Involvement of Ethylene in Responses of Etiolated Bean Hypocotyl Hook to Coumarin

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ABSTRACT
Coumarin, at concentrations between 1.0 and 0.1 mM, inhibited red light-induced opening of the etiolated bean hypocotyl hook. In addition, anthocyanin synthesis and geotropic bending were inhibited. Coumarin stimulated ethylene synthesis, and ethylene was shown to mediate the inhibitory actions of coumarin. This conclusion was supported by: (a) the parallel concentration dependence and time sequence of hook closing and ethylene synthesis, (b) the restriction of the bulk of coumarin-induced ethylene production to the curved portion of the hook where opening is expressed, (c) the ability of both coumarin and ethylene to reclose partially opened hooks, and (d) the ability of exogenous ethylene, in the amounts produced by coumarin-treated hooks, to duplicate the inhibitory effects of coumarin. There was an increasing stimulation of growth of the straight portion of the hypocotyl hook section as coumarin concentrations were increased from 0.1 to 1.0 mM. This action of coumarin was not duplicated by ethylene and occurred regardless of the presence or absence of added ethylene. The results of this study suggest that many actions of coumarin in growth systems are mediated by ethylene produced in response to the coumarin.

In the course of a study of bean and cotton seedling development (28), we observed that coumarin is a striking inhibitor of hypocotyl hook opening. Auxin is known to have a similar inhibitory effect on hook opening (17), a property which it shares with ethylene (12, 15). Further, endogenous ethylene has been shown to regulate the hook opening process (12, 13, 15).

An explanation of the growth-inhibitory effect of exogenous auxin has developed from the demonstration that auxins stimulate synthesis of ethylene (23, 24). This phenomenon was proposed to explain the similar effects of auxins and ethylene on several plant responses (14, 23, 24), thus repeating a hypothesis advanced in 1935 based on indirect evidence (32). Subsequently, induced ethylene synthesis was shown to mediate inhibition of growth of stem sections observed in response to supraoptimal levels of auxin (6).

The above facts suggest that coumarin may achieve part or all of its inhibitory action via modification of ethylene synthesis. This report describes several effects of coumarin on the bean hypo-

cotyl, some of which appear to be mediated by ethylene produced in response to the inhibitor.

MATERIALS AND METHODS
The culture of etiolated bean (Phaseolus vulgaris L., cv. Black Valentine) seedlings and handling of excised hypocotyl parts were described in detail previously (28) and will only be reviewed briefly. Seeds were germinated in the dark, and after 5 days hypocotyl parts were excised under a green safe light. Hooks were excised and placed at random into Petri dishes or Erlenmeyer flasks containing two pieces of Whatman No. 3 filter paper and 10 ml of liquid. In specific experiments the crooked portion of the hook was excised at a point opposite the apical end of the hook to yield a straight section of hypocotyl 20 mm in length. Ten hooks or hypocotyl sections were used per flask or dish representing one replication.

Coumarin was supplied in aqueous solutions, and controls received distilled water. Coumarin (K & K Laboratories, Inc., lot 39220L) was purified by recrystallizing it twice from an aqueous ethanol solution. The recrystallized material had a sharp melting point at 69 to 70°C and was used in all experiments subsequent to the initial test (Fig. 1). Exposure to red light (28) or darkness occurred after the tissue pieces were in the various solutions. The number of replications, concentrations of coumarin and duration of red light exposure, where applicable, are given in the various legends.

Hook angles were measured 22 to 24 hr after excision as previously described (17, 28). Fresh weights were obtained after other determinations. In some tests anthocyanin pigments were extracted with 3 volumes of 0.1 N HCl. The extract was brought to 12 ml/g fresh weight with 0.1 N HCl, and the relative pigment content was measured at 520 nm with a Beckman DB spectrophotometer.

When ethylene was to be measured, tissues were placed in 500-ml Erlenmeyer flasks closed with rubber stoppers in which the holes were fitted with serum bottle caps. Air samples of 10 ml volumes were withdrawn with gas-tight syringes at specified intervals, and ethylene was determined by gas chromatography (22). The identity of ethylene was verified as before (22).

Data were analyzed by calculating analysis of variance, Duncan's multiple range tests, and standard errors. Standard errors less than 1% are not shown.

RESULTS
Coumarin inhibited or reversed red light-mediated hypocotyl hook opening, confirming our previous observations (28), and the concentration dependence of the inhibition was determined (Fig. 1). At 0.1 mM coumarin caused little or no restriction of hook opening while at 1 mM it caused significant closing of the hook. Coumarin increased the fresh weight and length of hooks at all concentrations above 0.1 mM. The results in Figure 1 were

1 A contribution of the Texas Agricultural Experiment Station. Research supported in part by Grant GB-5640, National Science Foundation. A preliminary report of this study appeared in Plant Physiol. 43: S-45. 1968.
supported by other experiments, except that the decline in growth between 0.75 mm and 1 mm did not always occur.

In the course of this study we observed that the geotropic bending exhibited by excised bean hooks placed horizontally was inhibited by concentrations of coumarin which caused hook closing. Further, the synthesis of anthocyanin following exposure to room light was significantly inhibited by coumarin at 1 mM (Table 1). Inhibition of pigment synthesis occurred simultaneously with an increase in the fresh weight of the hooks (Table 1).

The effect of coumarin on ethylene production by etiolated hooks was tested by incubating hooks in flasks in the dark for 18 hr and sealing them during an additional 6 hr. Under these conditions there was a marked stimulation of ethylene synthesis (Fig. 2). The concentration dependence for hook closing and ethylene synthesis was very similar. Hook closing and a major increase in ethylene synthesis occurred between 0.25 and 0.5 mM coumarin. This was also the critical concentration step for closing of hooks exposed to red light (Fig. 1).

The time sequence of the effect of coumarin on ethylene production and hook closure suggested a causal relationship (Fig. 3). Ethylene production increased almost 4-fold during the first 6 hr hooks were in coumarin and reached a peak rate between hr 6 and 12. Closing of hooks had started by 6 hr and continued for 24 hr. The increase in ethylene synthesis appeared to precede hook closing.

By separating the crooked upper portion of the hook section from its straight portion, we determined that the major site of coumarin-induced ethylene synthesis was the hook (Table II). In contrast, the lower hypocotyl showed the largest growth promotion in response to coumarin.

While coumarin induced hook closing in either Petri dishes or closed flasks, there was a question of whether the amount of ethylene produced by coumarin-treated hooks was adequate to close nontreated hooks. Hooks were treated with levels of ethylene (0.4 to 1.0 μl/liter) previously found to accumulate within 6 hr in flasks containing treated hooks (Fig. 3). Actual ethylene levels were determined in all flasks at the end of the experiments. Ethylene in the range of concentrations produced by coumarin-treated hooks caused significant closing of hooks (Fig. 4). This was true despite the fact that hooks were given 2 hr, 18 hr, or con-

Table I. Effect of 1 mM Coumarin on Absorbance at 520 nm of 0.1 N HCl Extract of Bean Hypocotyl Hooks

<table>
<thead>
<tr>
<th>Absorbance units</th>
<th>Control</th>
<th>Coumarin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.18</td>
<td>0.03</td>
</tr>
</tbody>
</table>

| Weight/replication | 4.73 | 5.59 |

1 Hooks kept in dark for 20 hr then exposed to room light for 60 hr before extraction.
2 Average absorbance values at the time of hook excision were 0.006.
3 Difference from control is statistically significant at the 1% level of probability.
4 Fresh weight in g at time of extraction. Data are averages of three experiments with a total of 11 replications.

Fig. 1. Effect of coumarin on bean hypocotyl hook opening and total growth as measured by fresh weight and length. All hooks were exposed to red light for 2 hr after being excised. Data are averages of three replications per treatment. Standard errors for each point are indicated by vertical brackets.

Fig. 2. Effect of coumarin on ethylene production and opening of bean hooks in the dark. Flasks sealed the last 6 hr of the experiment to collect ethylene. Data are averages of three replications per treatment. Standard errors for each datum are indicated by vertical brackets.

Fig. 3. Time sequence of the effect of 1 mM coumarin on ethylene production and closing of bean hooks. Flasks were closed for only one 6-hr interval after which hook opening and ethylene content were measured. Data are averages of three replications per treatment per time. Standard errors for each datum are indicated by vertical brackets.
Table II. Effect of 1 mM Coumarin on Ethylene Production and Fresh Weight of Parts of the Bean Hypocotyl Hook

<table>
<thead>
<tr>
<th>Part</th>
<th>Treatment</th>
<th>Ethylene (µl/kg/hr)</th>
<th>Fresh Weight</th>
<th>Increase in Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete hook</td>
<td>Coumarin</td>
<td>23.5 ± 2</td>
<td>5.12 ± 2</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>1.6 ± 2</td>
<td>4.55 ± 2</td>
<td></td>
</tr>
<tr>
<td>Upper “crook”</td>
<td>Coumarin</td>
<td>28.0 + 1</td>
<td>2.08 ± 2</td>
<td>15</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>4.6 ± 2</td>
<td>1.84 ± 2</td>
<td></td>
</tr>
<tr>
<td>Lower hypocotyl</td>
<td>Coumarin</td>
<td>3.72 ± 3</td>
<td>3.37 ± 3</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td>0.8 ± 2</td>
<td>2.54 ± 2</td>
<td></td>
</tr>
</tbody>
</table>

1 Flasks closed 6 hr after plant parts were excised and ethylene was collected for 17 hr. Ethylene production data are averages of three replications from one experiment; growth data are averages of three experiments, three replications per experiment.

2 Difference from control is statistically significant at the 1% level of probability.

Fig. 4. Effect of ethylene on opening or closing of bean hooks. Hooks were excised, enclosed, and treated with air (control) or ethylene ranging from 0.2 to 1 µl/liter. Ethylene levels and hook opening were determined 20 to 24 hr later. Observed ethylene levels include added ethylene as well as that produced by the hooks. Hooks were exposed to red light for 2 hr (squares) or continuously (triangles) immediately after being cut or continuous red light 6 hr after being cut (circles). Data are from three experiments, three replications per treatment. Standard errors for each datum are indicated by vertical brackets.

Fig. 5. Effect of ethylene on absorbance at 520 nm of 0.1 N HCl extract of bean hypocotyl hooks. Hooks in Petri dishes were enclosed in 55-liter Plexiglas chambers for 80 hr in room light. Observed ethylene levels include added ethylene as well as that produced by the hooks. Hooks were treated and enclosed for total exposure time (circles) or opened each 24 hr, ventilated, and retreated (squares). All absorbance values for ethylene treatments are significantly different from their enclosed controls (ethylene levels < 0.1 µl/liter) at the 1% level of probability. Data are from two experiments, five replications per treatment.

Table III. Effect of Coumarin and Ethylene on Bean Hooks That Had Partially Opened in Response to Red Light

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Hook Angle (degrees)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>+63</td>
</tr>
<tr>
<td>Ethylene, 0.8 µl/liter</td>
<td>-12</td>
</tr>
<tr>
<td>Coumarin, 1 mM</td>
<td>-13</td>
</tr>
</tbody>
</table>

1 Data are averages of three experiments with a total of 11 replications.

2 Hooks exposed to red light for 2 hr and after 6 additional hr the average hook angle was +36°, at which time treatments were initiated. Hook angles were determined 29 hr after light exposure or 21 hr after treatment.

Burg and Burg (7) demonstrated that ethylene treatment reclosed hooks opened by light. If the action of coumarin in hook closure was mediated by ethylene, then coumarin as well as ethylene should reclose hooks that have opened in red light. We reclosed hooks with both coumarin (1 mM) and ethylene (0.8 µl/liter) (Table III).

The evidence indicated that the effect of coumarin on hook opening or closing was mediated by ethylene. On the other hand, our data did not indicate that the stimulatory effect of coumarin on hypocotyl growth was mediated by ethylene. Ethylene is considered to inhibit, not stimulate stem growth (18). To clarify this question, two kinds of experiments were conducted. First, etiolated bean hypocotyl sections (without coumarin) were fumigated with ethylene (0.2 to 0.8 µl/liter) for 24 hr. There were no significant differences in weight or length between these sections and the controls after 24 hr. At this time one-half of the replications of each initial treatment were exposed to 0.75 mM coumarin. Ethylene was removed by venting, and all flasks were left open for an additional 24 hr. Coumarin caused an increase in length (Fig. 6) in all hypocotyl sections regardless of prior treatment. Those sections not exposed to coumarin shrank slightly. Coumarin caused a similar increase in weight (0.20 to 0.35 g/10 sections) regardless of prior treatment. Thus, at the end of a period during which ethylene did not influence weight or length of hypocotyl
FIG. 6. Effect of 0.75 mM coumarin or water on growth in length of bean hypocotyls during the second 24 hr of a 48-hr experiment. Treatments during the first 24 hr after excision are indicated on the abscissa; an analysis of variance indicated that these treatments did not significantly alter fresh weights of sections at the 1% level of probability. Data are averages of four replications per initial treatment. A similar stimulatory effect of coumarin on growth as measured by change in fresh weight was obtained.

FIG. 7. Effect of coumarin on the growth in length of bean hypocotyl sections in the presence or absence of 1 μl/liter exogenous ethylene. Sections were enclosed in 500-ml flasks for 22 hr.

sections, coumarin could still cause growth as measured by either parameter. In a second experiment, we measured the response of hypocotyl sections to coumarin in the presence and absence of ethylene (1 μl/liter). The results (Fig. 7) demonstrated that the growth-stimulating action of coumarin occurred in the presence of ethylene concentrations high enough to inhibit hook opening (Fig. 4). We concluded that the stimulatory effect of coumarin on growth of the bean hypocotyl was not mediated by ethylene and could occur in presence or absence of ethylene (Figs. 6 and 7).

DISCUSSION

Coumarin’s activity as a growth inhibitor was first indicated by its toxicity to wheat plants (Reference 29 as cited in Reference 9). Subsequently, coumarin was shown to inhibit the growth of roots (3) and stem sections (30, 31). Growth promotion by coumarin was first noted by its synergistic action with IAA in Avena coleoptile sections (30). Subsequently, coumarin alone was shown to stimulate growth of stem and coleoptile sections (26, 27), and, to a lesser degree, roots (Reference 11 and reviews in References 11, 27).

Since the bean hypocotyl hook exists because the cells on the upper side are longer, i.e., growing faster, than those in the lower side (16), hook closing by coumarin can be viewed as a selective growth inhibition which magnifies the existing difference in growth of cells on the upper and lower sides of the hook. Hook opening occurs in red light because of accelerated growth of cells on the lower side of the hook (16). Therefore, prevention of red light-induced hook opening by coumarin can also be viewed as a growth inhibition.

There were several inhibitory effects of coumarin observed in this study: inhibition of hook opening, geotropic curvature, and anthocyanin synthesis. The hook opening process was studied in greatest detail. The data indicate that this effect of coumarin was mediated by the increased amount of ethylene produced in response to the inhibitor. This conclusion is supported by the following observations: (a) the close agreement between the concentration dependence of coumarin-induced ethylene production and hook closing (Figs. 1 and 2); (b) the parallel time sequence of ethylene synthesis and hook closing (Fig. 3); (c) the restriction of the bulk of coumarin-induced ethylene synthesis to the curved portion of the hypocotyl hook (Table II) while ethylene synthesis was elevated in the lower hypocotyl to rates approaching those in etiolated hooks (Reference 15 and Table II); and (d) the duplication of coumarin effects with exogenous ethylene (Fig. 4) in the levels produced by coumarin-treated hooks within 6 hr of enclosure in flasks (Fig. 3).

That the amount of ethylene produced is adequate to account for the inhibitory effects of coumarin seems assured. The bean hook normally remains almost completely closed in the dark when it produces ethylene at the rate of 3.4 μl/kg hr, and it opens in red light when the ethylene production drops to 1 μl/kg hr (15). Goeschl et al. (12, 13) reported a similar response of pea epicotyl ethylene production and growth to red light. Hooks treated with coumarin produced ethylene at 23 to 28 μl/kg hr in some of our tests following 18-hr exposure to the inhibitor (Table II). The average ethylene production rates during the first 6-hr period of the experiment in Figure 3 were 2.7 and 9.2 μl/kg hr for control and coumarin-treated hooks, respectively. In the periods which followed, ethylene production by control hooks declined while that for the coumarin-treated hooks increased to 19 to 22 μl/kg hr. Since these ethylene production rates by coumarin-treated hooks are so much higher than amounts naturally required to keep the hook closed, it is not surprising that the treated hooks close even further. It is apparent that coumarin is affecting the synthesis of ethylene and not simply the release of ethylene already present, because relatively high rates of production are sustained for at least 24 hr.

Ethylene has been shown previously to inhibit various plant movements (4, 10, 32). Since we found that ethylene in amounts produced by coumarin-treated hooks will inhibit geotropic bending and anthocyanin synthesis (Fig. 4 and text), we concluded that the coumarin-induced inhibition of these processes (Table I) was also mediated by ethylene.

The involvement of ethylene in bean hypocotyl hook responses to coumarin suggests that many other “ethylene-like” actions of coumarin are mediated in part or completely by ethylene. This proposal would include growth inhibition by supraoptimal concentrations of coumarin (11, 30) as well as release of apical dominance (8). Coumarin action via ethylene synthesis extends the hypothesis that some actions of auxin are mediated by ethylene produced in response to the auxin (6, 23, 24, 32). Based on
data here and the effect of many abscission rate modifiers on ethylene synthesis (1), it appears that many growth regulators modify ethylene synthesis.

Owing to the complexity of many hormone-regulated processes, we do not suggest that all responses to coumarin can be explained by a single auxin-like capacity to stimulate ethylene synthesis. For example, coumarin stimulated ethylene synthesis and abscission of orange fruits, occasionally the latter without the former (20); yet in other studies coumarin retarded aging and abscission of debladed petals (2).

Our data indicate that the growth-promoting effect of coumarin on bean hypocotyl sections is not mediated by ethylene and can occur in spite of the presence of ethylene (Tables I and II, Figs. 1, 6, and 7). Similarly, part of the stimulatory effect of auxin on growth is not prevented by ethylene (6). Thus one can theorize that both auxin and coumarin stimulate growth and ethylene synthesis independently of each other. On the other hand, since coumarin is an inhibitor of IAA oxidase (Reference 5 as cited in Reference 21), its effect noted here could be the result of sparing of auxin, which in turn modifies ethylene synthesis or growth. One argument against this possibility is the observation that IAA and coumarin did not act synergistically when applied to sections which had depleted their auxin supply and stopped growing, but both stimulated growth to the same degree alone (27). Further, coumarin-induced growth is more sensitive to the inhibitory action of dioscuraric and thioracil while IAA-induced growth is more sensitive to inhibition by actinomycin, puromycin, and chloramphenicol (19). This situation is not likely if coumarin's auxin action is mediated by sparing of IAA. The question of whether the present effects of coumarin are direct or mediated through auxin sparing will be investigated further.

Our data on ethylene production by parts of the bean hypocotyl hook (Table II) are in agreement with the proposed regulatory role of ethylene in that tissue (15) and the pea epicotyl (12, 13).

The relationship of the present findings to the extensive literature on coumarin as a germination inhibitor (see reviews in References 3, 21) is obscure. However, we have observed that coumarin inhibits the red light-mediated germination of lettuce seed and prevents the rise in ethylene production which parallels germination (25). This suggests an action of coumarin in germination of lettuce seeds that is not mediated by induction of ethylene production.

LITERATURE CITED