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

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**ABSTRACT**


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. *Cylindrocladium* was produced by *C. scoparium* both from actively growing mycelium (direct) and enzymatically from culture filtrates (indirect). Both direct and indirect systems were light-mediated, and appeared to involve a fungal metabolite, a flavin-like compound (FLC).

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

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 produced by the interaction between the fungal pathogen *Cylindrocladium* and azalea (*Rhododendron obtusum* (Lindl.) Planch.) tissue, and was most likely involved in the leaf abscession that occurs with the disease. He further showed that abscessed infected leaves play a key role in the epidemiology of the root rot and wilt phase of the disease (15). 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* Sober & 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-week 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 d-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 6,694 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 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 3900 gas chromatograph (Perkin-Elmer Corp., Norwalk, CT 06852) equipped with a flame-ionization detector and a 2.4-m Porapak 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 No. 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 analyzed with a statistical equation (17) that gave

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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 D-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 volumecalibrated 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 μm 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 dark, and cultures 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. Methionine concentration was 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 hr, with the flasks sealed during the last hour before the ethylene analysis. Inhibitory compounds tested were CuSO₄ (Sigma Chemical Co.) and amino-ethoxyvinylecycine (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 sterile 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 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 (L.S.D., F = 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.

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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 
found that it was involved in the *Cylindrocladium* ethylene biosynthesis.

Yang et al. (28) described a nonenzymatic 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 characterized 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 flavin-like 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 FLC derivative after 24 hr of 
illumination and produced an absorbance spectrum similar to 
those produced by *C. floridanum* and *C. scoparium* cultures.

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 μM FMN and 10 mM methionine after 2 hr of incubation 
under fluorescent plus incandescent light (62 nL/h). The FLC 
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 FLC in sterile modified Pratt's medium (Fig. 5A), we noted 
that without methionine, and after 4 hr of illumination, FMN lost 
its peak at 430 nm, but retained a narrower double peak between 
345 and 410 nm. Over time, the absorbance slightly decreased, 
probably owing to degradation of the FLC 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 nonenzymatic 
pathway for ethylene production described by Yang et al. (28) 
was inhibited by CuSO₄. A preliminary test of a range of CuSO₄ 
concentrations with the FMN reaction showed that when methionine and CuSO₄ concentrations were equal (10 mM), 
maximal inhibition occurred. Thus, 10 mM CuSO₄ was tested as 
an inhibitor of ethylene production by *C. floridanum* and *C. scoparium.* Although CuSO₄ 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
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 (10⁻⁷ M) 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* (18), *Aspergillus* and *Mucor* spp. (7), and *Pseudomonas solanacearum* (2).

Our results suggest that *Cylindrocladium* may produce ethylene by at least two pathways. One pathway, possibly chemical, is mediated by light and appears to involve a flavin-like 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)

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

<table>
<thead>
<tr>
<th>Treatments</th>
<th>Ethylene* (nl/hr)</th>
<th>Decrease in ethylene production compared with untreated culture (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cylindrocladium</em> floridanum</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated culture</td>
<td>174</td>
<td>...</td>
</tr>
<tr>
<td>Culture filtrate</td>
<td>99</td>
<td>43 ± 13</td>
</tr>
<tr>
<td>Heat-killed, original medium</td>
<td>83</td>
<td>33 ± 4</td>
</tr>
<tr>
<td>Heat-killed, renewed medium</td>
<td>7</td>
<td>96 ± 1</td>
</tr>
<tr>
<td><em>Cylindrocladium</em> scoparium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Untreated culture</td>
<td>70</td>
<td>...</td>
</tr>
<tr>
<td>Culture filtrate</td>
<td>29</td>
<td>26 ± 11</td>
</tr>
<tr>
<td>Heat-killed, original medium</td>
<td>39</td>
<td>46 ± 8</td>
</tr>
<tr>
<td>Heat-killed, renewed medium</td>
<td>2</td>
<td>97 ± 0</td>
</tr>
</tbody>
</table>

*Data represents the mean of two experiments, each experiment had six replications per treatment.

*Standard deviation of the percent decrease in ethylene production.

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**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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ethylene (nl/hr)*</th>
<th>Decreased ethylene production (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 mM CuSO₄</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 mM CuSO₄</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FMN 0.25 M</td>
<td>78</td>
<td>13</td>
</tr>
<tr>
<td><em>C. floridanum</em></td>
<td>122</td>
<td>46</td>
</tr>
<tr>
<td>Culture</td>
<td>70</td>
<td>27</td>
</tr>
<tr>
<td>Culture filtrate</td>
<td>174</td>
<td>75</td>
</tr>
<tr>
<td><em>C. scoparium</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Culture</td>
<td>64</td>
<td>51</td>
</tr>
</tbody>
</table>

*Data represents the mean of two experiments, each experiment had five replications per treatment.

*Standard deviation of the percent decrease in ethylene production.

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**Fig. S.** 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 systems 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 does not seem to parallel the system of P. digitatum except in methionine dependency and ethylene production by the culture filtrates. It does, however, show similarities to the systems 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 filtrates 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"02addine 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; NH₃ 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 CuSO₄, 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 Struss (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 flavilike compound although the accumulation of an additional metabolic involved in ethylene synthesis also may occur. Perhaps 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 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-pathogen interaction leading to pathogenesis.

LITERATURE CITED

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