting off the water, and resuspending the mycelium in sterile medium.

The effects of light and dark incubation on cultures and culture filtrates of *Cylindrocladium* were determined. The experiments were conducted under continuous light and flasks were wrapped in aluminum foil for dark treatment. Flasks were sealed with serum caps during the last hour of all treatments.

Light absorbance of static (still) culture filtrates of *C. floridanum* and *C. scoparium* and known flavin mononucleotide (FMN) (Sigma Chemical Co.) suspended in modified Pratt's medium was measured on a Shimadzu Bausch and Lomb Spectrophotometer (Spectronic 200 Uv, Shimadzu Seisakusho, Ltd., Kyoto, Japan; Bausch and Lomb, San Leandro, CA 94577) with modified Pratt's medium used as the reference.

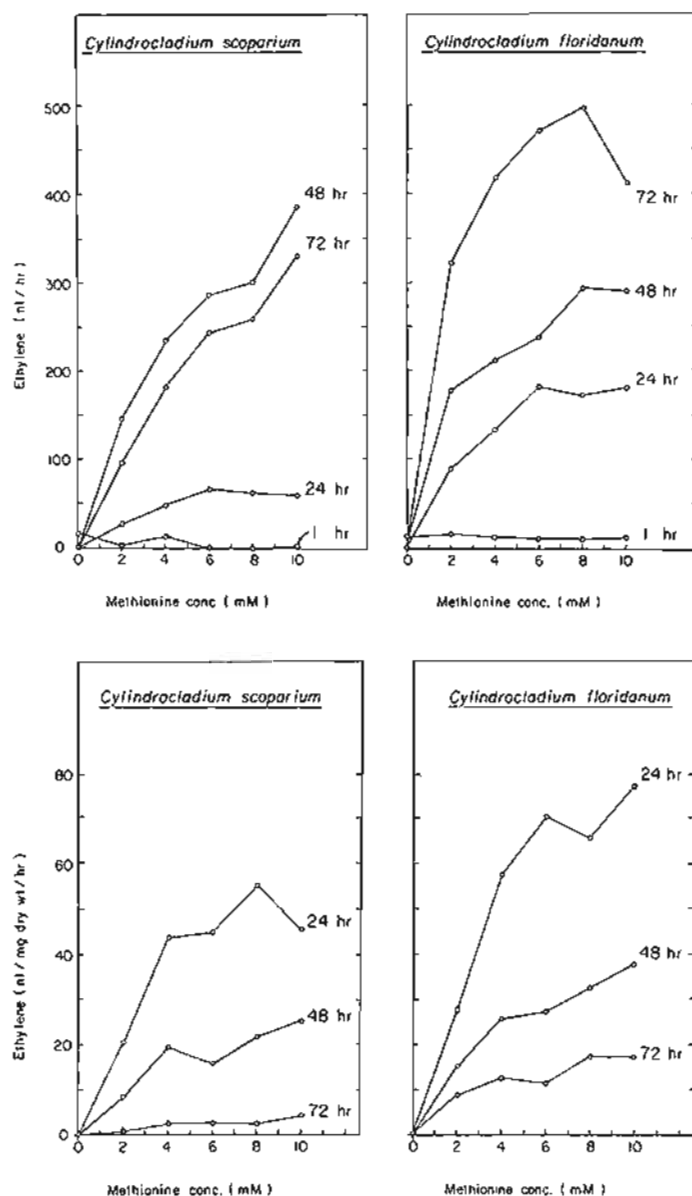


Fig. 1. The effect of methionine concentration on ethylene production by *Cylindrocladium floridanum* and *C. scoparium* when expressed as A, nanoliters per hour, and B, as nanoliters per milligram (dry wt) mycelium per hour. Data points are the means of four replications.

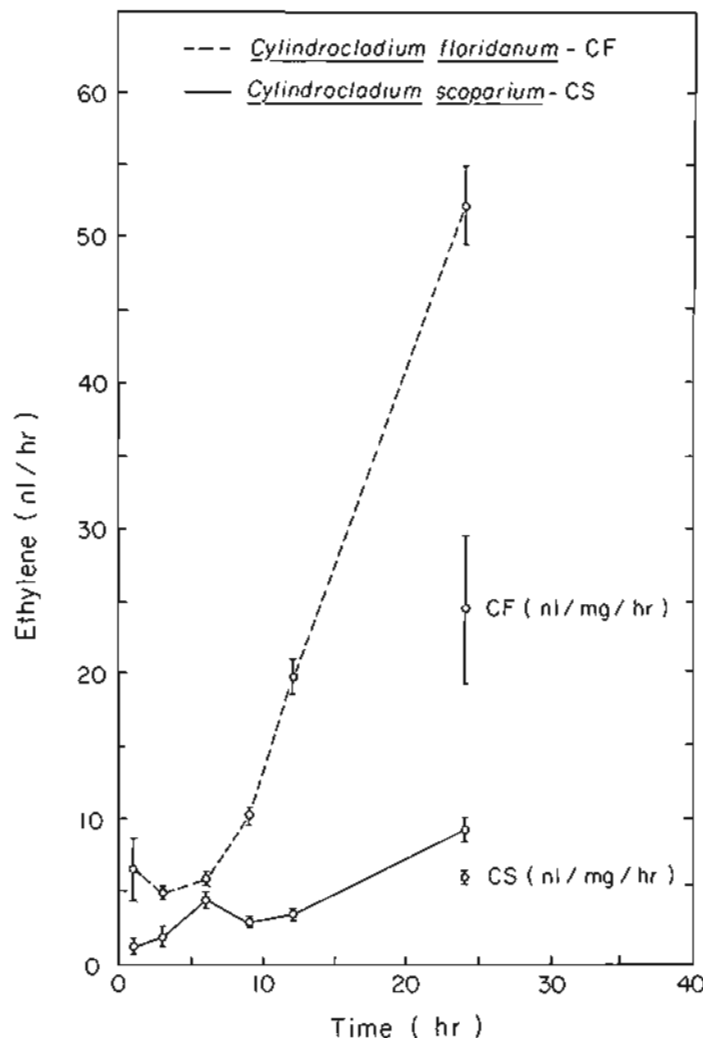


Fig. 2. Ethylene production by *Cylindrocladium floridanum* and *C. scoparium* during the first 24 hr after inoculation of culture vials. Data points are the means of two experiments with six replications each. Bars represent the standard error of the mean. Individual readings, at 24 hr, are ethylene production per milligram of mycelium (dry weight).

Compounds potentially inhibitory to ethylene production were added to cultures after 24 hr of growth. Cultures were then incubated 2.0 hr, with the flasks sealed during the last hour before the ethylene analysis. Inhibitory compounds tested were CuSO_4 (Sigma Chemical Co.) and amino-ethoxyvinylglycine (AVG) (Hoffman LaRoche Co., Nutley, NJ 07110). AVG is structurally related to rhizobitoxine and often referred to in the literature as the ethoxy analogue of rhizobitoxine. Rhizobitoxine inhibits ethylene production in shake cultures of *Penicillium digitatum* (5).

In all experiments the amount of ethylene produced by control flasks inoculated with spore inoculum filtrate was subtracted from the ethylene produced by *Cylindrocladium*.

RESULTS

Values for ethylene production by *Cylindrocladium* varied from experiment to experiment. This was most likely due to variation in spore inoculum concentration and, therefore, the amount of growth present. Trends within experiments, however, remained constant.

Effect of methionine concentration on ethylene production. Ethylene production by cultures of *C. floridanum* and *C. scoparium* occurred and was methionine-dependent (Fig. 1A and B). Increasing concentrations of methionine, within a range of 2–10 mM, generally increased ethylene production. Duplicate experiments at most sampling times exhibited no consistently significant difference in ethylene production between 6, 8, and 10 mM methionine.

Ethylene production over time. Ethylene production by cultures of *Cylindrocladium* was determined during the first 24 hr after

inoculation of Pratt's medium in vials (Fig. 2). Spore germination was zero 2 hr after inoculation, 50–80% by 4.5 hr and a maximum of 80–87% by 7 hr. No peak in ethylene production corresponded with germination of conidia. *C. floridanum* began producing ethylene several hours after conidial germination started, however, and produced more ethylene than *C. scoparium* within the first 20 hr of growth. The difference was significant by the 9-hr reading (LSD, $P=0.01$), and was not due solely to a faster growth rate by *C. floridanum*, because a significant difference existed 24 hr after inoculation when ethylene production was expressed as nanoliters ethylene per milligram (dry weight) of mycelium per hour (Fig. 2).

Maximum ethylene production by *Cylindrocladium* expressed on a mycelium dry weight basis (Fig. 1B) occurred in culture flasks at 24 hr followed by a decline. These results suggest that the fungus growth rate exceeds or is independent of the rate of ethylene production.

Cylindrocladium grows very rapidly, colonizing 40 ml of liquid medium within 3–4 days. A crust of mycelium formed at the surface within 72 hr but not uniformly in all flasks. The crust greatly increased mycelium dry weight and may have interfered with available oxygen, implicated as a necessary component in ethylene biosynthesis (3,25). For these reasons all further experiments involving ethylene production by mycelium were conducted within a 48-hr period to avoid an oxygen deficiency.

The pH of the culture medium became more acidic with continuous growth of *Cylindrocladium*. After 1, 3, and 11 days of incubation, medium pH was 4.35, 3.37–3.42, and 2.77–2.79, respectively. This decrease in pH may have affected the rate of ethylene production by *Cylindrocladium*.

Ethylene production by cultures, culture filtrates, and heat-killed mycelium. Ethylene was produced in culture both biologically from viable mycelium, and nonenzymically (as determined by heat

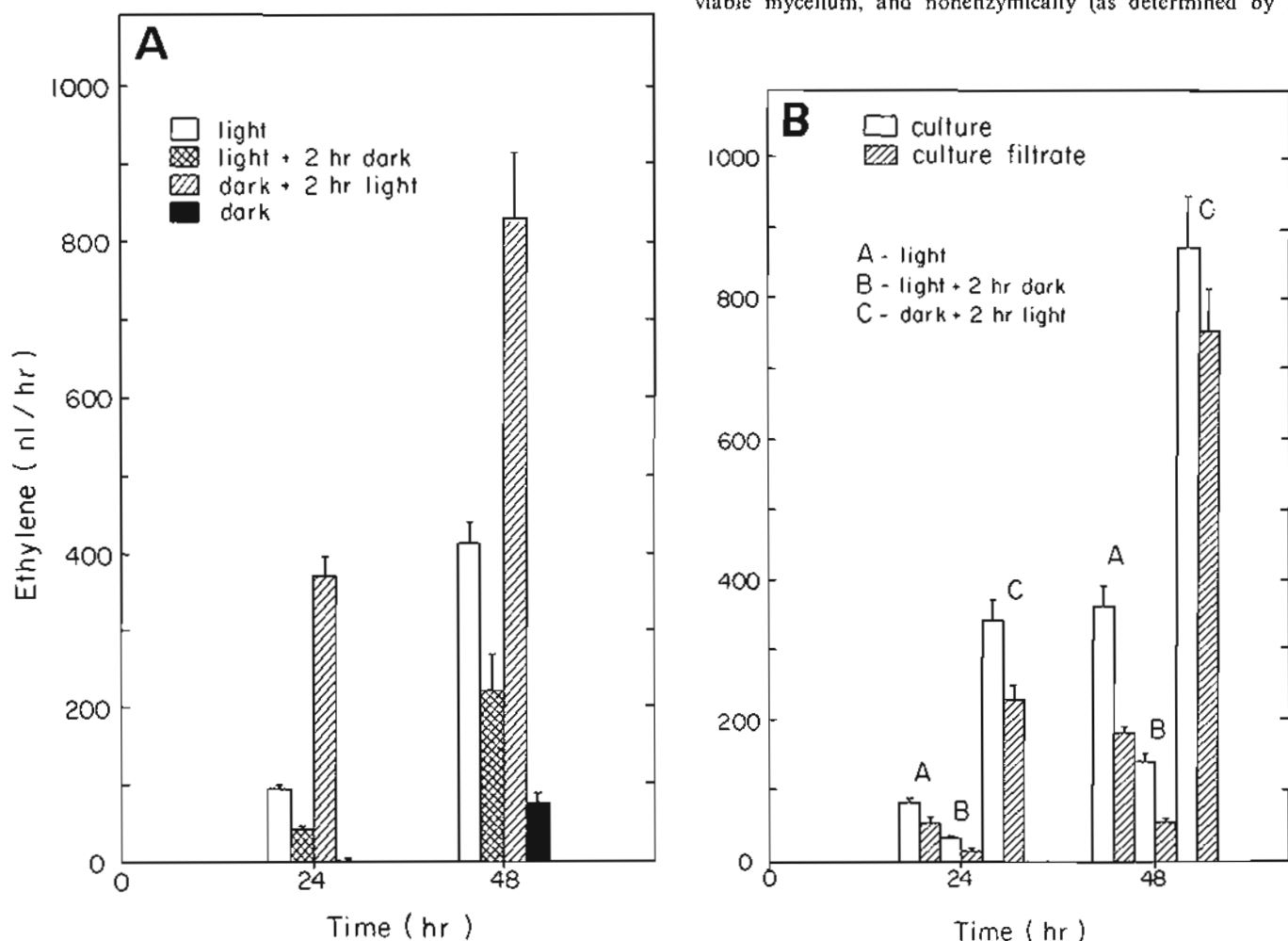


Fig. 3. The effects of light and dark on ethylene production by *Cylindrocladium floridanum* cultures and culture filtrates. Data represented are the means of six replications and bars represent the standard error of the mean. F test was significant, $P = 0.01$.

treatment) from culture filtrates. An increasing amount of ethylene was produced by filtrates over time, and after 48 hr production by the filtrate contributed 75% of the total ethylene production by cultures of *Cylindrocladium* (Axelrod-McCarthy and Linderman, unpublished). Heat-killed mycelium in the original culture medium produced a quantity of ethylene similar to that produced by the culture filtrate (Table 1). Ethylene production was negligible from heat-killed mycelium if the original growth medium was renewed with sterile medium; therefore, viable mycelium was necessary for continued ethylene evolution.

These results suggest that a fungal metabolite was continuously secreted into the medium, accumulated there over time, and was involved in ethylene production. Boiling the culture filtrate for 10 min did not decrease ethylene production; therefore, the possibility of an enzymic pathway was diminished.

Effect of light/dark on ethylene production. Incubation in light and dark influenced ethylene production in both cultures and culture filtrates of *C. floridanum* (Fig. 3A and B). Cultures grown in light for 24 hr followed by 2 hr of dark, produced less ethylene than cultures grown in total light (Fig. 3A). In contrast, cultures incubated for 24 hr in the dark, and then illuminated for 2 hr produced more ethylene than did cultures grown in continuous light, apparently due to the accumulation of a fungal metabolite in the medium. No ethylene was produced in total darkness after 24 hr, but by 48 hr some ethylene was detected (Fig. 3A).

Ethylene production by culture filtrates of *C. floridanum* was similarly influenced by light (Fig. 3B).

Similar results were obtained when the experiments were repeated with *C. scoparium* cultures, except no ethylene was produced at 48 hr in the dark. An 11-day-old dark-grown culture of *C. scoparium*, however, did produce a minimal amount of ethylene (22 nl/hr).

Preliminary absorbance spectra of culture filtrates (from dark-grown 11-day-old cultures) suggested that a metabolite from *Cylindrocladium* had accumulated during dark incubation. We hypothesized that it was involved in the *Cylindrocladium* ethylene biosynthesis.

Yang et al (28) described a nonenzymic pathway for ethylene production that required methionine, flavin mononucleotide (FMN), and light. We therefore compared the absorbance spectra of the culture filtrates produced by *C. floridanum* and *C. scoparium* after 11 days of growth in the dark (to allow for accumulation in the medium) to FMN suspended in methionine-supplemented modified Pratt's medium. Absorbance was measured only on culture filtrates, not on any isolated compound. FMN characteristically had absorption maxima at 370 and 450 nm at initial illumination in the spectrophotometer. The metabolite produced by *C. floridanum* and *C. scoparium* (hereafter referred to as a flavinlike compound, FLC) peaked at 310 nm (Fig. 4), although the peak shapes for the two species were slightly different. More FLC was produced by *C. floridanum* than by *C. scoparium*. FMN appeared to change to an FMN derivative after 24 hr of illumination and produced an absorbance spectrum similar to those produced by *C. floridanum* and *C. scoparium* culture filtrates.

The pH of culture filtrates of *C. floridanum* and *C. scoparium* was 2.60–2.94, whereas the pH of FMN in modified Pratt's medium was 4.2–4.4. Raising the pH of culture filtrates to 4.2, or lowering the pH of FMN to 2.6 did not change the absorbance spectra.

Ethylene was produced from culture medium supplemented with 0.25 μ M FMN and 10 mM methionine after 2 hr of incubation under fluorescent plus incandescent light (62 nl/hr). The FMN derivative formed in the medium after 24 hr illumination, produced 32 nl of ethylene during the last hour of the 24-hr incubation.

During the study of the influence of methionine on the pigment shift of FMN in sterile modified Pratt's medium (Fig. 5A), we noted that without methionine, and after 4 hr of illumination, FMN lost its peak at 450 nm, but retained a narrower double peak between 345 and 410 nm. Over time, the absorbance slightly decreased, probably owing to degradation of the FMN in light. FMN underwent a more dramatic change to a derivative peaking at 315 nm after 24 hr in the presence of both light and methionine.

Methionine affected the absorbance spectrum of the FLC produced by *C. floridanum* (Fig. 5B). When methionine was present, FLC initially had an absorption maximum at 315 nm, and after illumination the peak changed shape slightly and decreased in absorbance. In the absence of methionine at initial illumination, FLC had a broad absorbance peak that ranged from 360 to 390 nm. The peak decreased and narrowed to the 379–390 nm region after illumination. FLC peaked in the range of the narrower double peak of FMN after 4 hr of illumination in the absence of methionine. Although FMN and the FLC of *Cylindrocladium* were not identical, they appeared to have some similarities. A measurable concentration of FLC failed to accumulate in the *C. scoparium* culture filtrate, and therefore the effect of methionine on the FLC produced by *C. scoparium* is unknown.

The absorbance of *C. floridanum* culture filtrate decreased and a spectral shift occurred during dark incubation in the presence of methionine compared with the culture filtrate in the absence of methionine (Fig. 5B). This may have been associated with the ethylene that was produced in the dark, but the determination of this as well as the identification of FLC is dependent upon isolation of the compound from culture filtrates.

Effect of inhibitors on ethylene production. The nonenzymic pathway for ethylene production described by Yang et al (28) was inhibited by CuSO_4 . A preliminary test of a range of CuSO_4 concentrations with the FMN reaction showed that when methionine and CuSO_4 concentrations were equal (10 mM), maximal inhibition occurred. Thus, 10 mM CuSO_4 was tested as an inhibitor of ethylene production by *C. floridanum* and *C. scoparium*. Although CuSO_4 reduced ethylene production by *C. floridanum* and *C. scoparium* cultures and culture filtrates, the reduction was significantly less than that with FMN (Table 2). This

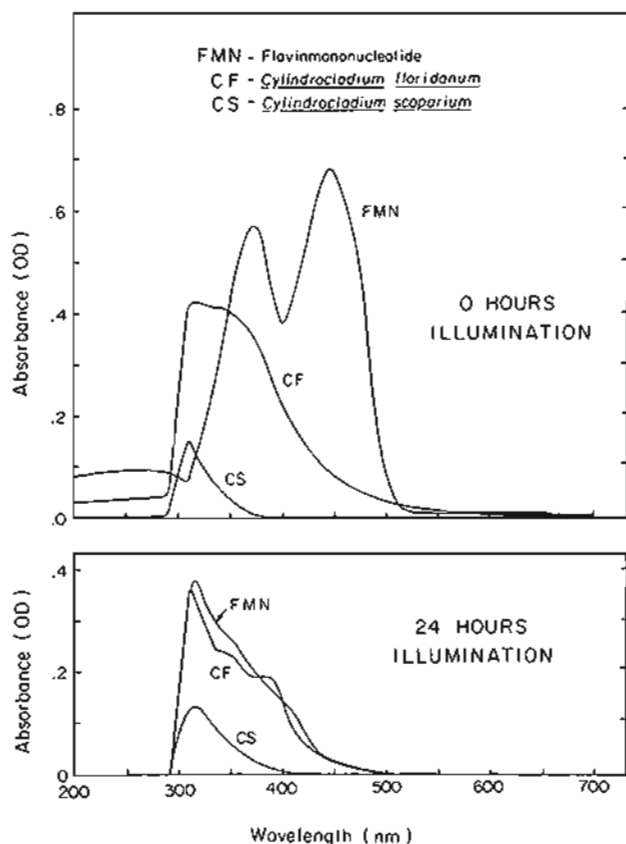


Fig. 4. The effect of light on the absorbance spectra of FMN (suspended in modified Pratt's medium supplemented with methionine) and culture filtrates of 11-day-old *Cylindrocladium floridanum* and *C. scoparium* cultures. Data represented are the means of four replications. Similar results were obtained when the experiment was repeated.

suggests that an additional pathway for ethylene production by *Cylindrocladium* not involving FLC may also be operative. Growth of the fungus also was greatly inhibited by 10 mM CuSO₄.

An ethoxy analogue of rhizobitoxine (structurally related to amino ethoxyvinyl glycine, AVG) has been shown to inhibit ethylene production in *Penicillium digitatum* (5). We made a single experiment on the effect of AVG (10⁻⁴M) on ethylene production by *Cylindrocladium* and found no effect.

DISCUSSION

The basis for the present study was the question of whether *Cylindrocladium* was capable of producing ethylene, since ethylene has been reported as a product of the interaction between *Cylindrocladium* spp. and azalea tissue (14). We have clearly shown that *Cylindrocladium* can produce ethylene in culture, but only when methionine is present. Furthermore, ethylene is produced very soon after conidia germinate and in high enough concentrations to be considered significant in the host-pathogen interaction.

Maximum ethylene production by *Cylindrocladium* (expressed on a mycelium dry weight basis) occurred in culture flasks at 24 hr and was followed by a decline. When expressed as nanoliters of ethylene per hour, however, ethylene was still increasing after 24 hr. A possible explanation for the apparent decline in production after 24 hr and thereafter may be that the fungus growth rate exceeds or is independent of the rate of ethylene production. For some microorganisms, *Colletotrichum musae* (19) and *Ceratocystis fimbriata* (4), maximum ethylene production occurs during the active growth stage. Other workers report maximum production during the growth rate decline phase for *Penicillium digitatum* (23), *Mucor hiemalis* (16), *Aspergillus* and *Mucor* spp. (7), and *Pseudomonas solanacearum* (2).

Our results suggest that *Cylindrocladium* may produce ethylene

TABLE 1. Ethylene production from viable mycelium, culture filtrates, and heat-killed mycelium of two *Cylindrocladium* spp. after 24 hr of growth

Treatments	Ethylene ^a (nl/hr)	Decrease in ethylene production compared with untreated culture (%)
<i>Cylindrocladium floridanum</i>		
Untreated culture	174	...
Culture filtrate	99	43 ± 13 ^b
Heat-killed, original medium	83	53 ± 4
Heat-killed, renewed medium	7	96 ± 1
<i>Cylindrocladium scoparium</i>		
Untreated culture	70	...
Culture filtrate	29	59 ± 11
Heat-killed, original medium	39	45 ± 8
Heat-killed, renewed medium	2	97 ± 0

^aData represents the mean of two experiments, each experiment had six replications per treatment.

^bStandard deviation of the percent decrease in ethylene production.

TABLE 2. CuSO₄ inhibition of ethylene production by flavin mononucleotide (FMN), *Cylindrocladium floridanum*, and *C. scoparium* after 24 hr of growth in medium plus 10 mM methionine

Treatments	Ethylene (nl/hr) ^a		Decreased ethylene production (%)
	0 mM CuSO ₄	10 mM CuSO ₄	
FMN 0.25 M	78	13	83 ± 1 ^b
<i>C. floridanum</i>			
Culture	122	46	62 ± 12
Culture filtrate	70	27	61 ± 11
<i>C. scoparium</i>			
Culture	174	75	57 ± 10
Culture filtrate	84	51	39 ± 11

^aData represents the mean of two experiments, each experiment had five replications per treatment.

^bStandard deviation of the percent decrease in ethylene production.

by at least two pathways. One pathway, possibly chemical, is mediated by light and appears to involve a flavinlike compound (FLC) produced by *Cylindrocladium*. The other pathway occurs in the dark, displays a lag in production, yields less ethylene, and may or may not be related to the first pathway. Chalutz et al (6)

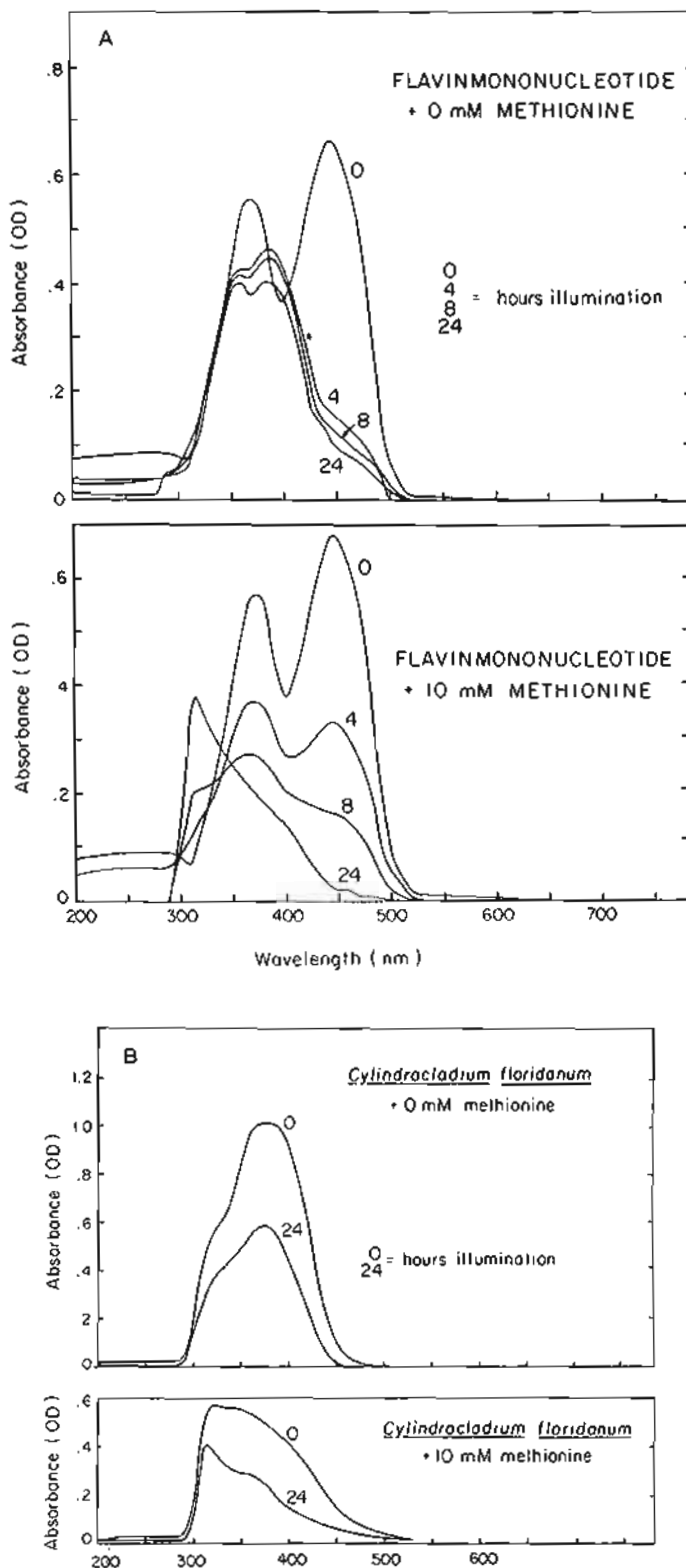


Fig. 5. The effect of light and methionine on the absorbance spectra of A, FMN (suspended in modified Pratt's medium) and B, culture filtrates of *Cylindrocladium floridanum*. Data represented are the means of four replications. Similar results were obtained when the experiment was repeated.

described three ethylene-generating systems operative in cultures of *P. digitatum*: an enzymic system in viable cells and two culture filtrate systems. Both of the culture filtrate systems depended on a fungal metabolite of methionine, one of which was nonenzymic. The system(s) of *Cylindrocladium* appears to largely involve FLC, either contained in fungal cells or released into the medium. The FLC apparently reacts with light and methionine to produce ethylene. This system doesn't seem to parallel the system of *P. digitatum* except in methionine dependency and ethylene production by the culture filtrate. It does, however, show similarities to the system of *Mucor hiemalis* reported by Lynch (15,16) in that ethylene production was methionine-dependent and light enhanced (less ethylene was produced by *M. hiemalis* in the dark). The fungal filtrate of *M. hiemalis* also produced ethylene, and it was proposed that a chemical intermediate released into the culture medium played a role in its production.

Primrose (22) also showed that light enhanced ethylene production by cultures and culture filtrates of *Escherichia coli*, and proposed that a light-sensitive intermediate formed by *E. coli* accumulated in the dark and decomposed to ethylene in both light and dark.

Yang et al (28) described a nonenzymic pathway for ethylene production in which methionine is converted to ethylene by flavin mononucleotide (FMN) and light, although they did not link their pathway to any microbial or higher plant system. Lynch (15) also proposed a pathway for *M. hiemalis* involving the flavin-adenine dinucleotide of an amino acid oxidase that reacts with light in the presence of methionine to form ethylene. The systems described by Yang et al (28), and probably that of Lynch (15), most closely parallel the *Cylindrocladium* system reported in this paper.

The similarities between the FMN-methionine-light system described by Yang et al (28) and the *Cylindrocladium* system include: dependency on light and methionine; NH_3 as a by-product of the FMN system and the *Cylindrocladium* system (determined by an amino acid analysis of culture filtrates done by the Biochemistry and Biophysics Department, Oregon State University, Corvallis); inhibition of both systems by CuSO_4 , the similarities of the absorbance spectra of FMN and the *Cylindrocladium* FLC after 24 hr of illumination; and similarity of FMN and the FLC spectral patterns of *C. floridanum* in the presence and absence of methionine, and the pigment shift to a flavin derivative in the presence of methionine. Nickerson and Strauss (18) showed that methionine can act as an activator for the photochemical reduction of riboflavin.

It is difficult, if not impossible, to compare ethylene production systems of microorganisms reported in the literature because the test conditions used by various workers are not the same. In the presence of methionine *Mucor hiemalis*, *E. coli*, *P. digitatum*, and *Cylindrocladium* all produce a metabolite that is released into the culture medium and is involved in ethylene production. Billington et al (1) identified 4-methylthio-2-oxobutanoate in culture fluids of *P. digitatum* and *E. coli* and suggested that it is an intermediate in ethylene biosynthesis from methionine. The metabolite produced by *Cylindrocladium*, both in the presence and absence of methionine, appears to be a flavinlike compound although the accumulation of an additional metabolite involved in ethylene synthesis also may occur. Perhaps if *M. hiemalis*, *E. coli*, *P. digitatum*, and *Cylindrocladium* spp. could all be compared under identical conditions, a common pathway might be elucidated, possibly involving the intermediate 4-methylthio-2-oxobutanoate as suggested by Billington et al (1) as well as displaying similarities to the pathways proposed by Yang et al (28), or Pegg (20), or both. Nonetheless, this report provides evidence of a methionine-light-FLC pathway for a fungal plant pathogen. Furthermore, the ethylene system of *Cylindrocladium* offers unusual opportunity for future biochemical analysis as well as analysis of the role ethylene plays in the host-parasite interaction leading to pathogenesis.

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