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Stimulation of Ethylene Production in Bean Leaf Discs by the Pseudomonad Phytotoxin Coronatine

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ABSTRACT

Coronatine is a toxin produced by *Pseudomonas syringae* pv. *glycinea* which induces the same chlorotic response in bean leaves as does infection by the bacterial pathogen. Although the structure of coronatine is known, the biological mode of action is not. One possible clue to its activity is the ethyl-substituted cyclopropane side chain of the molecule. This part structure (1-amino-2-ethycyclopropane-1-carboxylic acid or AEC) is an analog of the ethylene precursor 1-aminocyclopropane-1-carboxylic acid (ACC).

When coronatine was applied to bean leaf discs in solution, or to intact leaves through prick application, a substantial stimulation of ethylene production was measured. This stimulation was concomitant with an increase in ACC content of the tissue, and occurred under the same conditions as did the chlorotic response to the toxin. The stimulation of ethylene production was inhibited by aminoethoxyvinylglycine, an inhibitor of ACC synthesis. These results, along with those of experiments using L-[U-^{14}C]methionine, indicated that the stimulation involved *de novo* production of ethylene via the methionine pathway.

The whole, unhydrolyzed coronatine molecule is probably necessary to elicit both the ethylene and chlorosis responses since neither hydrolysis product (coronafacic acid and coronamic acid AEC]) is effective alone. A naturally occurring analog of coronatine, coronafacoylvaline, also stimulated ethylene production and caused chlorosis. However, the unrelated pseudomonad phytotoxin phaseolotoxin, which also causes chlorosis, did not stimulate ethylene production. Ethylene thus may have a specific role in the coronatine toxic syndrome.

Coronatine is a phytotoxin produced by several plant pathogenic *Pseudomonas syringae* pathovars (15, 19). The visible phytotoxic effect of coronatine after prick application to green leaves is chlorosis, a symptom often observed around natural infection sites of the coronatine-producing bacterial strains (6, 18, 19, 20). The biological mode of action of coronatine is not known. Some work has suggested that chlorosis is a result of inhibition of Chl synthesis rather than of increased degradation (7), but no work has been reported on the effects of purified coronatine on green leaf tissue. An auxin-like hypertrophic growth response in potato tuber tissue after treatment with coronatine has been recorded (22–24), and the toxin also inhibits root growth in wheat seedlings (22).

An enhancement of ethylene production has often been implicated in plant pathogenesis, both in terms of virulence and resistance (2, 21), and at least two bacterial toxins, rhizobitoxine and AVG[1] (12), are known to inhibit ethylene biosynthesis in plants. Both produce a chlorotic response in plant leaves (11; Mitchell, unpublished data), although a chlorotic response generally is not necessarily associated with ethylene synthesis (see results, below, with the phaseolotoxin). Coronatine (Fig. 1) has a structural region (AEC) which is similar to ACC, an intermediate in the biosynthetic pathway of ethylene in plants. Recent work by Hoffman *et al* (8) shows that a diastereoisomer of AEC (coronamic acid) is converted enzymically to 1-butene, probably by the same enzyme system that converts ACC to ethylene. These structural similarities and analogies indicated the possibility that coronatine *in vivo* may interfere with the regulation of ethylene production.

We have therefore tested the hypothesis that coronatine may influence ethylene production in bean leaf tissue. Results, principally from bean leaf discs provided with solutions of coronatine, show that the toxin causes a marked stimulation of ethylene synthesis. Further results indicate that the intact molecule is necessary both for the ethylene response in discs, and for the chlorotic response in the intact leaf; neither coronamic acid nor coronafacic acid, the two products of hydrolysis of coronatine, were active in these respects.

MATERIALS AND METHODS

Plant Material. Bean (*Phaseolus vulgaris* L. cv 'Seminole') seedlings were grown in nutrient solution for up to 14 d at 23°C with a 16-h photoperiod, light intensity 150 μE m^{-2} s^{-1}.

Ethylene Production. Discs (1.1 cm diameter) were cut from primary, unifoliate leaves and placed 12 per flask (0.2–0.3 g fresh weight) into 50-ml conical flasks containing 1.5 ml 0.05 M phosphate buffer (pH 6.5), with test compounds where required. All toxins and hydrolysis products were first dissolved in 200 μl ethanol and then made to volume in 0.05 M phosphate buffer. A CO_2 trap of 1 ml 12% KOH was included in each flask and the flasks were stoppered with rubber septa. Unless otherwise stated, all experiments were performed in the dark in a shaking water bath at 25°C, and in replicates of 6 or 12 flasks. In experiments using prick applications of toxin to bean leaves still attached to the plant, 3-μl droplets of coronatine or phaseolotoxin were applied, and the leaf punctured through the droplet. After 1 h, when the solution had been taken up by the leaf, discs were cut to include the site of application. The discs were placed on moist filter paper in 25-ml flasks, eight to a flask. Incubation was as above.

Ethylene was sampled by removing 1-ml gas samples from the sealed flasks and the ethylene measured by GC using flame ionization detection.

Measurement of ACC. Combined samples of leaf discs, of about 1.5 g fresh weight, were extracted with 6 ml 70% ethanol. After centrifugation, the extracts were dried under vacuum at 40°C, taken up in 1 ml water, and 200 μl samples assayed for ACC by the method of Lizada and Yang (13).

[1] Abbreviations: AVG, aminoethoxyvinylglycine; ACC, 1-aminocyclopropane-1-carboxylic acid; AEC, 1-amino-2-ethylcyclopropane-1-carboxylic acid; TLE, thin layer electrophoresis.

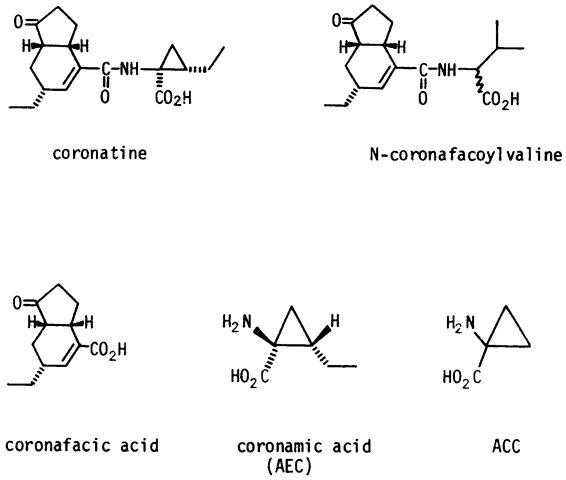

FIG. 1. Chemical structures of coronatine and related compounds.

[^{14}C]Methionine Feeding Experiments. Discs were incubated as above in 1.5 ml of buffer which included 1 μCi L-[U-^{14}C] methionine (290 μCi μmol^{-1}) per flask of 12 discs in the presence or absence of coronatine. After 24 h incubation, duplicate 3-ml gas samples were taken from the flasks and injected into scintillation vials containing 0.5 ml 0.1 M mercuric acetate in methanol. The vials were incubated at 0°C and shaken vigorously for 2 h; then 3 ml of scintillation fluid (ACS II, Amersham) were added, and the ^{14}C measured by liquid scintillation spectrophotometry. Replicate experiments without added [^{14}C]methionine were conducted concomitantly to assess unlabeled ethylene production as measured in the flask headspace.

Preparation of Toxins and Related Compounds. Coronatine, *N*-coronafacoylvaline and coronafacic acid were all prepared from liquid cultures of *Pseudomonas syringae* pv. *atropurpurea* strain 4328 as described by Mitchell (16). Each purified compound was homogeneous on TLC, and on GLC as its methyl ester. Coronatine was recrystallized from ethyl acetate and coronafacic acid from petroleum ether-benzene.

Phaseolotoxin was prepared from liquid cultures of *Pseudomonas syringae* pv. *phaseolicola* strain 4419 and purified as described by Mitchell (14). The product was homogeneous on two-dimensional TLE/TLC.

Coronamic acid was obtained from the acid hydrolysis of coronatine. Coronatine (15 mg) dissolved in 100 μl ethanol, and 1.1 ml 6 M HCl, were combined and sealed in a glass tube, then heated in steam for 16 h. The product was diluted with 4 ml water and extracted with ethyl acetate (4 × 10 ml). The aqueous phase was then evaporated, and contained the single product, coronamic acid (7.8 mg) as the hydrochloride, which was homogeneous on two-dimensional TLE/TLC.

RESULTS AND DISCUSSION

Effect of Coronatine on Ethylene Production in Bean Leaf Discs. Leaf discs from 1-week-old plants undergo a wound response wherein about 25 nl ethylene g^{-1} fresh weight is accumulated in 4 h (Fig. 2a); thereafter, the rate of production of this wound ethylene declines. The presence of coronatine (5 × 10^{-5} M) stimulated production of ethylene in 1-week-old leaf discs, this being noticeable after 8 h; the declining rate of wound ethylene production was reversed after 8 h in the coronatine-treated discs such that, by 24 h, there was almost a 10-fold increase in the rate and accumulation of ethylene (Fig. 2, a and b). An increase in ACC concentration in extracts of the leaf discs was also apparent 8 h after the coronatine treatment (Fig. 2c). The ACC accumulation was concomitant with ethylene production and did not appear to substantially precede it in toxin-treated tissue. There was an accumulation of ACC associated with the decline in ethylene production in control tissue (Fig. 2, b and c). This was most noticeable from leaf discs from 1-week-old plants.

There was a substantial quantitative difference in the stimulation response between discs taken from expanding leaves (1-week-old plants), and those from fully expanded leaves (2-week-old plants). The stimulation by coronatine in discs from expanded leaves was only about half that in discs from expanding leaves. This was evident in ethylene accumulation, in the rate of ethylene production, and in the concentration of ACC (Fig. 2, a–c). This result provides an indirect link between ethylene stimulation and the chlorotic response of intact leaves to the toxin, since we have observed that chlorosis is greater in younger than in older leaves. A transportable toxin, which is likely to be coronatine, has also been shown to cause extensive chlorosis in young soybean leaves after infection of old leaves by the bacterium (5).

Most of the disc experiments assessing ethylene response were done in the dark, whereas the toxin-induced chlorosis is commonly observed in leaves exposed to conventional photoperiods. We found that coronatine stimulated ethylene production in the dark equally to that in discs under a 16-h photoperiod. In the dark, control and coronatine-induced ethylene rates over 24 h were 2.43 ± 0.37 and 23.29 ± 3.23 nl g^{-1} h^{-1}, respectively, and under the 16-h photoperiod, were 2.39 ± 0.50 and 22.95 ± 4.14 nl g^{-1} h^{-1}, respectively.

Although in most experiments a coronatine concentration of 5 × 10^{-5} M was used, an effect on ethylene was found with coronatine concentrations as low as 5 × 10^{-7} M (Table I). Chlorosis can be elicited in intact leaves by prick application of amounts of coronatine in the region of 100 ng (0.313 nmol) in solutions of about 10^{-4} M (15). A solution of 5 × 10^{-7} M coronatine provides about 240 ng in 1.5 ml of medium for our leaf disc experiments. The extent of uptake in both systems is unknown. However, ethylene stimulation does appear to be produced by concentrations of coronatine which may be encountered *in vivo*. The lack of a strong dose response (Table I) may indicate an indirect relationship between ethylene production and coronatine, or a triggering effect of the toxin.

When leaf discs were taken from the site of prick application of coronatine on intact leaves and maintained in humid conditions for 48 h, ethylene production was again elevated above that of the control (Table IIa). However, this ethylene response took longer to appear and was quantitatively less when compared with the response to coronatine of discs in solution. At both concentrations of coronatine used, ethylene production preceded any visible signs of chlorosis. With the 0.65 nmol (207 ng) treatment, chlorosis was weak and not apparent until about 48 h. With the 3.9 nmol (1244 ng) application, chlorosis was strong and developed between 24 and 48 h. These results indicate that ethylene production is not a reaction subsequent to chlorosis.

To test whether ethylene production was a general response to toxins from pathovars of *Pseudomonas syringae*, a similar experiment was conducted involving prick application to intact leaves, using 0.78 nmol phaseolotoxin per application. There was no effect on ethylene production from leaf discs over 48 h (Table IIb), although strong chlorosis developed after 24 h.

Origin of Ethylene in Coronatine-Treated Bean Leaf Discs. There are three possible reasons for the stimulatory effect of coronatine. Endogenous ethylene could be released by the toxin; the toxin, or some part of the molecule, could be converted to ethylene in the plant; coronatine could stimulate ethylene production through the common methionine pathway.

Ethylene synthesis by the methionine pathway is inhibited by AVG, specifically by blocking the conversion of *S*-adenosylmethionine to ACC (1, 12). In bean leaf discs, AVG completely

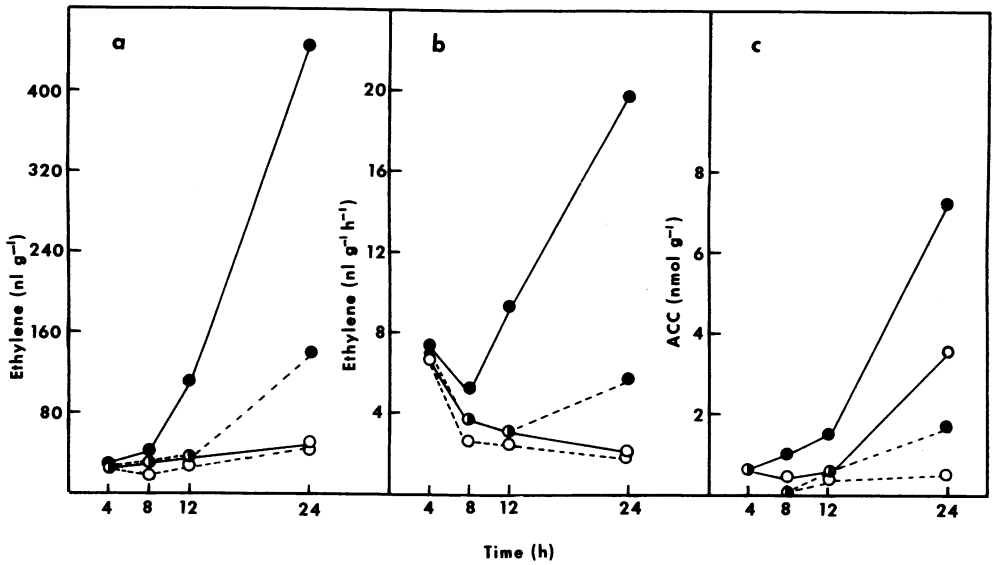

FIG. 2. Effect of coronatine on ethylene (a, b) and ACC (c) production in bean leaf discs. Each point represents collection of ethylene or accumulation of ACC for the number of hours indicated. Discs were taken either from expanding leaves of 1-week-old plants (——) or expanded leaves of 2-week-old plants (- - -). (○), Control; (●), + 5×10^{-5} M coronatine. All data points are the means of at least six replicates and have SE less than 15%.

Table I. *Effect of Coronatine Concentration on Ethylene Production Collected over 24 Hours in Bean Leaf Discs*

Data are accompanied by ± SE.

Coronatine	Ethylene
M	*nl g⁻¹ h⁻¹*
Control	2.43 ± 0.37
5×10^{-7}	15.59 ± 2.80
5×10^{-6}	17.54 ± 2.41
5×10^{-5}	22.95 ± 4.14

Table II. *Ethylene Production Induced by Coronatine (a) or Phaseolotoxin (b) after Prick Application of the Toxin to Intact Bean Leaves*

After application, discs were excised and incubated, and ethylene was collected for two 24-h periods. The flasks were flushed with air after the first 24 h. Data are accompanied by ± SE.

Toxin	Ethylene	
	24 h	48 h
	nl g⁻¹ h⁻¹	
(a) Coronatine		
Control	2.35 ± 0.31	0.87 ± 0.10
0.65 nmol	4.46 ± 0.85	5.65 ± 0.83
3.90 nmol	8.14 ± 0.96	36.60 ± 4.16
(b) Phaseolotoxin		
Control	1.29 ± 0.20	0.60 ± 0.06
0.78 nmol	1.14 ± 0.05	0.65 ± 0.06

Table III. *Effect of 10^{-3} M AVG on Ethylene Production Collected over 24 Hours when Stimulated in Bean Leaf Discs by Coronatine, ACC, and IAA*

Data are accompanied by ± SE.

Treatment	Ethylene	
	− AVG	+ AVG
	nl g⁻¹ h⁻¹	
Control	2.95 ± 0.40	0
5×10^{-5} M coronatine	16.01 ± 4.61	0
5×10^{-5} M ACC	84.28 ± 5.05	70.96 ± 3.93
5×10^{-4} M IAA	143.66 ± 10.36	0.57 ± 0.05

Table IV. *Effect of 5×10^{-5} M Coronatine on the Conversion of L-[U-^{14}C]Methionine to [^{14}C]Ethylene in Bean Leaf Discs*

Ethylene was collected over 24 h. Data for unlabeled ethylene production in a replicate experiment are also included. [^{14}C]Methionine was provided carrier-free. Data are accompanied by ± SE.

Treatment	Ethylene	
	nl g⁻¹ h⁻¹	*cpm g⁻¹ h⁻¹*
Control	6.35 ± 2.24	659 ± 42
+ Coronatine	18.65 ± 4.15	1154 ± 89

inhibited both the control, and the coronatine-induced ethylene production (Table III). It had little effect on ACC-stimulated ethylene production, but inhibited that elicited by IAA (Table III). These results are consistent with the evidence that AVG acts prior to the conversion of ACC to ethylene and that the stimulatory activity of auxin on ethylene synthesis is the result of stimulation of ACC synthase activity (27, 28). By comparison with these two stimulants, coronatine, although less effective, has characteristics like that of auxin, and would appear to act on the methionine-ethylene pathway prior to the conversion of ACC to ethylene. It is unlikely that coronatine has an effect on the methionine pool itself; the latter does not appear to be limiting since the provision of leaf discs with 1 mM methionine had no effect on the control or coronatine-induced ethylene production (data not given). A similar result has been found in tobacco leaves, where TMV infection induces ethylene production (4). In bean and tobacco, as in many plant tissues (3), the rate-limiting step in ethylene biosynthesis is likely to reside in the synthesis of ACC.

Further evidence for the involvement of the methionine pathway comes from labeling studies. When [^{14}C]methionine was supplied to leaf discs, the additional presence of coronatine brought about a 75% increase in [^{14}C]ethylene collected after 24 h (Table IV). This stimulated [^{14}C]ethylene production is not as extensive as found for unlabeled ethylene derived from the endogenous methionine pool. However, we have not assessed the efficiency of uptake or incorporation into the methionine pool in this tissue.

In conclusion, it is unlikely that coronatine releases endogenous ethylene bound in the tissue, or is itself in any way converted directly to ethylene. This is further supported by the results reported below, and the results of Hoffman *et al.* (8), who showed that an isomer of one hydrolysis product of coronatine, AEC (coronamic acid), was converted to 1-butene through the same enzyme system as used for ACC conversion. AEC did not itself

Table V. *Comparison of the Effect of Coronatine on Ethylene Production in Bean Leaf Discs over 24 Hours with That of the Hydrolysis Products (Coronafacic Acid and Coronamic Acid), and the Valine Analog Coronafacoylvaline*

All concentrations were 5×10^{-5} M. Data are accompanied by ± SE.

Treatment	Ethylene	Chlorosis
	$nl\ g^{-1}\ h^{-1}$	
Control	8.39 ± 2.62	–
Coronatine	39.77 ± 8.74	+
Coronafacic acid	4.09 ± 0.73	–
Coronamic acid	10.38 ± 3.78	–
Coronafacoylvaline	31.47 ± 3.84	+

produce ethylene in fruit tissue. Our results suggest that coronatine probably causes stimulation of ethylene synthesis by acting on the methionine pathway between methionine and ACC.

Structure and Activity Relationships. The coronatine molecule consists of two structural components, coronafacic acid and coronamic acid, linked by an amide bond. One of these components, coronafacic acid, is released into the growth medium by *P.s. atropurpurea* in culture, as is coronatine (16, 19). Although it is probable that the plant is exposed to coronafacic acid upon infection, it is less likely to be exposed to coronamic acid, since the probable biosynthetic pathway of coronatine is through the coupling of coronafacic acid with isoleucine followed by cyclization of the side chain (17). One possible source of coronamic acid *in situ* would be from coronatine as a result of enzymic hydrolysis. However, we found no evidence of ethylene stimulation in bean leaf discs when they were treated with either hydrolysis product (Table V). Furthermore, the configuration of our AEC as obtained from the hydrolysis of coronatine (10) would not be the same as that of the stereoisomer which Hoffman *et al.* (8) found to act as substrate for the ethylene-forming enzyme. There is further evidence that the functional structure involves the intact molecule. *Pseudomonas syringae* pv. *atropurpurea* produces a valine analog of coronatine, *N*-coronafacoylvaline (Fig. 1), which also produces chlorosis in bean leaves. When this was tested on bean leaf discs, we found substantial ethylene stimulation (Table V). This also suggests that the ethylcyclopropyl moiety of the coronatine structure is not a specific requisite for eliciting the ethylene response. A further link between the ethylene response in excised leaf tissue, and chlorosis after prick application on intact leaves, is demonstrated by the data in Table V, where only those compounds stimulating ethylene production also produce chlorosis.

Our conclusion is that the intact coronatine molecule is necessary to elicit both the chlorotic response and ethylene stimulation. This is further supported by the activity on both these responses of the analog *N*-coronafacoylvaline which does not have the cyclopropane moiety. Shiraishi *et al.* (25) also reached a similar conclusion with respect to the hypertrophic response of potato tubers and synthetic analogs of coronatine.

General Discussion. An important question, still to be resolved, is whether ethylene plays a direct part in the toxin syndrome, or is an indirect consequence of earlier responses such as a possible stimulation of auxin activity. The prick application of coronatine to bean leaves showed that a stimulation of ethylene was evident before visible symptoms of chlorosis were manifest. This suggests that ethylene production and, perhaps, ACC accumulation are relatively early events in the coronatine reaction. The primary effects of such a toxin are likely to occur within a short time, such as is seen with phaseolotoxin and tabtoxin, two other pseudomonad toxins. Inhibition of ornithine carbamoyltransferase, the major metabolic response to phaseolotoxin, can be detected *in situ* within 10 min of toxin treatment, whereas chlorosis can be detected by Chl assay only after about 10 h (J.G. Turner, personal communication). After treatment of tobacco leaves with tabtoxin, the target enzyme glutamine synthetase had lost all but 5% of its activity after 4 h, well before chlorosis was detectable (26). With coronatine, the only evidence we have that indirectly links chlorosis and ethylene production is that only those chemical species which stimulate ethylene also induce chlorosis, and that there is a weaker response of older leaf tissue to both effects of the toxin. An obvious experiment would be to test whether AVG blocks symptom expression. However, AVG itself induces chlorosis (unpublished data) and cannot be used in this respect.

The chlorosis response of bean leaves to phaseolotoxin does not have an associated change in ethylene production. This result, together with the evidence showing the coronatine response to be specifically related to the coronatine structure, invites the suggestion that ethylene plays some part in the plant response to coronatine. This response appears to be in some way associated with the amide bond linking coronafacic acid to the coronamic acid or valine part structures.

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