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Ethylene Synthesis and Growth of Tomato Hypocotyls: Induction by Auxin and Fusicoccin and Inhibition by Vanadate

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Abstract. The effects of fusicoccin (FC) on growth and ethylene synthesis of tomato (*Lycopersicon esculentum* Mill.) hypocotyls were compared to those of indole-3-acetic acid (IAA). Fusicoccin promoted both growth and ethylene production maximally at $<2\mu M$. Growth was stimulated to a slightly greater extent by FC as compared to IAA, while ethylene synthesis rates in response to FC were about 50% less than those induced by IAA. Cycloheximide (0.5 μM) inhibited auxin-induced growth by 80% but had no effect on FC-induced growth; ethylene production was inhibited to the same extent (58%) when induced by either IAA or FC. Both IAA and FC caused tissue contents of 1-aminocyclopropane-1-carboxylic acid (ACC) and malonyl-ACC to increase, indicating that like IAA, FC induces ethylene synthesis by stimulating the formation of ACC. Orthovanadate, a potent inhibitor of proton-translocating plasma membrane ATPases, reduced both IAA- and FC-induced growth and ethylene synthesis at concentrations less than 1 mM, with ethylene synthesis being approximately 10 times more sensitive to inhibition than growth. Vanadate did not affect tissue ACC levels, slightly reduced total ACC production, and inhibited conversion of ACC to ethylene. However, significant inhibition of in vivo ethylene-forming enzyme activity required high concentrations of vanadate (1 mM) and was less effective than inhibition by cobaltous ion. The site of action of vanadate in inhibiting ethylene synthesis remains unclear, but the ion did not prevent the elevation of tissue ACC levels in response to IAA or FC. It is unlikely, therefore, that stimulation of plasma membrane H^+-ATPase activity is required for the induction of ACC synthase by IAA and FC.

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The fungal toxin fusicoccin (FC) and the plant hormone indole-3-acetic acid (IAA) have similar effects on several physiological processes, including membrane transport, elongation growth of stems and seedlings, and regulation of stomatal aperture (Marré 1979). An early, common event in these processes may be the stimulation of a proton-translocating ATPase (H^+-ATPase) associated with the plasma membrane (Marré 1979; Rayle and Cleland 1977; Yamagata and Masuda 1975). It is controversial whether activation is direct, or indirect via effects of these compounds on cytoplasmic pH (Kurkdjian and Guern, 1989).

We have documented stimulation of ethylene synthesis in tomato hypocotyls by both FC and IAA (Kelly and Bradford 1986), indicating the possibility that changes in the activity of the plasma membrane H^+-ATPase or cytoplasmic pH may be involved in the induction of ethylene synthesis. A role for cytoplasmic pH changes in response to IAA or FC in the regulation of ethylene synthesis has been proposed (Göring and Zoglauer 1979; Zoglauer et al. 1987). Testing of these hypotheses requires further characterization of FC-induced ethylene synthesis. While IAA is known to stimulate ethylene synthesis by inducing 1-aminocyclopropane-1-carboxylic acid (ACC) synthase activity (Yoshii and Imaseki 1982; Yu and Yang 1979), the effect of FC on endogenous ACC levels has not been reported. In wheat coleoptiles, FC did not influence the conversion of ACC to ethylene (Zoglauer et al. 1987). An influence of transmembrane proton gradients, but not membrane electrical potential per se, on the conversion of ACC to ethylene has been observed both in vivo (John et al. 1985) and in isolated vacuoles (Mayne and Kende 1986).

In the current study, we compared IAA- and FC-induced growth of tomato hypocotyls with respect to concentration dependence and responses to inhibitors. Ethylene synthesis rates in response to

IAA and FC were characterized, and tissue ACC levels and in vivo ethylene-forming enzyme (EFE) activity were measured. The possible role of plasma membrane H^+-ATPase activity in FC- and IAA-induced ethylene synthesis was examined using vanadate, an inhibitor of this enzyme in vitro (Sze 1985).

Materials and Methods

Plant Material

Tomato (*Lycopersicon esculentum* Mill. cv. VFN8) seeds were surface sterilized for 20 min in 1% NaOCL solution (20% commercial bleach) and rinsed thoroughly. Approximately 100 seeds were sown on moist blotter paper in 10 × 10 × 3-cm plastic boxes at 27°C in the dark. After 6 to 7 days, 3-, 6-, or 12-mm hypocotyl segments were excised with a double razor blade cutter. Cuts were made as close to the hook as would yield straight segments. Immediately after cutting, segments were floated on 2.5 mM KH_2PO_4/K_2HPO_4 buffer (pH 6.5 or 5.2, depending on the pH at which the experiments were performed) for 0.5–2 h.

Solutions

Treatments were made in a basal medium of 2.5 mM KH_2PO_4/K_2HPO_4 buffer (pH 5.2 or 6.5), 2.5 mM KCl, 1 mM $Ca(NO_3)_2$, and 3% (wt/vol) sucrose. Chemicals of interest were added in small volumes from concentrated stock solutions stored in the freezer or refrigerator. Fusicoccin was obtained from Italchemia, Milan, Italy. Indole-3-acetic acid solutions were prepared daily. Stock solutions of vanadate were prepared from V_2O_5 (Aldrich Chemical Co., Milwaukee, WI, USA, "gold label") in 20 mM NaOH as described by Gallagher and Leonard (1982). Vanadate (HVO_4^{2-}) concentrations reported are twice the molar concentrations of V_2O_5 used to prepare the stock solutions. Experiments using vanadate or molybdate were performed at pH 6.5, and the final Na^+ concentration was adjusted to 2.0 mM in both control and vanadate solutions. The remaining experiments were performed at pH 5.2.

Hypocotyl Elongation Assay

Samples of 20 6-mm sections were selected at random and floated on 5 ml of solution in 10-ml beakers on an orbital shaker (60 rpm) at 27°C under laboratory light. After 6 h, segment lengths were measured to within 0.1 mm with a dissecting microscope equipped with an ocular micrometer. Pretreated segments were incubated for 2 h in vanadate solutions under identical conditions before addition of IAA or FC.

Ethylene Synthesis

Fifteen 12-mm segments were chosen at random, blotted to remove excess solution, and floated on 1 ml of solution in a 10-ml bottle. Bottles were loosely capped for 2 h to allow dissipation of the wound ethylene response, flushed with air, and sealed with a serum cap. The ethylene concentration of the gas phase was measured 3 h later by gas chromatography with an activated alumina column and a flame ionization detector. Background ethylene (in the absence of tissue) was measured periodically and was consistently negligible in this system. The CO_2 and O_2 concentrations have previously been demonstrated to be nonlimiting for ethylene synthesis in this system (Kelly and Bradford 1986).

Ethylene-Forming Enzyme (EFE) Activity

Hypocotyl segments (3 mm) were chosen at random and incubated on a shaker in 10 ml of 2.5 mM KH_2PO_4/K_2HPO_4 buffer (pH 6.5) with or without 0.75 mM ACC. After 1 h, segments were surface-dried in a vacuum funnel for 30 s, washed in 25 ml of buffer for 1 min, then surface dried with vacuum for another 30 s. For each sample, 15 segments were transferred to 1 ml of buffer solution without ACC in a 10-ml bottle. The bottles were capped immediately, and the ethylene concentration of the gas phase was measured at several intervals. Prior to each sampling, air was injected into the bottle to keep the gas pressure constant. Corrections were made for the dilution due to sampling and replacement of the air. The absolute ethylene production rates were variable among experiments; the results reported are consistent with the overall pattern observed.

Measurement of ACC and Malonyl-ACC Content

Ethylene synthesis rates were measured as described above. Immediately after measurement, hypocotyl segments from two or three replicates of 15 segments each were combined, dropped into 3 ml of boiling 80% ethanol for 2 min, and incubated at 65°C overnight. The ethanol solution was decanted, and the tissue was incubated for 1 h in a second 3 ml of boiling 80% ethanol. The ethanol extracts were combined, evaporated to dryness, and resuspended in 1 ml of water. Aliquots of this extract were either analyzed for ACC content according to the method of Lizada and Yang (1979), or incubated in 2 N HCl at 110°C for 3 h before ACC analyses. The additional ACC released by hydrolysis was assumed to be malonyl-ACC (Yang and Hoffman 1984).

Results

Comparison of IAA- and FC-Induced Growth and Ethylene Synthesis

We previously reported that growth and ethylene production in VFN8 tomato hypocotyls were saturated at 1 and 10 μM IAA, respectively (Kelly and Bradford 1986). Growth and ethylene synthesis were saturated at 1–2 μM FC, and the dependence of both processes on FC concentration was identical (Fig. 1). Averaged over a number of experiments, the maximal growth of tomato hypocotyls in response to FC was slightly greater than the maximal growth in response to IAA, while the most rapid FC-induced ethylene synthesis rates were about 50% of the maximal IAA-induced rates.

Stimulation of growth by auxin and FC can be distinguished by their differential sensitivities to in-

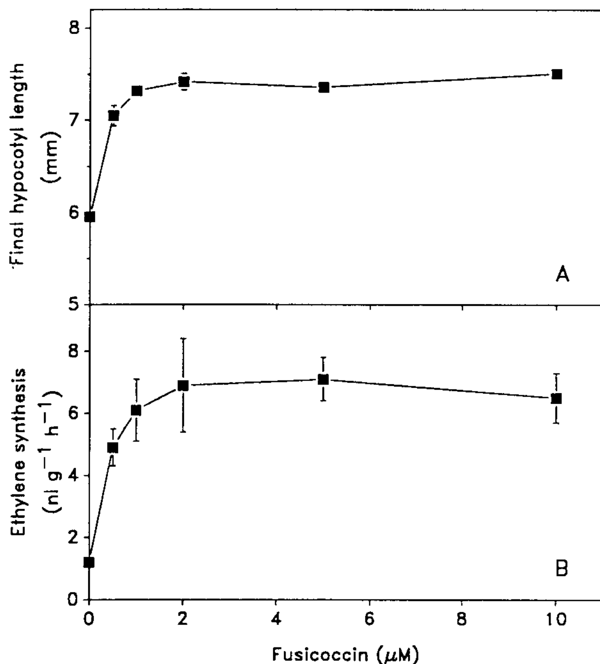

Fig. 1. Dependence of (**A**) growth and (**B**) ethylene synthesis of tomato hypocotyl segments on FC concentration. For growth measurements, 6-mm segments were incubated on the indicated concentration of FC for 6 h. Ethylene synthesis was measured during the period 2–5 h after treatment using 12-mm segments. Standard errors are shown where they exceed the size of the symbol (n = 20 segments for growth; n = three replicates of 15 segments each for ethylene synthesis).

Table 1. Effect of cycloheximide (CHM) on IAA- and FC-induced elongation and ethylene synthesis in tomato hypocotyl segments.

Treatment	CHM (μM)	Final hypocotyl length (mm)	Ethylene synthesis (nl/g h)
Control	0	6.00 ± 0.02	0.9 ± 0.1
IAA (10 μM)	0	6.80 ± 0.11	15.5 ± 2.1
IAA (10 μM)	0.5	6.16 ± 0.06	6.5 ± 6.5
FC (1.5 μM)	0	6.79 ± 0.08	7.1 ± 1.6
FC (1.5 μM)	0.5	6.81 ± 0.08	3.1 ± 0.3

Segment lengths were measured after 6 h of incubation. Ethylene was trapped during the 2nd to 5th hours of incubation. Elongation data are the means ± SE of 18–22 segments. Ethylene synthesis rates are the means ± SE of three replicates of 15 segments each.

hibition by the protein synthesis inhibitor cycloheximide (CHM):FC-induced growth is relatively insensitive to CHM, while IAA-induced growth requires protein synthesis (Bates and Cleland 1979; Kutschera and Schopfer 1985). This relationship was confirmed for tomato hypocotyls (Table 1). A low concentration of CHM (0.5 μM) reduced growth in response to IAA by 80%, but had no effect on FC-dependent growth. Ethylene synthesis was inhibited 58% by the same concentration of CHM, regardless of whether it was induced by IAA or FC (Table 1). At the much higher concentration of CHM used in studies of grass coleoptiles (36 μM; Bates and Cleland 1979; Kutschera and Schopfer 1985), neither growth nor ethylene synthesis occurred in response to IAA or FC.

The endogenous levels of ACC and malonyl-ACC increased following treatment with either IAA or FC (Table 2). Ethylene production increased by 8.7- and 5.7-fold for IAA and FC, respectively, while ACC levels increased by only 2.2-and 3.6-fold. Malonyl-ACC content approximately doubled after treatment with either IAA or FC. Total ACC (ethylene + ACC + malonyl-ACC) produced was similar for the IAA and FC treatments, although the ethylene production rate was 50% grreater for the IAA-treated hypocotyls (Table 2). This suggests that FC and IAA were equally effective in increasing ACC-synthase activity, but that EFE activity might be depressed by FC. This was tested by determining ethylene synthesis rates in the presence or absence of FC in segments preloaded with ACC. (Segments were preloaded with ACC to avoid possible effects of FC on ACC uptake). Fusicoccin slightly increased ACC-dependent ethylene synthesis, but this could be due to stimulation by FC of endogenous ACC accumulation. When aminoethoxyvinylglycine (AVG), an inhibitor of ACC synthase, was included in the medium to block ACC synthesis, no effect of FC on ACC-dependent ethylene synthesis was detected (unpublished observations).

Effect of Vanadate on IAA- and FC-Induced Growth and Ethylene Synthesis

Vanadate, an inhibitor of the FC-sensitive plasma membrane H^+-ATPase (reviewed in Sze 1985), has previously been used to imply the involvement of an H^+-ATPase in IAA-induced growth (Jacobs and Taiz 1980). Both IAA- and FC-dependent growth of tomato hypocotyls were inhibited by <1 mM vanadate (Fig. 2). Pretreatment of the tissue with vanadate for 2 h before IAA or FC addition shifted the concentration dependence to somewhat lower values, but did not alter the overall trends (Fig. 2). Growth induced by IAA was completely inhibited by 0.5 mM vanadate (in pretreated tissues), while 28% of the FC-dependent growth response remained even at 1 mM vanadate.

Ethylene synthesis was more sensitive to inhibition by vanadate than was growth (Fig. 3). Although the maximum rate of ethylene synthesis was greater

Table 2. Ethylene production and ACC and malonyl-ACC contents of 12-mm tomato hypocotyl segments treated with IAA, FC, vanadate, or Co^{2+}.

Treatment	No. of replicates	Ethylene produced (nmol/g)	ACC (nmol/g)	Malonyl-ACC (nmol/g)	Total ACC (nmol/g)
Control	8	0.21 ± 0.03	0.75 ± 0.10	3.18 ± 0.61	4.14
IAA (50 μM)	8	1.83 ± 0.42	1.68 ± 0.23	6.21 ± 0.84	9.72
FC (2.5 μM)	6	1.20 ± 0.27	2.71 ± 0.33	6.89 ± 0.72	10.8
IAA (50 μM) + vanadate (200 μM)	4	0.60 ± 0.12	2.41 ± 0.42	5.05 ± 0.35	8.06
FC (2.5 μM) + vanadate (200 μM)	4	0.27 ± 0.00	2.60 ± 0.27	3.98 ± 0.41	6.85
IAA (50 μM) + Co^{2+} (100 μM)	5	0.24 ± 0.03	5.59 ± 1.02	5.74 ± 0.72	11.6
FC (2.5 μM) + Co^{2+} (100 μM)	3	0.24 ± 0.03	6.16 ± 0.20	7.44 ± 0.49	13.8

Ethylene synthesis was measured 2–5 h after treatment. The ethylene data are expressed as total nmol/g released over the 3-h period to allow molar comparison with the ACC contents. For comparison with ethylene synthesis rate data, 1 nmol/g here is equivalent to 7.4 nl/g h. ACC and malonyl-ACC were extracted immediately after ethylene synthesis measurement. Total ACC is the sum of the ethylene evolved + ACC + malonyl-ACC. Values are means ± SE for the indicated number of replicates.

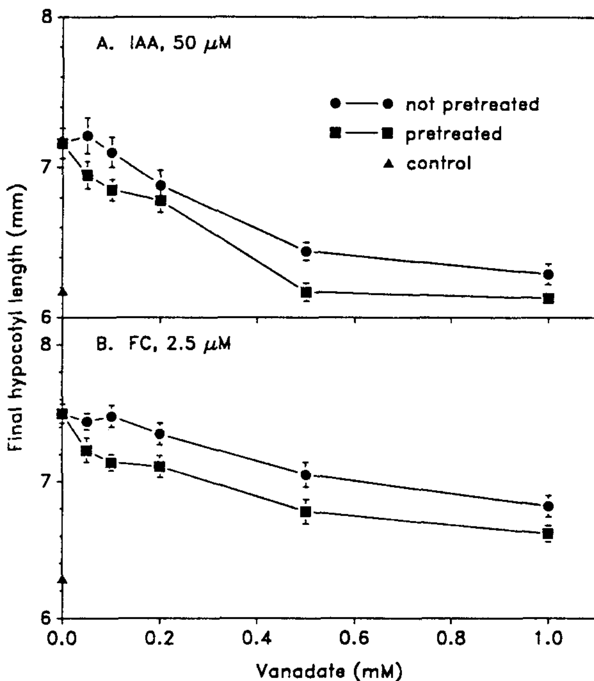

Fig. 2. Effect of vanadate on growth of 6-mm hypocotyl segments in response to (A) IAA or (B) FC. Pretreated tissues were incubated in vanadate for 2 h prior to transfer to solutions containing vanadate plus IAA or FC. Standards errors are shown where they exceed the size of the symbol (n = 20 segments).

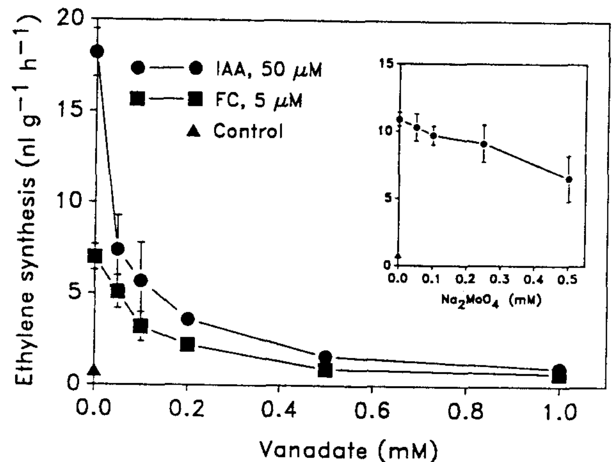

Fig. 3. Inhibition of IAA- and FC-induced ethylene production by vanadate in 12-mm hypocotyl segments. Ethylene synthesis was measured during 2–5 h after treatment. The inset shows a similar experiment using molybdate, which was much less effective than vanadate in inhibiting ethylene production induced by IAA. Standard errors are shown where they exceed the size of the symbol (n = three replicates of 15 segments each).

with IAA, IAA- and FC-dependent ethylene synthesis rates were 50% inhibited at 0.04 and 0.08 mM vanadate, respectively, and completely inhibited at 0.5 mM. Molybdate, an inhibitor of acid phosphatases (which may also be inhibited by vanadate) but not of H^+-ATPases (Gallagher and Leonard 1982; O'Neill and Spanswick 1984), had much less effect on ethylene production than did vanadate at comparable concentrations (Fig. 3, inset), indicating that the vanadate effect was not a consequence of nonspecific phosphohydrolase inhibition. The ACC content of hypocotyls treated with IAA or FC was not significantly affected by vanadate concentrations up to 1 mM, and malonyl-ACC content was reduced to the control level only at the highest vanadate concentration (unpublished observations). Vanadate (0.2 mM) caused some decrease in malonyl-ACC levels and total ACC production, more so for FC than for IAA.

The effect of vanadate on in vivo EFE activity was tested in 3- or 12-mm hypocotyl segments preloaded with 0.75 mM ACC. In 3-mm segments, vanadate significantly reduced in vivo EFE activity,

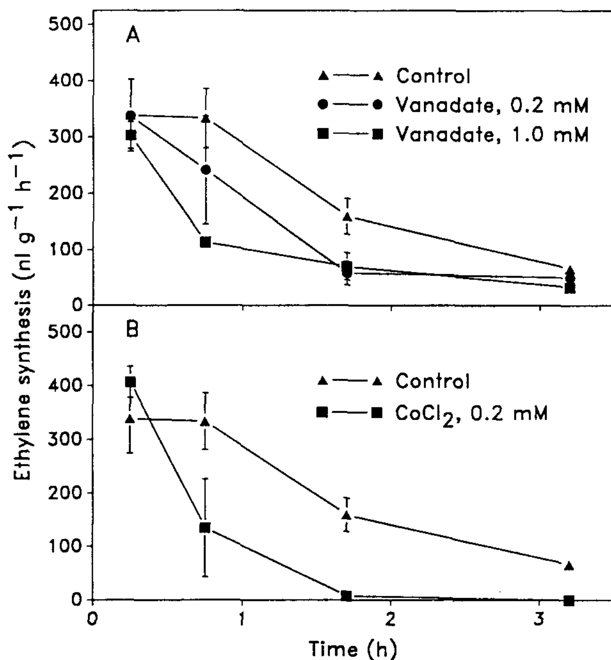

Fig. 4. Effects of (A) vanadate and (B) Co^{2+} on in vivo EFE activity. Three-millimeter hypocotyl segments were preloaded with 0.75 mM ACC, then transferred to buffer, with or without vanadate or Co^{2+}. Three millimeter hypocotyl segments were used to improve penetration of vanadate into the segments. Standard errors are shown where they exceed the size of the symbol (n = three replicates of 15 segments each).

but inhibition was incomplete even with 1 mM (Fig. 4A). Vanadate was only slightly inhibitory in 12-mm segments even at 1 mM (unpublished observations). In comparison, 0.2 mM $CoCl_2$, which is known to inhibit EFE activity (Yu and Yang 1979), rapidly and completely blocked ACC-dependent ethylene synthesis (Fig. 4B). Inhibition of EFE activity by Co^{2+} was also more rapid in 3-mm than in 12-mm segments (unpublished observations), indicating that these inhibitors penetrate more readily into the tissue in the smaller segments. When ethylene production was inhibited by Co^{2+}, ACC levels increased two- to threefold, which was not observed with vanadate (Table 2).

Discussion

The generality of FC induction of ethylene synthesis remains to be established. Fusicoccin stimulated ethylene synthesis in citrus leaves (Feldman et al. 1971), but had no effect on ethylene synthesis in pea (Branca and Ricci 1983; Theologis 1987), rice (Bottalico 1975), wheat (Zoglauer et al. 1987), or cucumber (our unpublished results). In fact, FC inhibited auxin-induced ethylene synthesis in wheat (Zoglauer et al. 1987) and pea (Branca and Ricci 1983), although we observed stimulation of ethylene synthesis rates by FC in IAA-treated tomato hypocotyls (unpublished results). In tomato, the similarity of the concentration dependencies of FC-induced growth and ethylene synthesis (Fig. 1) suggests that FC may act via the same mechanism in both responses.

We previously showed that AVG inhibited FC-induced ethylene synthesis in tomato hypocotyls (Kelly and Bradford 1986) and suggested that FC-induced ethylene synthesis proceeds via the ACC pathway, which is induced by IAA and most other promoters of ethylene synthesis (Yang and Hoffman 1984). Measurement of elevated tissue ACC contents in both IAA- and FC-treated hypocotyls (Table 2) confirms the involvement of ACC synthase. Further, the effects of CHM (Table 1) indicate that FC- and IAA-induced ethylene synthesis have similar requirements for protein synthesis, a requirement that can be attributed to de novo synthesis of ACC synthase (Yang and Hoffman 1984). Ray (1987) showed that protein synthesis is necessary for promotion of β-glucan synthase activity by both FC and auxin. In agreement with results for wheat coleoptiles (Zoglauer et al. 1987), we found no effect of FC on EFE activity in tomato hypocotyls preloaded with high ACC concentrations.

Although the mechanisms of action of IAA and FC differ, both compounds stimulate vanadate-sensitive proton efflux (Cleland 1982). Therefore, we investigated the influence of vanadate on IAA- and FC-induced growth and ethylene production in tomato hypocotyls. Hypocotyl growth induced by either IAA or FC was reduced linearly with increasing concentrations of vanadate to 0.5–1.0 mM (Fig. 2). Vanadate at 1 mM also inhibited auxin-stimulated growth and proton efflux of oat coleoptiles, pea stems, and cucumber hypocotyls (Brummell et al. 1986; Jacobs and Taiz 1980). It is interesting that IAA-induced growth was more sensitive to inhibition by vanadate than was FC-induced growth (Fig. 2). We are unaware of any other reports where the effectiveness of vanadate as an inhibitor of IAA- and FC-induced growth has been compared, although its relatively sluggish and incomplete action in vivo, in contrast to its effectiveness in blocking in vitro H^+-ATPase activity, has been noted (Felle 1987; Sanders and Slayman 1982). Our attempts to measure H^+ extrusion (medium acidification) by tomato hypocotyls were inconsistent, perhaps because of the use of tissues with intact cuticles. Since our interest was in ethylene synthesis and since abrasion would inevitably involve wounding and associated ethylene production, we

elected not to utilize abraded hypocotyls. Thus, we could not determine whether the difference in sensitivity of growth to inhibition by vanadate was related to differential rates of proton extrusion (see Kutschera and Schopfer 1985).

Vanadate was a potent inhibitor of ethylene synthesis at concentrations approximately tenfold less than those required to inhibit growth (Fig. 3) and similar to those which inhibit in vitro plasma membrane H^+-ATPase activity (O'Neill and Spanswick 1984). While effects of vanadate on processes other than H^+-ATPase activity cannot be excluded, including involvement in protein phosphorylation (e.g., Tracey and Gresser 1986), the low concentrations of vanadate required and the relative ineffectiveness of molybdate (Fig. 3, inset) both indicate that H^+-ATPase activity may be necessary for ethylene synthesis. This possibility cannot be evaluated further with the present data because the site of action of vanadate in the ethylene-biosynthetic pathway is unclear. Tissue ACC content was unchanged by vanadate, suggesting an inhibition of further ACC metabolism (Table 2). However, inhibition of EFE activity often results in an increase in ACC levels (Yu and Yang 1979), as we observed with Co^{2+} (Table 2). Due to the absence of ACC accumulation in the presence of vanadate, an effect on ACC synthesis cannot be excluded. Nonetheless, since ethylene production rates were reduced in the presence of vanadate with little change in tissue ACC levels, the major effect of vanadate would appear to be on EFE activity. Failure to unequivocally demonstrate inhibition of ACC-dependent ethylene synthesis by vanadate might be explained in two ways. First, our data indicate that ACC and vanadate are differentially accessible to hypocotyl tissues. Second, vanadate may inhibit transport of ACC into the vacuole, a possible control point in ethylene biosynthesis (Guy and Kende, 1984; Saftner and Baker, 1987). This effect would not be readily apparent in an assay in which tissues are preloaded with ACC, as they should already contain a high vacuolar ACC content (Mayne and Kende, 1986).

In summary, both IAA and FC stimulate ethylene production in tomato hypocotyls by elevating tissue ACC levels. Vanadate was a highly effective inhibitor of IAA- or FC-induced ethylene synthesis, but did not prevent the elevation of ACC levels. We conclude that vanadate-sensitive processes, most likely H^+-ATPase activity, are not required for the induction of ACC synthase by IAA or FC, but may be involved in the subsequent metabolism of ACC.

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